ANNUAL REPORTS IN MEDICINAL CHEMISTRY Volume 24

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

Editor-in-Chief: **RICHARD C. ALLEN**

HOECHST-ROUSSEL PHARMACEUTICALS, INC.
SOMERVILLE, NEW JERSEY

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers

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Volume 24

Academic Press Rapid Manuscript Reproduction

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ACADEMIC PRESS, INC.,
Harcourt Brace Jovanovich, Publishers
San Diego New York Berkeley Boston
London Sydney Tokyo Toronto

ACADEMIC PRESS, INC.
San Diego, California 92101

United Kingdom Edition published by
ACADEMIC PRESS LIMITED
24-28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 66-26843

ISBN 0-12-040524-5 (alk. paper)

PRINTED IN THE UNITED STATES OF AMERICA
89 90 91 92 9 8 7 6 5 4 3 2 1

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PREFACE

Volume 24 of *Annual Reports in Medicinal Chemistry* is similar in format to previous volumes of this series. The 31 chapters of this volume are distributed among seven sections: CNS Agents; Cardiopulmonary and Vascular Agents; Chemotherapeutic Agents; Endocrinology, Immunology, and Metabolic Disorders; Topics in Biology; Topics in Chemistry and Drug Design; and Trends and Perspectives.

Traditional annual updates include chapters on antipsychotics, antihypertensives, antianxiety agents/anticonvulsants, antifungals, antivirals, and new NCE introductions. Chapters on antibiotic transport into bacteria (Chapter 15) and neutrophil chematoxis (Chapter 25) complement the annual general update on antibiotics (Chapter 11).

A number of chapters deal with topics of high current interest including central muscarinic ligands and receptors (Chapter 4); excitatory amino acids (Chapter 5) and a complementary one (30) on polyamine spider toxins; potassium channel openers (Chapter 10); second generation recombinant proteins (Chapter 23); hypercholesterolemia (Chapter 16); and transgenic animal models of disease (Chapter 22). Chapters on leucotriene inhibitors, PAF antagonists and phospholipase A_2 inhibitors cross various therapeutic areas, some of which, including pulmonary and antiallergy agents, inflammatory bowel disease, and dermatology, are treated more globally as separate monographs. Complementing this latter offering are ones on alopecia (Chapter 20) and dermal wound healing (Chapter 24).

Recent advances in the design of peptidomimetics and structure determination of peptides by MS are reviewed in sequential chapters 26 and 27. Complementary chapters 28 and 29 address aspects of MRI and PET of interest to medicinal chemists. Reviews on recent developments in antitumor therapy and proliferative prostatic disease round out this year's edition.

With this volume I conclude my eight-year editorial association with *Annual Reports in Medicinal Chemistry,* including two years as Editor-in-Chief. I would like to take this opportunity to express my sincere appreciation to the section editors, authors, secretaries, and countless others for their contribution to making my tenure a rewarding one. Finally, I would like to acknowledge the support and encouragement I have received from the management of Hoechst-Roussel Pharmaceuticals, Inc., specifically, Drs. Grover C. Helsley and Victor J. Bauer.

Richard C. Allen
Somerville, New Jersey
May 1989

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Section I- CNS Agents

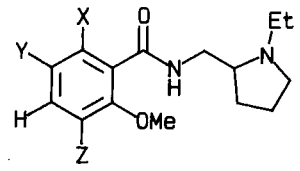
Editor: Joel G. Berger, Schering-Plough Corporation
Bloomfield, NJ 07003

Chapter 1. Antipsychotic Agents

James S. New and Katherine S. Takaki
Bristol-Myers Co., Wallingford, Ct 06492

Introduction - The ongoing analysis of dopamine (DA) D1 and D2 receptor functions has recently focussed on the pharmacology derived from their mutual interaction, and what possible role this may play in the pharmacotherapy of schizophrenia. The more controversial sigma receptor, which has been implicated in the biological mechanisms for certain antipsychotic agents, is suggested to mediate the motor dysfunctions accompanying antipsychotic drug use in man (1-3). The sigma receptor has also been shown to undergo up-regulation in rats treated subchronically with the ligand 3-(3-hydroxyphenyl)-N-n-propylpiperidine (3-PPP) (4). Although not conclusive, these results strongly argue for a functional significance of the sigma receptor in the CNS. The dopamine hypothesis of schizophrenia has been critiqued, and the relevance of serotonin (5-HT) and 5-HT2 antagonists in the treatment of schizophrenia reviewed (5,6).

D2 Selective Antagonists - The second generation sulpiride analogues, remoxipride (1) and raclopride (2), represent refined prototypes of the substituted benzamide chemical class which derive their antipsychotic activity from selective interaction with the D2 receptor. The antipsychotic efficacy of 1 has been confirmed in a comparative double-blind study with thioridazine in acute schizophrenic patients (7). Multiple dosing in healthy males indicates that remoxipride is generally safe and, although akathesia was reported at the higher dose, no EPS was observed (8). A single-blind, placebo controlled study of patients with persistent tardive dyskinesia demonstrated that 1 reduced the dyskinesia score without an increase in parkinsonism (9).



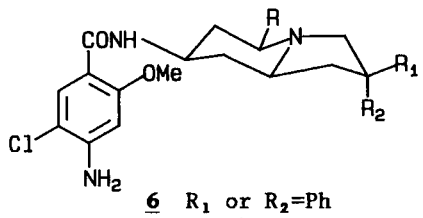
	X	Y	Z
1	OMe	H	Br
2	OH	Cl	Cl
3	H	I	H
4	H	SO_2NH_2	H
5	OH	I	H

Interestingly, evaluation of the binding characteristics of a number of structurally diverse antipsychotics has shown that these compounds share significant affinity for sigma receptors and that, among those tested, 1 had a 15-fold greater affinity for the sigma site than for the D2 receptor (10). The research strategy and medicinal chemistry utilized in the development of the DA D2 selective 2,6-dioxygenated benzamides has been thoroughly reviewed (11).

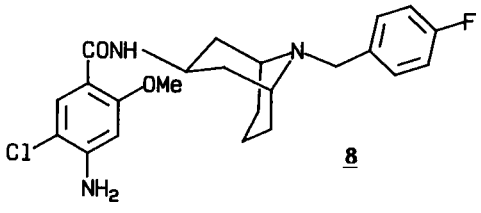
A study of the *in vitro* selectivities of a number of benzamides has indicated that 2 showed the best combination of potency and selectivity for D2 receptors (12). In addition, other characteristics including low non-specific binding and blood/brain barrier permeability make 2 a useful probe for the study of D2 receptors both *in vitro* and *in vivo* (13,14). ^{11}C-2 has been used to successfully label D2 receptors in positron emission tomography (PET) studies in monkeys and in man (15,16). In healthy humans highly stereoselective binding was demonstrated by PET for ^{11}C-2 over its enantiomer ^{11}C-FLB 472 (16). This stereoselectivity has also been demonstrated by *in vivo* labelling of rat brain D2 receptors (17). A study of DA receptor subtypes in human post-mortem brain using ^{3}H-SCH-23390 (26) and ^{3}H-2 has shown that the distribution of D1 and D2 receptor subtypes coincides with results from PET studies utilizing the ^{11}C labelled version of these ligands (18,19). A brief review of some of the methods developed to specifically and quantitatively study DA receptor function in living subjects by PET has appeared (20).

The syntheses of two radioiodinated benzamides which may prove useful for *in vivo* studies utilizing single photon emission computed tomography have been described (21,22). In iodopride (3), replacement of the hydrophilic aminosulphonyl group of sulpiride (4) with iodine maintains the D2 affinity and selectivity of the molecule, but renders it 40 times more lipophilic (23). The corresponding iodinated salicylamide IBZM (5) is also highly D2 selective and readily localizes in D2 rich areas of the brain (24-26).

The indolizidines 6 are benzamides with conformationally restricted side chains in which the stereochemistry of the phenyl substituent dictates whether the molecule will have gastro-prokinetic properties or central DA activity (27). In particular, 7 showed good D2/D1 receptor selectivity and inhibited amphetamine-induced locomotor activity in rats more potently than it induced catalepsy. The azabicyclononyl benzamide BRL 34778 (8) is a selective D2 antagonist which shows potent, prolonged



6 R_1 or R_2=Ph
7 R_1=Ph R_2=Me R=H



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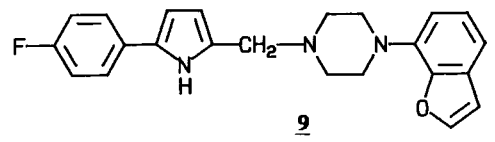
duration of action in *in vivo* models of antipsychotic activity but is no longer under development due to toxicological findings (28). Continued investigations of 2-phenylpyrrole derivatives as conformationally restricted benzamides has led to the development of benzofuran 9. Compound 9 has affinity and selectivity for D2 receptors superior to the original prototypes (*e.g.* 10), and it does not induce catalepsy at doses 10 times the effective dose in the conditioned avoidance response test (CAR) (29).



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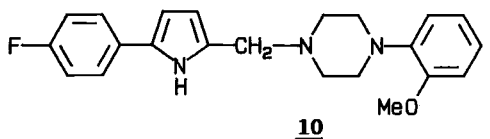
A QSAR analysis of aromatic substitution in the orthopramides indicates that an electron withdrawing substituent at position 5



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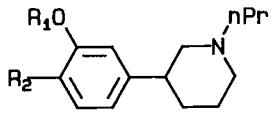
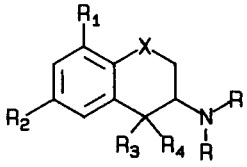
of the aromatic ring enhances D2 receptor affinity, but it decreases affinity if placed at position 4 (30,31). Conformational studies of **2** employing crystallographic and molecular modeling techniques suggest that benzamides with a N-ethyl-2-pyrrolidinylmethyl side chain adopt a folded or half-folded side chain conformation when interacting with the DA receptor (32). Good structural overlap is achieved between the half-folded conformer of **2** and one of the two low energy conformations calculated for the phenylpyrrole **10** (11).

DA-Autoreceptor Agonists - A theoretical alternative to post-synaptic D2 antagonists in the pharmacotherapy of schizophrenia is the reduction of dopaminergic neurotransmission evoked by DA autoreceptor agonists. (-)-3-PPP (**11**), a well-studied autoreceptor agonist, has been recently shown to suppress neuroleptic-induced abnormal movements in monkeys without inducing parkinsonism (33). Synthesis and evaluation of the 0-methylated catechols **12** and **13** has demonstrated that these compounds are minor metabolites of **11** which are unlikely to contribute to its pharmacological profile (34).



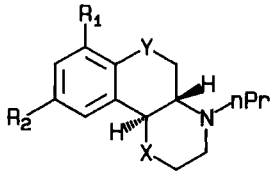
11 $R_1=R_2=H$
12 R_1=Me, R_2=OH
13 R_1=H, R_2=OMe

Extensive SAR investigations have focussed upon modifications of the DA ligands 5-OH- and 7-OH-DPAT (**14** and **15**). The pre-synaptic DA receptor agonist, *R*-**14**, demonstrates low bioavailability that does not appear to be due to its metabolic conversion to the catechol (35). Improved delivery to the brain is achieved if the methylene group of **14** is replaced by an oxygen atom as in analogue **16**. This interchange renders **16** slightly more selective for DA autoreceptors than **14**, although its postsynaptic DA agonist effects are weaker (36). The isomer **17** demonstrates a 60-fold greater relative affinity for DA agonist vs. antagonist labeled receptors and it is active in the gamma-butyrolactone (GBL) model for autoreceptor activity (37).



	R	R_1	R_2	R_3	R_4	X
14	nPr	OH	H	H	H	CH_2
15	nPr	H	OH	H	H	CH_2
16	nPr	OH	H	H	H	O
17	nPr	H	OH	H	H	O
18	alkyl	OH/OMe	H	Me	Me	CH_2
19	alkyl	OH/H	H/OH	=O		CH_2

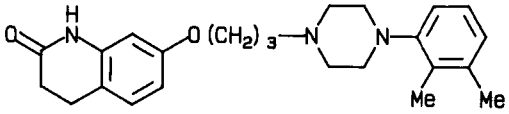
A series of C_1-dimethylated analogs of 5-OH- and 5-OMe-aminotetralins **18** has been synthesized, but none of the compounds demonstrated strong DA D2 affinity in binding studies (38). Introduction of a carbonyl group at C_1 to give 2-amino-1-tetralone derivatives **19** also produced compounds which were inactive in DA binding assays (39). The hexahydronaphthoxazine **20** is an orally active DA agonist which demonstrates autoreceptor selectivity (40). Replacement of a methylene group by oxygen produced compound **21** which was inactive in both *in*



	R_1	R_2	X	Y
20	H	OH	O	CH_2
21	H	OH	O	O
22	OH/H	H/OH	S	CH_2
23	OH/H	H/OH	NH	CH_2

vitro and *in vivo* tests of DA receptor activation (41). This inactivity may result from inadequate protonation of the compound at physiological pH due to its low pKa. Previous work employing both dopamine agonists and antagonists suggest that it is the charged species of the dopaminergic ligand which actually binds to the DA D2 receptor (42,43). The synthesis of the thio (22) and amino (23) analogs of 20 have also recently been reported (44).

A series of neurochemical and behavioral tests indicate OPC-4392 (24) acts as a DA agonist at presynaptic autoreceptors and a dopamine antagonist at postsynaptic receptors (45-48). In contrast to conventional antipsychotics, OPC-4392 dose-dependently decreased serum prolactin levels in healthy volunteers (49).



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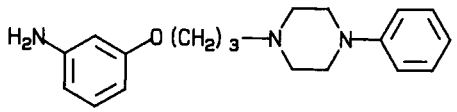
The aminoalkoxyaniline 25 has been shown to be an orally active DA agonist whose autoreceptor selectivity was demonstrated in the inhibition of exploratory locomotor activity and the GBL model in rats (50). Compound 25 was active in the Sidman avoidance test and did not induce dystonias or dyskinesias in sensitized monkeys suggesting it may possess limited side effects.



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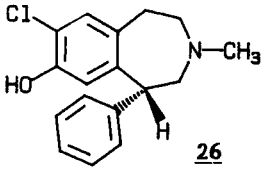
Mechanistic Studies and D1 Antagonists - The selective D1 receptor antagonist SCH 23390 26 continues to serve as the best prototypical agent to investigate the full range of behavioral and neurochemical issues associated with the D1 receptor function in the CNS. Photoaffinity labels based on SCH 23390 have been prepared, and specifically label peptides of M_r = 51-75 Kd as the putative ligand binding subunits of the D1 DA receptor (51,52). Partial purification of this molecular entity was achieved by affinity chromatography of solubilized striatal membranes using a benzazepine derivatized column matrix (53). Similar to typical neuroleptics, SCH 23390 antagonizes apomorphine (APO)-induced climbing and sniffing behaviors in mice; however, the production of this climbing behavior is believed to require both D1 and D2 receptor activation (54,55). The complex interaction of these DA receptors suggest the D1 receptor performs an "enabling" role in the functional expression of D2 receptors, although the reciprocal relationship has not been convincingly demonstrated (56,57). The selective inactivation of either D1 or D2 receptors in the rat does not have identical effects on the stereotypies induced by selective D1 or D2 agonists, and the detection of D1 receptor supersensitivity in behavioral studies may be reliant on D2 stimulation (58-60). The D1/D2 receptor interaction also seems to differ in the lesioned vs. nonlesioned side of a rat rendered supersensitive by unilateral lesions with 6-hydroxydopamine (6-OHDA). The stimulation of either subtype alone in the dopaminergic denervated side can produce rotation, whereas simultaneous stimulation of both DA receptor subtypes on the innervated side is required for the production of turning behavior (61). In addition, the contralateral rotation produced by selective D1 or D2 agonists is shown to be specifically inhibited by DA antagonists selective for those receptor subtypes (62). Whether in the normosensitive or supersensitive receptor state in mice, the D1 receptor is thought to play a key



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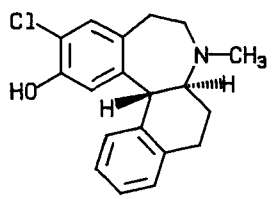
role in mediating locomotor responses controlled through DA stimulation (63). Other studies suggest the D1 receptor may be uniquely involved with the motivational effects of opioids, or that its location in the nucleus accumbens mediates the reinforcement produced by ventral tegmental (VT) stimulation (64,65).

The acute effects of SCH 23390 in the rat indicate the compound has only modest effects on DA metabolism and release (66,67). In a study comparing the acute effects of both typical and atypical antipsychotics on various parameters of DA metabolism, SCH 23390 resembles the neurochemical profile of the atypical agents (68). Chronic treatment of rats with SCH 23390 decreased DA metabolism in the caudate nucleus, and decreased the number of spontaneously active DA neurones in both the substantia nigra pars compacta (SNC or A9 DA neurones) and the ventral tegmental area (VTA or A10 DA neurones) (69,70). Although these responses are similar to what is observed with the classical neuroleptics, chronic treatment of rats with SCH 23390 effected an up-regulation of only D1 receptors and not D2 receptors (71). This result was confined to the nigrostriatal system, and was not observed in the mesolimbic-cortical areas. In contrast, another study demonstrated that chronic SCH 23390 produced depolarization inactivation in only A10 and not A9 cells, predictive of an atypical antipsychotic profile in man (72). In rats with unilateral quinolinic acid-induced striatal lesions, D1 receptor supersensitivity led to behavioral subsensitivity in response to D2 agonist mediated turning behavior (73). SCH 23390-induced catalepsy (s.c. administration) in rats has been characterized as potent, but brief (<90 min.), as opposed to the catalepsy induced by mixed D1/D2 antagonists which is more slowly rising and prolonged (>240 min.) (74). Stereotaxic injections of SCH 23390 suggest that its catalepsy-induction properties are related to occlusion of DA D1 receptors in the forebrain, particularly the striatum (75). Different mechanisms may be facilitating SCH 23390-induced catalepsy versus the catalepsy caused by raclopride (76). DA D1 receptor up-regulated rats chronically treated with SCH 23390 developed tolerance only to the catalepsy-induction properties of spiperone, but not SCH 23390. Conversely, rats chronically treated with spiperone developed tolerance only to the catalepsy-induction properties of spiperone, and not SCH 23390 (77). Autoradiographic studies in human brain demonstrate that high densities of both D1 and D2 receptors are found in the nucleus caudatus, putamen, and nucleus accumbens. The cerebral cortex is also rich in D1 receptors, while low concentrations of D2 receptors are restricted to the entorhinal and cingulate cortex (78). Preliminary studies of the serotonergic properties of SCH 23390 indicate that the putative 5-HT2 antagonist and 5-HT1 agonist properties of the compound are much weaker than its dopaminergic properties (79-82). SCH 39166, (27), a conformationally rigid analogue of SCH 23390, antagonizes APO-induced stereotypy in rats (M.E.D. = 10 mg/kg, p.o.), and does not cause catalepsy at doses up to 10 times its minimal effective dose in the rat CAR (83). The extended duration of action which SCH 39166 demonstrates in both rodent and primate models of antipsychotic activity make it a useful D1 antagonist prototype to evaluate in the clinic. A review on the therapeutic potential of D1 agonists and antagonists in psychiatry, and full accounts of D1 receptor mediated pharmacology, have appeared (84-86).



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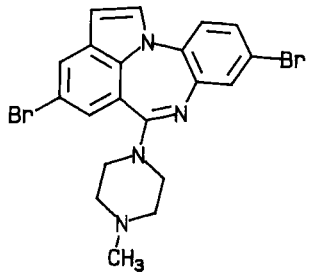
Mixed D1/D2 Antagonists and Atypical Agents - Positron emission tomography studies have documented a 65% to 85% occupancy of DA D2 receptors using a variety of typical and atypical neuroleptics, including clozapine (87).

New autoradiographic studies are also consistent with previous reports that indicate an elevation of D2 receptor density occurs in the striata of many schizophrenics (88). The atypical antipsychotic clozapine has shown efficacy in ameliorating both the positive and negative symptoms of schizophrenia in treatment-resistant patients, while results exploring the use of carbamazepine for this indication were equivocal (89-91). D1/D2 antagonists, or selective D2 antagonists, also lead to an increase in neurotensin-like immunoreactivity in the nucleus accumbens, while SCH 23390 had the opposite effect (92). Separate reviews explore the pathophysiology behind the neuroleptic-induced side-effect of tardive dyskinesia, and the symptomatology which results from the withdrawal of antipsychotic drug therapy (93,94). Other than its lack of anticholinergic activity, the receptorology of the atypical antipsychotic HP 370 (**28**) closely resembles that of clozapine (95). HP 370 has potent activity in several *in vivo* rodent paradigms predictive of antipsychotic activity, but demonstrates relatively weak catalepsy-induction properties (96). Chronic administration of HP 370 to rats attenuated DA neuronal activity only in the VTA and had no effect on SN DA neurons, suggesting the compound has mesolimbic selectivity (96). Likewise, chronic administration of BMY 14802-1 (**29**) reduced the number of spontaneously active A10 DA cells in the rat without affecting the number



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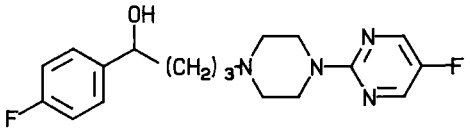

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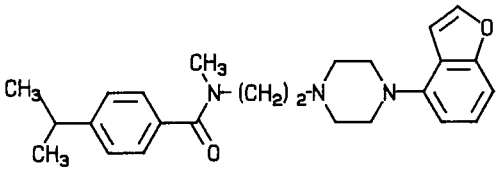
of active A9 DA cells (97). BMY 14802-1 has no affinity for DA receptors, but binds to the sigma receptor in a stereoselective manner (98). The *in vivo* antipsychotic-like activity of this compound may be expressed through a nondopaminergic mechanism. The differential responses of A9 and A10 cells, and the role of D1 and D2 receptors in the regulatory control of DA neurons, has been comprehensively reviewed (99). Befiperide (**30**) blocks the CAR and APO or amphetamine (AMP)-induced stereotypies while having only weak affinity for DA D2 receptors (K_i = 200 nM) (100). The specific antiaggressive activity of befiperide could be a useful adjunct to its



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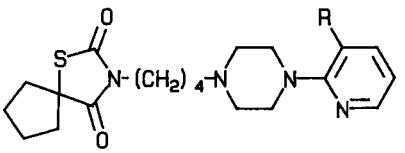

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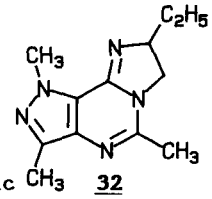
antipsychotic profile in treating agitated schizophrenics. The SAR in a series of BMY 13980 (**31**) (R = CN) derivatives defined a correlation between the electronic and lipophilic characteristics of the substituents on the pyridine ring with the desired biological properties of the lead compounds (101). A survey of various atypical antipsychotic agents and their pharmacological profiles has appeared (102).

CI-943 (**32**) has no binding affinity for dopamine, serotonin, or adrenergic receptors, but yet is active in a variety of *in vivo* paradigms predictive of antipsychotic activity (103). In addition, CI-943 does not induce dystonias in Cebus monkeys sensitized to the dystonic effects of

chronic haloperidol administration, and therefore may have a low liability to induce extrapyramidal symptoms in man. Tefludazine (±-33) is a structurally novel neuroleptic which dose-dependently decreases the activity of DA containing neurons in CNS, although at all doses its effects were more pronounced in the VTA area (104). Earlier studies indicate that the neuroleptic properties of racemic (33) reside in the 1R,3S enantiomer, while the 1S,3R enantiomer has DA and norepinephrine uptake inhibiting activity (105).



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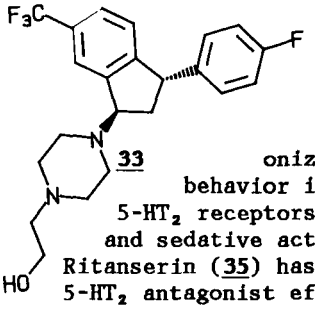

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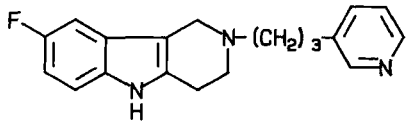
Wy 47,384 (34) is more potent in antagonizing apomorphine-induced climbing than stereotyped behavior in mice, and has only modest affinity for DA D2 and 5-HT_2 receptors (106). Wy 47,384 demonstrated some cataleptogenic and sedative activity, but lacked anticonflict effects (107,108). Ritanserin (35) has weak DA antagonist properties combined with potent 5-HT_2 antagonist effects. It was found to improve the negative and affective symptoms of schizophrenia, and to decrease neuroleptic-induced EPS (108). Risperidone (36) is also a mixed serotonin-dopamine antagonist with a relatively long duration of action in *in vivo* screens predictive of



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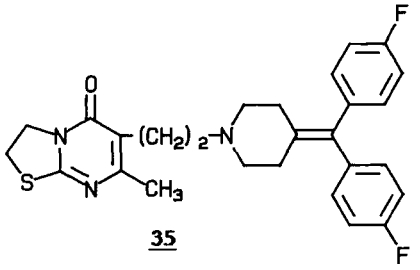

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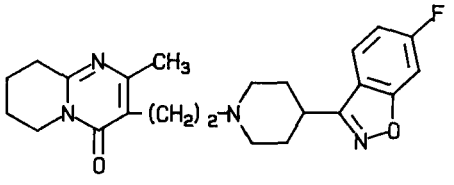
antipsychotic activity (109,110). Early clinical experience with risperidone suggests the compound may be effective against both the positive and negative symptoms of schizophrenia, and could be suitable for extrapyramidal symptom-free maintenance therapy (110). The SAR of a series of timelotem (37) derivatives is reported, and only the (+) enantiomer of this putative antipsychotic agent has potent *in vitro* affinity for the DA D2 receptor (111,112). Despite timelotem's benzodiazepine (Bz) structure, the compound neither binds to Bz receptors nor inhibits pentetrazole-induced convulsions.



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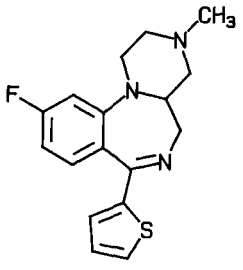

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Chapter 2. Antianxiety Agents and Anticonvulsants

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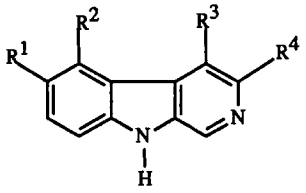
ANTIANXIETY AGENTS

Introduction - An estimated 3.5 million patients in the United States alone suffer from anxiety (1); while a clear understanding of the biological basis of this family of disorders remains elusive, current knowledge has been reviewed (2) and reports of differential effects of therapeutic agents on Generalized Anxiety Disorder (GAD) and Panic Disorder (PD) continue to appear (3). The range of drugs employed in anxiety therapy includes antidepressants, antipsychotics and antihistamines (4). The role of β-adrenoreceptor antagonists in anxiety was reviewed (5). A book on animal models (6) and a review on anxiety research (1) have appeared. A number of animal models detect both traditional benzodiazepine (BZ) and newer serotonin (5-HT) mediated agents (7-12). The conditioned defensive burying test (CDB), reported unreliable, was modified and has been reported effective (13).

BZ Receptor Mediation of Anxiety - Reviews have appeared on the behavioral effects of BZs (14), trends in BZ research (15), and the BZ agonist-inverse agonist continuum (16). Investigations into the structure and functional interrelationships of the BZ-GABA-Cl^- ionophore complex continue. A partial structure for the $GABA_A$ receptor was derived from the nicotinic acetylcholine receptor (17). Polyclonal antibodies specific for agonist BZs have been raised and may be useful for investigating the nature of agonist binding to the receptor complex (18). Effects on GABA-mediated Cl^- influx in rat brain synaptoneurosomes confirm BZ receptor modulation of Cl^- transport (19).

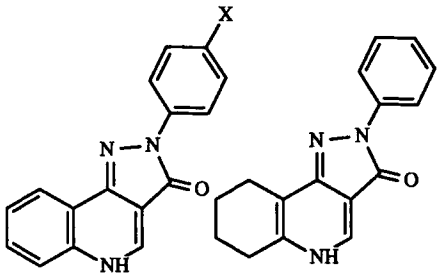
While the pharmacological significance of BZ receptor subtypes has not been demonstrated, the hypothesis that anxiolytic vs. sedative effects could be associated with BZ_1 and BZ_2 receptor activation, respectively, was examined (20). Modulation of [^{35}S]-TBPS binding by agents with different subtype affinities revealed that BZ_1 activation alone was sufficient for Cl^- channel activation (21). Renaming the BZ_1, BZ_2, and "peripheral" BZ_3 subtypes as ω_1-ω_3 was proposed (22). A "central" BZ agonist (anxiolytic) augmented uptake of NaCl solution in rehydrating rats; a "peripheral" agonist (non-anxiolytic) did not (23). A different EEG profile in animal neocortex was seen with agents selective for BZ_1 vs. non-selective agents (24). Development of tolerance and kindling appear to involve independent mechanisms (25). Clonidine's synergistic effect on BZ behavioral effects was evidence for noradrenergic (NA) system involvement (26). Interaction of BZs with the adenosine system was reviewed (27). Tracazolate and other pyrazolopyridines were more potent than theophylline at both A_1- and A_2-adenosine receptors (28). Full and partial agonists and pure antagonists were distinguished by microelectrode recordings on mouse spinal cord neurones (29).

BZ Receptor-Related Compounds - Results from clinical studies with metaclazepam (30, 31) and alprazolam (32) have appeared. Tolufazepam showed anticonvulsant and anxiolytic activity in animal models (33). A comparison of suriclone vs. diazepam (DZ) in neurotic anxiety reported both to be effective (34); single-dose comparison of their neurologic effects in normal volunteers was reported (35). First results from healthy subjects with the β-carbolines ZK 91296 (**1**), ZK 95962 (**2**) (partial agonists) and ZK 93426 (**3**) (antagonist) were reviewed (36). A review of the European clinical studies with alpidem has appeared (37). Zolpidem (BZ_1-selective) shows specificity for GABAergic function (38).



	R^1	R^2	R^3	R^4
1	H	OBn	CH_2OMe	CO_2Et
2	H	O-iPr	CH_2OMe	CO_2Et
3	H	O-iPr	Me	CO_2Et
4	OBn	H	CH_2OMe	CO_2Et
5	H	H	H	CONHMe

CGS 8216 (6) (antagonist/inverse agonist) decreased rate of responding to a fixed-interval schedule in squirrel monkeys (39). It was selective in precipitating only seizures without other signs of precipitated abstinence in DZ-dependent dogs (40) and produced withdrawal symptoms different from flumazenil's in DZ-dependent baboons (41). Pharmacokinetics of the anxiolytic BZ agonist CGS 9896 (7) in cynomolgus monkey was studied (42).



6 X = H

7 X = Cl

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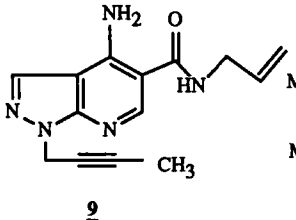

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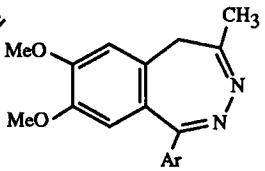
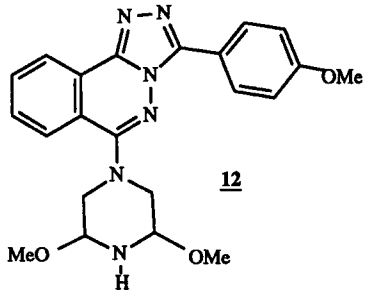


10 Ar = p-NH_2-C_6H_4

11 Ar = m-Cl-C_6H_4

ICI 190,622 (9) showed anxiolytic activity in conflict models; its *in vitro* profile suggested BZ_1 selectivity (43). The 2,3-BZs GYKI-52322 (10) (44) and GYKI 51189 (11) (45) showed anxiolytic activity in animal models. Triazolophthalazine 12 was active in the Vogel rat but inactive in anticonvulsant and antiaggression assays (46). Ketone 13 was orally active in a licking/conflict screen (47). Pipequaline (PK 8165, mixed agonist/antagonist) did not cause impairments in episodic memory in healthy volunteers (whereas DZ did); in combination, it did not antagonize any DZ effects (48).



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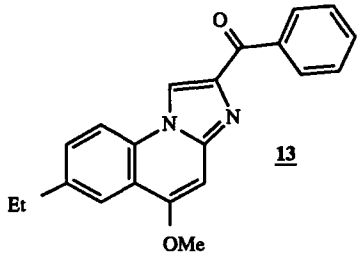

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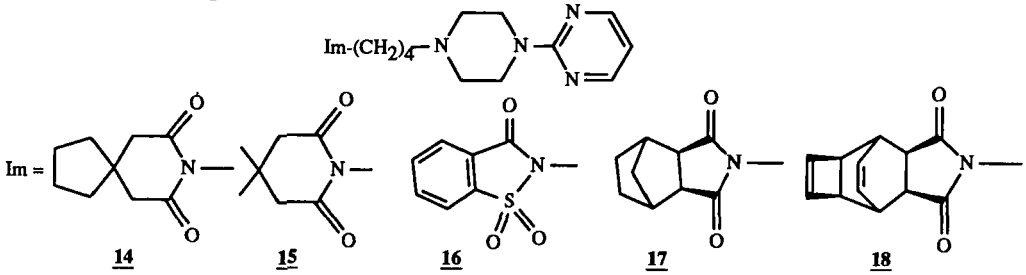
5-HT Receptor Mediation of Anxiety - Modulation of the 5-HT system in anxiolysis has been reviewed (49), as has the role of 5-HT reuptake inhibitors in PD (50). A computer-generated pharmacophore and 3-D map of the $5\text{-}HT_{1A}$ antagonist receptor site was reported (51). The role of 5-HT in regulation of β-adrenoreceptors was addressed (52) and interactions of 5-HT anxiolytics with the α-adrenergic receptor were examined (53). $5\text{-}HT_{1A}$ agonists increase plasma concentration of ACTH in rats (54).

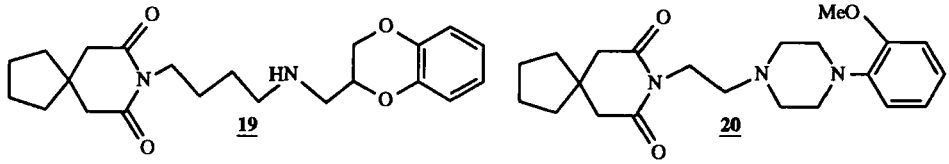
In studies of increased punished responding in pigeons, cerebrospinal fluid (CSF) samples showed decreased 5-HT metabolite levels with buspirone (BP, 14), gepirone

((GP, **15**), and 8-OH-DPAT (DPAT), but only BP increased dopamine (DA) metabolites (55). Also, BP showed DA-antagonist-like effects, decreasing unpunished responding in pigeons, while DPAT and GP increased responding (56). Discriminative cues (DC) produced by BP in pigeons generalized to 5-HT_{1A} compounds but not 5-HT_2 antagonists, although both increased punished responding (57). While BP produces a poor DC in rats, a DC for GP generalized to BP, DPAT and SM-3997 (**17**) (58). DCs to DPAT or ipsapirone (IP, **16**) in rats generalized to the α_2-antagonist yohimbine (59). Both BP and GP reduced the fear-potentiated acoustic startle response; the anxiolytic effects of BP in this model may not be 5-HT-mediated (60). In a learned helplessness model, BP, GP, IP and DPAT all eliminated escape failures (61).



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

5-HT Receptor-Related Compounds - Reviews have appeared on the prototypical 5-HT_{1A} anxiolytic BP (62,63). Clinical studies showed significant improvement in long-term treatment of GAD (64). BP was less effective in patients with a history of anxiolytic drug therapy (65). In rats, BP in combination with antidepressants was reported to induce myoclonic seizures (66). Actions of GP (BMY 13805) (67) and its clinical profile (68) were reviewed. Electrophysiological studies showed IP to be a 5-HT_{1A} antagonist with partial agonist properties (69). IP accelerated DA turnover and reduced 5-HT turnover (70). SM-3997, as potent as BP in rat water/lick conflict, has high affinity for 5-HT_{1A}, low for D_2 and 5-HT_2 and none for BZ, GABA, 5-HT_{1B} or adrenergic receptors (71). WY-47,846 (**18**) shows high affinity for 5-HT_{1A}, none for BZ, NA or opiate sites, and inhibits dorsal raphe firing (72). In conditioned avoidance procedures, it supressed avoidance and increased escapes; it lacks anticonvulsant activity, showed no anxiolytic activity in conflict models, but was weaker than DZ in producing sedation or ataxia (73).



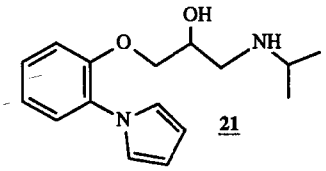
19 **20**

DPAT was active in animal models of anxiety (74). MDL 72832 (**19**) acts as a mixed agonist/antagonist at 5-HT_{1A} receptors. The (-)-isomer increased food intake 33-fold more potently than (+)-isomer (75). BMY 7378 (**20**) (5-HT_{1A} antagonist) enhances 5-HT transmission *in vivo* (76). The anxiolytic β-blocker isamoltane (CGP-361A, **21**) binds to 5-HT_{1B} receptors and increases 5-HT release from cortical slices (77). A clinical study showed comparable improvements with ritanserin (5-HT_2 antagonist) and lorazepam (78). Preclinical pharmacology of LY 281067 (**22**) showed a long-duration 5-HT_2 antagonist activity (79). Anxiolytic effects of 5-HT_3 antagonists zacopride (**23**) (80), odanserin (GR38032F, **24**) (81) and ICS 205,930 (**25**) (82) in animal models were reported.

Miscellaneous - Early Phase II results for enciprazine (WY-48,624, **26**), report no anxiolytic or sedative effects but possible improved goal orientation and self-control (83). ST-1112 (**27**) showed activity in animal models, and has no affinity for BZ, 5-HT_1 or 5-HT_2 receptors (84). (R)-(-)-NO-328 (**42**), a potent, non-competitive mixed type GABA-uptake inhibitor, was active in a modified Vogel assay, and does not generalize to a DZ cue (85). EGYT-3886 (**28**) was more active than DZ in the Vogel assay, and less so

in decrease of spontaneous locomotor activity (86).

L-pyroglutamic acid may be an endogenous anxiolytic; active in the Vogel assay, it was not antagonized by flumazenil nor did it change levels of 5-HT or 5-HIAA in rat cortex and hippocampus (87). NMDA antagonists were reported active in animal assays (11, 88). Corticotropin releasing factor (CRF) and amphetamine exaggerated the partial agonist effects of flumazenil (89). Cholecystokinin tetrapeptide (CCK-4) induced anxiety and panic attacks in healthy volunteers (90).



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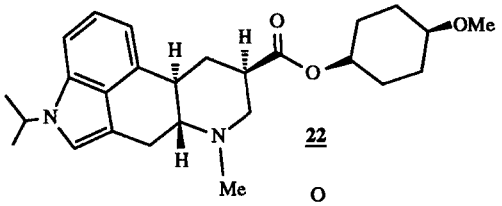

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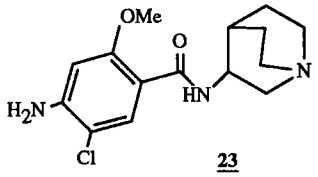

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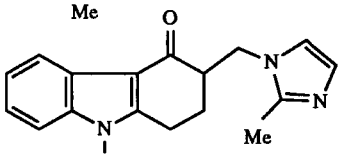

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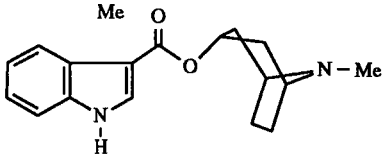

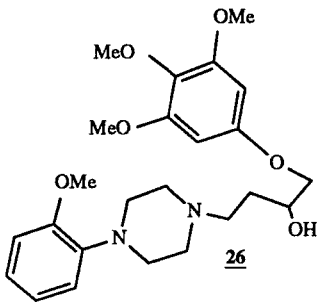

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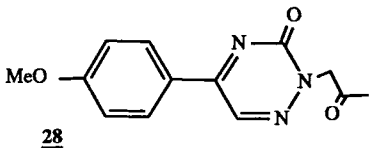

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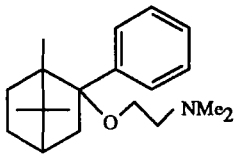
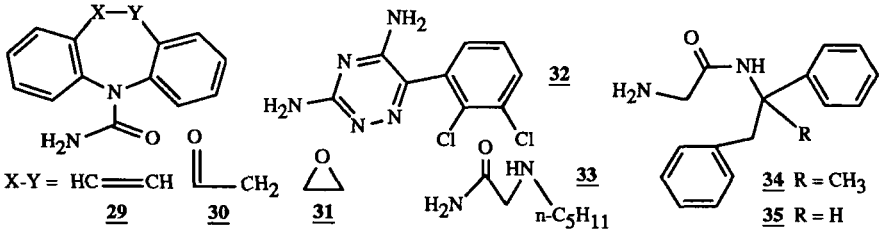


ANTICONVULSANTS

Introduction - Although seizure disorder therapy has progressed in the past decade, only approximately 60% of the patients are completely controlled by the available anticonvulsant (AC) drugs (91). Inadequate seizure control leads to increased, frequently changed dosages of multiple ACs, with an increased emergence of idiosyncratic and toxic effects (92).

Abstracts or proceedings of several meetings and symposia on epilepsy (93-97) appeared, and a number of books (98,99) were published. Numerous reviews describing the modern treatment of epilepsy (92), antiepileptics (100), new antiepileptic agents (101), the pharmacological basis of antiepileptic therapy (102), purinergic mechanisms in epilepsy (103), the comparative efficacy of AC drugs (104), the role of adenosine in the central actions of benzodiazepines (105), benzodiazepine interaction with adenosine systems (27), the cellular basis of epileptogenesis (106) and the cognitive hazards of seizure disorders (107) also appeared. Excitatory amino acids and their antagonists are discussed in chapter 5 of this volume.

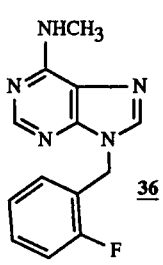
Highlights - Compounds Under Clinical Investigation - Addition of carbamazepine (29) to phenytoin (PHT) therapy resulted in significantly altered PHT pharmacokinetics (108), while the combination of 29 and phenobarbital (PB) had no advantage over each drug alone (109). Administration of oxcarbazepine (30) circumvents exposure to the epoxide (31) metabolite of 29, which may be partly responsible for dermatological and teratogenic side effects, and in a double blind crossover-designed trial, 30 was as effective as 29 with slightly better tolerability (including the disappearance of an eczema-like skin reaction) (110). Tolerance development limited the use of clobazam monotherapy (111), while marked therapeutic effects were noted with clobazam as adjunct medication in therapy-resistant patients (112). However, tolerance does not appear universally and some types of patients are more likely to have a sustained response to clobazam (113).



Denzimol and nafimidone prevented the epoxidation of 29, possibly by binding with hepatic cytochrome P-450 (114,115), and nafimidone markedly inhibited 29 and PHT elimination (116). Flumazenil, a BZ receptor antagonist, was clinically well-tolerated and produced significant AC effects, presumably by acting as a partial agonist at the "AC" receptor (117). The pharmacokinetic properties of lamotrigine (32) in normal volunteers were also reported (118). The antagonism of the milacemide-induced (33) increased seizure threshold to hyperbaric oxygen by l-deprenyl suggests that the conversion of 33 to the inhibitory neurotransmitter glycine is mediated by monoamine oxidase B and that the activity of 33 is mediated by glycine formation (119). The favorable oral efficacy/safety ratios and low toxicity led to Phase I clinical testing of PR 934-423 (34) and to preclinical evaluation of its optical antipodes (120). The preclinical profile of the des-methyl analogue PR 1013-708 (35) suggested utility for generalized tonic/clonic seizures (121).

A dual regimen of stiripentol and 29 may be synergistic, permitting lower doses of 29 to be employed (122). Valproic acid hepatotoxicity was reviewed (123), and a QSAR study of valproic acid, related carboxylic acids, and tetrazoles was reported (124). A glc-ms determination of valproic acid and five metabolites in CSF of epileptic children indicated that the role of active metabolites may be insignificant with respect to drug effect (125). In patients with complex partial epilepsy, vigabatrin elevated total CSF GABA levels, without any clear or consistent changes to cholinergic and aminergic neurotransmission or effect on cyclic nucleotides (126). However, negative feedback of highly elevated GABA levels may decrease the AC efficacy of vigabatrin and the choice of chronic dosage regimens is more critical with vigabatrin than other ACs (127). Clinical trials of the Ca^{2+} antagonists nifedipine and flunarizine as add-on therapy afforded significant reductions in seizure frequency (128,129), confirming many predictions based on preclinical AC activity of several Ca^{2+} antagonists. Corn oil successfully substituted for the more expensive medium chain triglyceride oil in the ketogenic diet, which is sometimes employed for intractable seizures in children (130).

Preclinical Highlights - BW A78U (36) emerged from a series of purines as an orally active nontoxic AC, which may be useful in disorders where PHT is indicated (131). CGS 9896 (7) and CGS 17867A (8) are BZ agonists, which markedly antagonize pentylenetetrazol-induced seizures (PTZ) without inducing tolerance, withdrawal symptoms and sedative/muscle relaxant effects (132). The AC profile, including the activity of 7 in a spontaneous absence model (133), indicates that 7 and 8 may be effective in absence-type seizures (132). The antagonism of kainic acid-induced seizures, in combination with a slower onset and longer duration of action than DZ, suggests that CL 218-872 (37) may be useful for the treatment/prophylaxis of status epilepticus (134).



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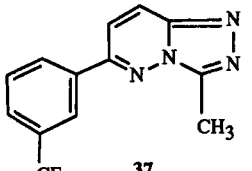

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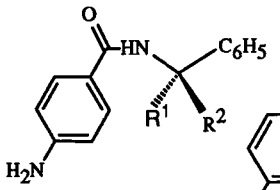
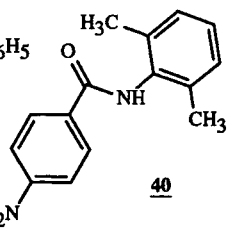


38 R^1=CH_3, R^2=H

39 R^1=H, R^2=CH_3



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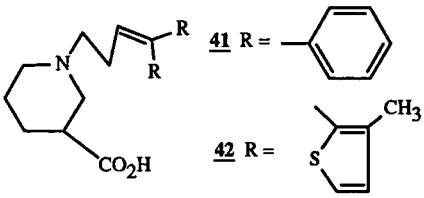
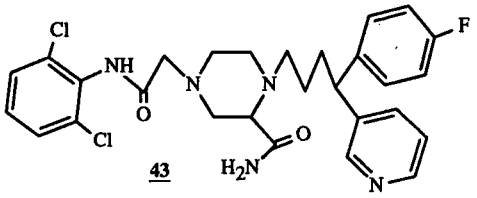
The ability of dextromethorphan to prevent the development of full kindling in rats, to decrease seizure intensity in fully kindled animals and its apparent safety in antitussive doses suggests that clinical AC testing is warranted (135). However, both *in vitro* and *in vivo* data suggest that the AC effects of dextromethorphan and dextrorphan (the major metabolite of dextromethorphan) are probably mediated by different mechanisms (136). The optical antipodes of LY188544, which displayed a profile similar to PHT and PB (137), were evaluated. Although LY188545 (38, S-isomer) was more potent than LY188546 (39, R-isomer) against maximal electroshock-induced seizures (MES), the overall results suggest that 39 is the antipode with a promising AC profile (138). LY201116 (ADD75073, 40) displayed potent efficacy against MES and was predicted to be effective against partial seizures and generalized tonic-clonic seizures (91,139). Desirable characteristics of 40 include good separation between doses producing AC effects versus neurological impairment, lack of tolerance development, and lack of effects on hexobarbital-induced sleeping times (91). In support of the hypothesis that BZ receptor partial agonists may induce less tolerance and/or dependence than full agonists, the AC protection against PTZ afforded by RO 16-6028 did not change significantly during a ten day treatment regimen (140).



41 R =

42 R =



43

GABA uptake inhibitors are of interest as potential ACs. SKF 89976-A (41) and related analogues are competitive inhibitors of both neuronal and astroglial GABA uptake (141). On oral administration, 41 inhibited various parameters of kindled seizure activity, with the d-isomer being more potent than the l-isomer intraperitoneally. The results suggest that 41 may prevent generalized seizure activity (142). The GABA uptake inhibitor 42 may also be useful in the treatment of epilepsy (143). BZ receptor ligands of the β-carboline class may provide a variety of beneficial drugs, including ACs (36). The full agonist ZK 93423 (4) and the partial agonists 1 and 2 suppressed spike and wave discharges in rats displaying spontaneous petit mal-like seizures, suggesting the involvement of the BZ-GABA receptor complex in the control of petit mal seizures (144). However, evaluation of 4 on amygdala-kindled seizures indicated that 4 has no advantage over BZs, in terms of AC potency, side effects, and tolerance development (145). Soluflazine (43), a specific nucleoside transport inhibitor in erythrocytes, may increase extracellular adenosine levels in the CNS and thus may be the prototype of a new class of ACs (146).

Mechanisms of Anticonvulsant Action - The correlations between animal models of epilepsy (MES, PTZ), proposed mechanisms of AC action, and clinical effectiveness for PHT, 29, valproate, ethosuximide and diazepam were reviewed. At least three mechanisms may be involved in the actions of ACs, including the reduction of sustained repetitive firing (SRF) at high frequency, enhancement of GABAergic synaptic transmission, and decreased neuronal excitability secondarily to blocking Na^+ and Ca^{2+} channels (147). The AC effects of PHT, PB, 29 and chlormethiazole probably do not

involve excitatory amino acids (148). The observation that the L-glutamate evoked release of [^{3}H]GABA from cultured avian retina cells is not dependent on the activation of excitatory amino acid receptors supports the concept that a glutamate-GABA exchange operates in the CNS, which may be important in regulating neuronal excitability (149). Ca^{2+}-dependent mechanisms in neurons are also affected by ACs. PHT blocks type I Ca^{2+} channel currents, which could suppress an important component of the inward current that underlies epileptiform cellular bursting, thereby limiting the spread of seizure activity (150). The development of tolerance to DZ was not affected by chronic treatment with the partial inverse BZ-agonist FG 7142 (**5**), suggesting that kindling by **5** and tolerance to DZ depend on different mechanisms (151). Adenosine, hypothesized as an endogenous AC, may be more generally viewed as being involved in neuroprotection during seizures, ischaemia and other metabolic insults (152). N^6-L-phenylisopropyladenosine (L-PIA) prevents spreading of penicillin-induced epileptic activity, supporting the hypothesis that adenosine regulates seizure spreading (153). The enhancement of the protective effect of L-PIA on caffeine-induced convulsions by an ineffective AC dose of **29** suggests that the AC properties of **29** may be partially explained by an influence on the purinergic system (154). Antagonism of electroshock-induced seizures by intranigral vigabatrin requires α_2-mediated noradrenergic transmission, which does not involve a drug-induced increase of noradrenergic activity (155). Sympathomimetic agents enhanced the AC effectiveness of PB, PHT and **29**, further supporting the view that the noradrenergic system may play an essential role in inhibiting seizure activity (156). CSF taken from MES-treated rats caused significant elevations in seizure thresholds (naloxone reversible) in naive recipient rats, which was attributed to a heat- and trypsin-sensitive opioid peptide(s) (157).

Seizure Models - Two reviews of models utilized in the search for new ACs were published (158,159), and the relevance of kindling to human epileptogenesis was discussed (160). NMDA receptors also become more progressively involved during the development of kindling (161,162). The genetically epilepsy prone rat, an important and increasingly utilized model of genetically-determined epilepsy, was reviewed (163). Epileptic gerbils appear to predict AC efficacy of GABAmimetic drugs better than other available models (127). A model of human secondarily generalized convulsive status epilepticus was described (164), and an *in vitro* model utilizing rat frontal cortex slices for the study of epileptiform activity was validated (165).

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Chapter 3. Antidepressant Agents

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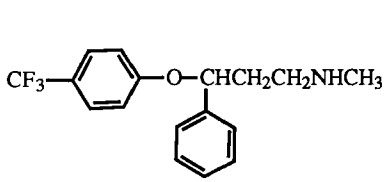
Introduction - Depression and related affective disorders remain one of the largest mental health problems in the U.S. with nearly 5% of the adult population experiencing a depressive episode of such severity and duration in their lifetime to warrant antidepressant drug therapy (1). The development of more efficacious and safer antidepressants offers new opportunities to provide therapy for a large number of untreated individuals as well as treatment resistant patients (2). Important advances in the field of antidepressant research have occurred since the area was last reviewed in this series (3). This progress is detailed in several recent reviews which cover various aspects of depression and antidepressant research (4-8). The milestone in antidepressant research this past year, however, is the successful introduction of fluoxetine, the first of the selective serotonin (5HT) uptake inhibitors to reach the U.S. market. The full therapeutic potential of the 5HT uptake inhibitors remains to be defined by expanding patient experience with fluoxetine and the market introduction of newer, and perhaps superior, members of this class. The following review highlights the recent advances in antidepressant discovery and development for each mechanistic class of agents.

Monoamine Uptake Inhibitors - During the past decade many compounds have been described as selective inhibitors of 5HT uptake relative to their effects on the uptake of dopamine (DA) or norepinephrine (NE). This effort has led to the successful development of selective serotonin reuptake inhibitors (SRIs) as antidepressants (4, 9-12). The strategy of employing drugs with selective effects on 5HT uptake is supported by a large body of literature indicating that brain 5HT plays a pivotal role in affective disorders (4,13). However, published reports during the past two years largely have focussed on those agents that have progressed to advanced stages of clinical development and registration. As an example, the biology and efficacy of fluoxetine (1) have been extensively reviewed (4,14-18). The results with fluoxetine seem to characterize the SRIs as a whole; efficacy equivalent to tricyclic antidepressants (TCAs) but often with better toleration because of a



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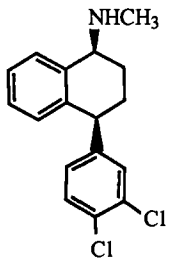

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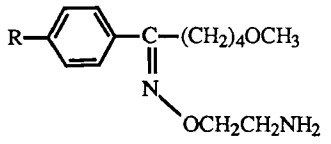
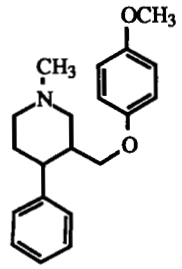


3 R= CF_3
4 R= Cl

different spectrum of side effects (4)(12). Thus the SRIs produce nausea, dizziness and nervousness as typical side effects but not the pronounced sedative, anticholinergic or cardiovascular effects observed for TCAs. The advantage offered by fluoxetine, therefore, relates to a more benign side effect profile, which makes it a more useful agent for some depressed patients (4,19). The X-ray structure of fluoxetine has been determined (20) as

have the absolute configurations of its optical isomers (21), both of which are pharmacologically active. There are a number of other SRIs in advanced stages of development. Sertraline (2) has proven to be a well tolerated and efficacious antidepressant in a number of trials and its preclinical biology has been reviewed (22,23). The superior potency of sertraline versus fluoxetine in various animal tests is not evident in the clinical data published to date. Although minimal doses for effective antidepressant therapy have not been extensively studied for SRIs, recent data for fluoxetine suggest that efficacy may be seen at surprisingly low doses, 5 - 20 mg/day (24). Fluvoxamine (3) was found to be effective against both endogenous and neurotic depression with comparable efficacy to oxaprotiline (14) , a specific blocker of NE uptake (25), and equally effective with imipramine in a separate trial (26). Replacement of the fluorine in fluvoxamine with chlorine yields clovoxamine (4), which loses both potency and selectivity as a SRI but retains some antidepressant activity (27,28). Equivalent efficacy was reported for femoxetine (5) and imipramine in a controlled study with better toleration and less toxicity associated with femoxetine (29). Paroxetine (6), one of the more potent 5HT uptake inhibitors *in vitro* (30), has shown antidepressant activity in a controlled clinical trial with imipramine (31). Like femoxetine, but unlike other SRIs, paroxetine exhibits moderate affinity for muscarinic receptors *in vitro*, although there seems to be no evidence for anticholinergic activity *in vivo* at doses relevant to antidepressant activity (14). Citalopram (7) and ifoxetine (8) are additional examples of SRIs being evaluated as treatment for depression (32,33). Tianeptine (9) is an interesting compound with antidepressant activity thought to be related to increased rather than decreased 5HT uptake (34).



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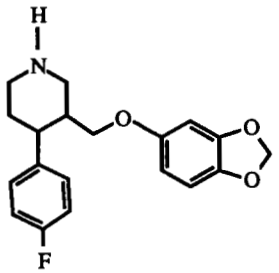

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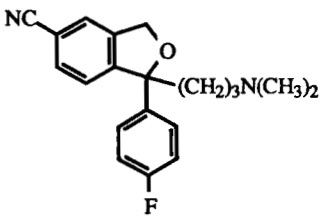
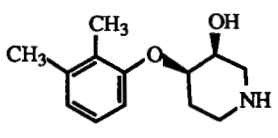


7

More recently, selective 5HT uptake inhibition has been found with new structural classes. A series of alkyl-3-piperazinoisoquinolines with SRI activity has been reported, of which 10 and 11 are the most potent (35). The naphthylpiperidine SL 81.0385 (12) has been shown to be a very potent and selective inhibitor of 5HT uptake in rat brain synaptosomes and human platelets (36). A number of potent uptake inhibitors have been reported in a series of pyrroloisoquinolines, some of which (e.g., 13) show a limited degree of selectivity for the 5HT uptake system (37).



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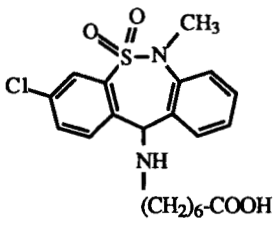

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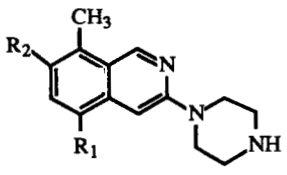
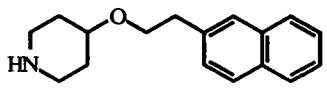


10 $R_1 = H$, $R_2 = CH_3$
11 $R_1 = CH_3$, $R_2 = H$



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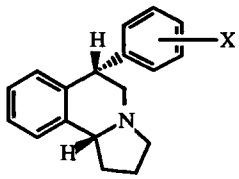
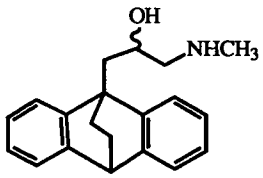


13 X= p-SCH_3
18 X= o-Cl

In addition to providing an alternative to treatment with conventional TCAs, the potent and selective SRIs have been useful tools for mechanistic studies aimed at elucidating the biological basis for antidepressant efficacy and at securing a better understanding of the etiology of depression. Tritiated cyanoimipramine, paroxetine and citalopram are superior to [^{3}H]-imipramine for labelling 5HT uptake sites in brain (38-40). Results obtained with these radioligands have questioned the validity of the two site (transporter and allosteric modulator) model originally proposed to account for the binding of [^{3}H]-imipramine. Studies have examined chronic versus acute effects of SRIs and discovered adaptive changes in neuronal systems that may account for the latency to therapeutic effect seen in the clinic. Over a period of weeks, SRIs appear to produce desensitization of 5HT autoreceptors (41) and downregulation of $5HT_2$ (42) and ß-adrenergic receptors (43,44), although there is lack of consensus with regard to the latter finding (45). In the case of $5HT_2$ receptor down regulation, it is interesting to note that sertraline altered receptor-second messenger coupling without producing an actual change in receptor affinity or number (43). Indirect modulation of noradrenergic neurotransmission by SRIs is of considerable interest for understanding their antidepressant activity and this concept may provide a mechanistic link between SRIs and the nonselective TCAs (46).

Perhaps the most exciting findings with the SRIs are their potential benefit in psychiatric disorders beyond depression. It is a consistent observation that SRIs produce positive results in treating obsessive compulsive disorder, whereas conventional TCAs are relatively ineffective (47). SRIs have been found to inhibit feeding and promote weight loss in laboratory animals through a serotonergic mechanism (48-51). These data are consistent with clinical observations that SRIs produce body weight loss, in contrast to TCAs which cause weight gain (52,53). Studies on the use of SRIs in treating non-depressed obese patients with serious health risk have been initiated (54,55). Animal studies with SRIs also have suggested a potential use for treating alcoholics (56,57), but this hypothesis lacks clinical validation.



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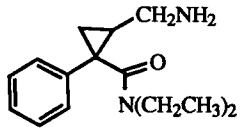

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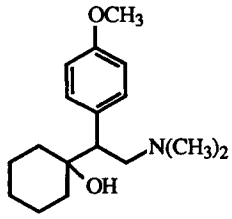
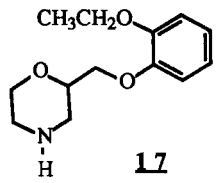


16

While the major focus of research on uptake blockers has been directed toward SRIs, a smaller effort continues in the area of catecholamine uptake inhibitors. Clinical investigation of the antidepressant properties of oxaprotiline (14), midalcipran (15), venlafexine (16), and viloxazine (17) seek to distinguish these newer agents from the traditional TCAs. Preclinical biology has suggested antidepressant activity for the combined 5HT-NE uptake

inhibitors McN-5707 **18** (58) and sibutramine **19** (59), the latter claimed to induce a potent and rapid down regulation of ß-adrenoceptors in brain (60).



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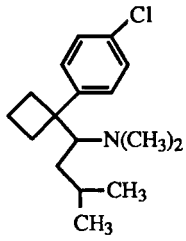
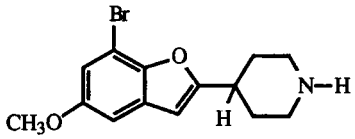


19

Monoamine Oxidase Inhibitors - Although one of the oldest classes of antidepressants, irreversible MAO inhibitors such as phenelzine and tranylcypromine have had limited use due to a severe hypertensive crisis following ingestion of tyramine rich foods, the so-called "cheese reaction." Research in the basic biology of MAO over the past few years has been extensive, affording new insight into the two main enzyme subtypes (MAO-A and MAO-B), substrate selectivity, and tissue distribution. Recent reviews have appeared which cover the current biological understanding of MAO and the role of MAO inhibition in the treatment of depression (61,62). Selective and reversible MAO inhibitors have also been recently reported which offer excellent tools for studying the full therapeutic potential of MAO inhibitors in depression. Current research now suggests that reversible and selective inhibition of MAO-A will afford antidepressant activity without hypertensive side effects. The efficacy of MAO-A inhibitors over that of MAO-B inhibitors is also reported for newer animal models such as the rat DRL 72-s model (63). The reversible MAO-A inhibitors brofaromine (CGP11,305A **20**) and moclobemide (**21**) are reportedly in human trials. Brofaromine has shown reduced side effects compared to tranylcypromine (64,65) with comparable efficacy in drug resistant depressed patients (66) while moclobemide also showed efficacy and improved safety in double blind trials in major depression (both unipolar and bipolar disorder) (67,68). The phenylpyridazine SR 95191 (**22**) is reportedly active in animal models of depression and has recently, contrary to *in vitro* results, shown to be a reversible MAO-A inhibitor *in vivo* (69). Interestly, SR 95191 also possesses dopamine stimulant properties, a profile distinct from other known selective MAO-A inhibitors (70,71). BW A616U (**23**) has also been reported to be an extremely potent (Ki = 16 nM) and selective inhibitor of MAO-A (72).



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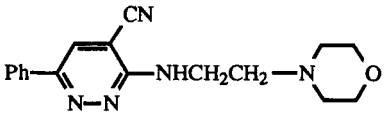

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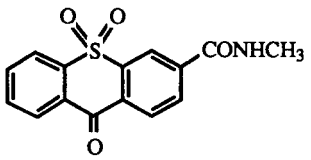
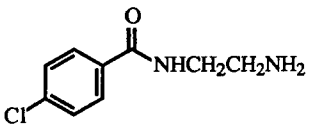


23

Despite the lack of positive clinical results with MAO-B inhibitors as antidepressants, research has continued in investigating the neurochemical role of MAO-B. Ro 16-6491 (**24**), a potent and reversible inhibitor (73) of MAO-B, has been used as a radioligand for tissue distribution studies (74) and, under reductive conditions, as an affinity label for MAO-

B in human brain and platelets (75). A series of aryl oxadiazinones are reportedly MAO-B inhibitors with **25** the most selective with Ki values of 0.15 and 11 uM for MAO-B and MAO-A, respectively (76). The excitotoxin quinolinic acid (**26**) also inhibits MAO-B selectively and reversibly but with a Ki > 1 uM (77). The SAR of MAO-B inhibitors modelled around deprenyl has also been reviewed (78).



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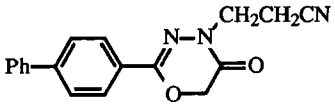

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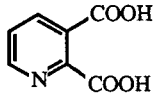
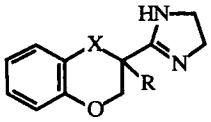
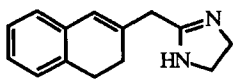


26

α–2 Adrenoreceptor Antagonists - The search for selective presynaptic α–2 antagonists has been quite active since the recent reviews on the SAR and clinical results of this class appeared (79-81). Idazoxan (**27**) continues to be extensively studied for selective α–2 antagonist behavioral effects in animals (82-85) and several reports of potential antidepressant compounds related to idazoxan have appeared including the thio analog (**28**), an overall weaker α–2 antagonist (86), and the resolved 2-substituted idazoxan analog (**29**) (87).



27 R=H, X=O
28 R=H, X=S
29 R=OCH_3, X=O



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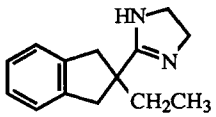
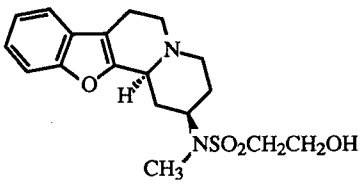


31

A series of arylimidazolines with potent α–2 affinity have been synthesized, incorporating the structural features of the agonist naphazoline and idazoxan (88). The preferred compound, napamezole (**30**), has a Ki of 23 nM in standard α–2 receptor binding, however its affinity is only 3.4 fold selective for α–2 over α–1 receptors (compared to 26 fold selectivity for idazoxan). A related compound, atipamezole (**31**) has been reported to be a potent α–2 antagonist with a ratio of 8500 for α–2/α–1 and overall claims of rapid onset of action (89,90). L-654,284 (**32**) has been reported to be an orally active, potent α–2 antagonist with a Ki of 0.8 nM (^{3}H-clonidine) and >100 fold selectivity over that for α–1 sites (91,92). L-654,284 is more potent and selective for α–2 receptors than the structurally similar series of benzoquinolizines such as WY 26703 (**33**).



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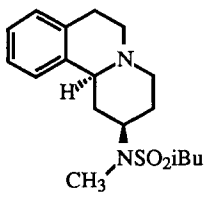
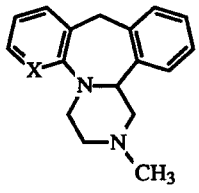


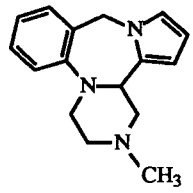
33

Mianserin (**34**), one of the earliest clinically effective antidepressants from this class, has served as a structural prototype distinct from idazoxan for developing new α–2 antagonist SAR. Org 3770 (**35**) is a potent α–2 antagonist with activity residing in the (+) isomer, a finding common to mianserin itself (93). Org 3770 reportedly enhances nocturnal

melatonin secretion in man, an effect that may serve as a clinical marker for α–2 antagonists (94). The fused pyrrole analog aptazepine (CGS-7525A **36**) is overall more potent than mianserin and may offer 10 fold more potency *in vivo* (2).

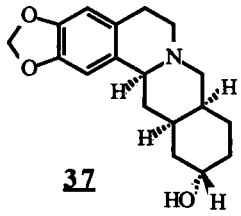


34 X= CH
35 X= N



36

New α–2 antagonists have been developed from the tetracyclic berbane class (95). The berbane (**37**) was the most selective α–2 antagonist with an α–2/α–1 affinity ratio of 1659. Substituent and stereochemical SAR for berbine (**38**) has also been reported (96). Finally, Org 6906 (**39**) has been reported to possess potential antidepressant activity with combined potent and selective α–2 activity and monoamine uptake inhibition (97).



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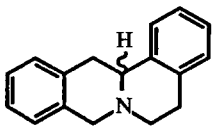

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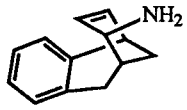
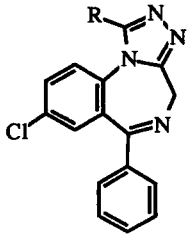
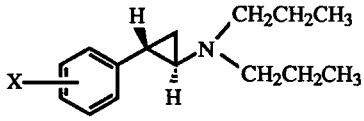


39

Benzodiazepines - The field of benzodiazepine research has been reviewed with only a brief note of the potential of this class as antidepressants (98). Results from 4 of 6 double blind clinical trials showed alprazolam (**40**) to be as effective as tricyclic antidepressants, but with



40 R= CH_3-
41 R= $(CH_3)_2NCH_2$-



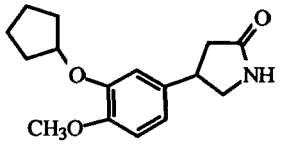
42 X= o-OH
43 X= m-OH

fewer side effects and greater tolerance (99). The success of **40** as both an anxiolytic and an antidepressant has spurred the clinical advancement of another triazolobenzodiazepine, adinazolam (**41**) (6).

5HT1A Agonists - Buspirone, gepirone, SM-3997, and ipsapirone represent a new class of non-benzodiazepine anxiolytics (see chapter 2 of this volume) which have also demonstrated promise as antidepressants. Early clinical reports of an open trial in major depression showed gepirone to have a significant overall antidepressant effect, with better efficacy in nonmelancholic patients (100). Gepirone is a partial 5HT1A agonist which potently inhibits serotonin cell firing, downregulates $5HT_2$ receptors and lacks the cholinergic side effect profile of conventional tricyclics (101). Buspirone and ipsapirone are also reported to be

effective in a stress-induced antidepressant model (102). Antidepressant activity for this class may be due in part to the potent α–2 adrenoreceptor antagonist activity of their common metabolite, 1-(2-pyrimidinyl)piperazine (103-105). A series of arylcyclopropyl amines, structurally related to known MAO inhibitors, has yielded **42** and **43** which lack MAO inhibition but demonstrate potent 5HT1A agonist activity. Interestingly, all 5HT activity resides in their 1R, 2S enantiomers (106).

Phosphodiesterase Inhibitors - Further reports on rolipram (**44**), the prototype of this new class of antidepressants, have been encouraging. Rolipram acts as an antidepressant by enhancing norepinephrine synthesis and release and by elevating cAMP levels through the inhibition of calcium independent phosphodiesterase. The effect of rolipram on cAMP appears to be stereospecific for the (-) isomer (107). Rolipram also reportedly lacks cholinergic activity as shown in three animal models (108) and in pilot safety trials in healthy elderly patients (109). Rolipram has shown antidepressant activity in open trials (110,111) and efficacy equal to amitriptyline (112) and superior to placebo (113) in two double-blind studies.



44

New Opportunities - The potential for new therapies of depression and affective disorders has emerged from research around lithium, corticotropin-releasing factor (CRF) and S-adenosyl methionine. Recent research suggests that lithium's effectiveness in mania and depression may be due to its selective inhibition of the inositol phosphate pathway and the overall suppression of muscarinic cholinergic responses mediated by the PI pathway (114-117). CRF, a hypothalamic hormone which modulates ACTH secretion from the anterior pituitary gland, has now been shown to be a primary mediator of the endocrine, autonomic and behavioral responses to stress (118,119). Additional compelling evidence suggests that CRF may actually be a causative factor in depression as evidenced by its hypersecretion in in the CNS of depressed patients and the apparent accompanying downregulation of CRF receptors in frontal cortex (120). The mood elevating effects of S-adenosyl methionine (SAMe) in depressed patients and its known pharmacological actions have been reviewed (121). Recent trials suggest that SAMe has antidepressant activity superior to placebo (122) and, in a double-blind trial of two weeks, efficacy equal to imipramine (123). Finally, in a broader context, it is worth noting that, to date, no antidepressant drug has been shown to be superior to electroconvulsive shock therapy (ECT) in the treatment of severe, suicidal depression (124).

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Chapter 4. Central Muscarinic Ligands and Receptors

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Introduction - Since the late seventies, it has generally been accepted (1-3) that there exist only two major categories of muscarinic receptor (MR), M_1 and M_2. However, recent experience with selective antagonists has raised the question of heterogeneity within the M_2 subtype (4-8). Over the last 18 months, molecular biological studies have revealed the presence of at least five gene products each of which encode for a distinct MR subtype. All are located in the brain. Much has been deduced regarding the structure and function of individual MR's both from direct mutagenesis studies and from data recorded with related receptors. This report will review these findings and also discuss the medicinal chemistry and biology of new ligands which offer comment on our understanding of MR classification and activation processes.

The peripheral actions of selective ligands (9) and their use as tools in receptor classification (10) have been discussed. General overviews of G-protein linked receptors have recently been presented (11,12). The evolution of neurotransmitter receptors including MR's highlighted the close homology of these systems (13-15). The proceedings of the 3rd International Symposium on MR subtypes were published (16). Three surveys on the use of MR agonists in cognitive disorders were presented (17-19).

MR Subtypes - Molecular biological techniques have furnished cloned porcine MR (mAChR I, II and III) which correspond to the pharmacologically defined M_1, M_2 ($M_{2\alpha}$), and M_3 ($M_{2\beta}$) receptors, respectively (9,20). Contemporaneously, two other groups analyzed human (21) and both human and rat (22) genomic clones which indicated that there exist at least four to six functional MR genes. The fourth receptor (called mAChR IV here to avoid confusion caused by the nomenclature of m4 (22) and HM3 (21) in the original reports), together with mAChR I and III more closely resembled M_1 in their binding profile and would probably have been previously classified as such. In situ hybridization revealed the presence of mAChR IV mRNA in cerebral cortex and hippocampus but its correspondence with functional peripheral receptors remains to be determined. Further analysis of the genomic blots has led to the isolation (23) of a fifth MR, mAChR V, which is closely related to mAChR III when expressed in mammalian cells. However expression of this mRNA in brain has yet to be observed. The distribution of mRNA's in rat brain has also been mapped (24,25) and the occurrence of mAChR III selectively in the cortex has raised speculation that an agonist for this subtype should produce the cognitive enhancement anticipated for cholinergic agents in AD with minimal side effects (25).

Receptor Effector Coupling - Electrophysiology and an antagonist binding profile of 4-DAMP > pirenzepine (PZ) > AF-DX 116 showed (26) that the endogenous MR in Xenopus oocytes is mAChR III and is linked to the stimulation of phosphatidyl inositol (PI). More interesting, however,

is the expression of homogeneous MR subtypes in stable cell lines lacking an endogenous MR to study specific effector systems (27-31). As a result, the conceptually appealing view (32) that distinct receptor subtypes are coupled to a single effector system has been replaced by an acceptance of promiscuity with several G-proteins.

It has been shown (27) that the porcine atrial receptor, mAChR II, when expressed in CHO cells and activated with carbachol, both inhibited adenylate cyclase and stimulated PI hydrolysis. Although the latter response was significantly less efficient, it remained unimpeded by concentrations of pertussis toxin sufficient to abolish the cyclase response, suggesting that these responses were transduced by different effector proteins, G_i and G_p. Extension of this work to all four cloned MR (28,29) revealed that mAChR I and III efficiently mediated the hydrolysis of PI and did not inhibit cyclase, whereas mAChR II and IV showed the reverse behaviour. At relatively high agonist concentrations however, I and III mediated an atropine sensitive increase in cAMP either through direct interaction with G_s or through the secondary effects of PI stimulation. In contrast, there was no evidence for mAChR III influence on cyclase activity in RAT-1 cells (30). Finally, mAChR I expressed in RAT-1 cells (31) was able to promote cyclase inhibition and, less efficiently, PI hydrolysis through different endogenous effector proteins.

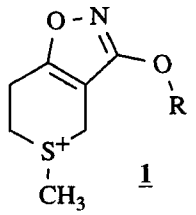
Electrophysiological and fluorescent dye studies (33-36) in A9L and NG107-15 cells have also been used to characterise the cloned receptors. Stimulation of mAChR I and III led to calcium release from intracellular stores and activation of a Ca^{2+}-dependent Cl^- current consistent with a PI involvement. mAChR II and IV principally induced activation of Na^+ and K^+ currents in a Ca^{2+}-independent manner. Using similar techniques, mAChR III was shown (37) to be 10-fold more sensitive to agonists than mAChR I. On the basis of a correspondence of EC_{50}'s in stimulating PI hydrolysis in partotid gland and cerebrum, it was suggested that the mAChR III receptor predominantly contributes to the former response while mAChR I is mainly involved in cerebral activation (37).

Receptor Structure - Site-directed mutagenesis of the MR remains to be published and much of our current knowledge is derived from an understanding of homologous proteins, particularly the beta receptor (11). Key experiments have identified the primary site(s) for ligand interaction and the location of regions involved in effector coupling. For the beta receptor, the anionic site which interacts with the cationic head group of the ligand had already been shown (38) to be an aspartic acid (ASP) residue buried within the membrane spanning domains of the protein (ASP-113 for beta). More recent evidence (39-41) has implicated two other ASP residues located in the same binding groove but having differing roles in agonist and antagonist binding. Since these ASP's are conserved throughout the MR subtypes, the corresponding residues (ASP's 105, 71 and 122 respectively) are candidates for the quaternary ammonium counterion. These results have been interpreted in terms of distinct binding sites for agonists and antagonists, a view which is supported by a binding model derived from SAR in a series of novel muscarinic ligands (42). Analysis of ACh induced current responses and antagonist binding properties of chimaeric mAChR have indicated (43) that the region adjacent to the carboxyl terminus of transmembrane domain V and the region adjacent to the amino terminus of VI are responsible for selective coupling of the MR with different effector systems. Thus, the mAChR I mutant having the V-VI loop

replaced by that loop present in mAChR II displayed a typical mAChR I binding profile whilst behaving functionally as an mAChR II receptor. In confirmation of the homology within this class of proteins, almost identical results have been achieved (44,45) with the beta receptor and rhodopsin (46).

Chemical studies have supported the functional role of ASP residues on the MR. The affinity label [^{3}H]-propylbenzylcholine mustard alkylated a carboxylate group (47,48) which was shown by peptide mapping to reside within the second and/or third putative transmembrane domain. The high pK_a of this group (6.2) and the ability of dicyclohexyl-carbodiimide but not a water soluble diimide to alkylate the receptor point to a hydrophobic environment for the ASP consistent with its buried location within the helical core of the receptor. The methylating reagent trimethyloxonium tetrafluoroborate (49) and EEDQ (50) also modified ligand binding to rat cortical and atrial receptors which could be protected by the presence of atropine or carbachol. Since the redox state of thiol groups present at, or near to, the binding site also influences the affinity of the MR for ligands (51), a thiol group is a candidate for this role. Irreversible inhibition of rat forebrain and cardiac receptors by the tyrosine directed alkylating agents (52) suggested the existence of a tyrosine residue group at the ligand binding site.

Muscarinic Agonists - The interest in the possible application of cholinergic agonists in the treatment of cognitive disorders such as Alzheimer's disease has stimulated substantial activity in the chemistry and properties of muscarinic agonists. Simple binding assays using [^{3}H]-N-methylscopolamine (NMS) and [^{3}H]-oxotremorine-M (Oxo-M) have now been developed both to measure affinity and to predict the relative efficacy of muscarinic agonists in the rat cerebral cortex (53,54). The ratio of the affinity constants of the two assays correlated with the ability of the agonists to stimulate cortical phosphatidyl-inositol turnover. A similar approach using [^{3}H]-quinuclidinyl benzylate (QNB) and [^{3}H]-cis-methyldioxolane binding has also been reported (55). Radioligand binding assays require cautious interpretation, since it is clear that muscarinic agonists can distinguish between tissues on the basis of receptor sensitivity and by the recognition of high and low agonist affinity states (56).



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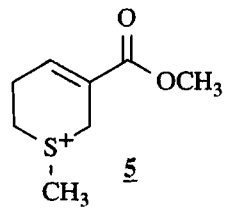

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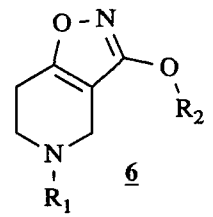


6

2 R = C_2H_5 3 R = $CH(CH_3)_2$

4 R = $CH_2C{\equiv}CH$

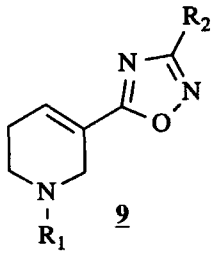
7 $R_1 = R_2 = CH_3$

8 R_1 = H; $R_2 = CH_2C{\equiv}CH$

The aim of much of the medicinal chemistry in this area was to discover compounds having differing levels of intrinsic cortical efficacy starting from arecoline. A series of bicyclic sulphonium arecoline biosteres 1 have been prepared and their efficacy ("M-agonist index") and selectivity ("M-2/M-1 index) estimated (57,58). A range of efficacy was observed with 2 being an antagonist whereas 3 and 4, for example, were non-selective partial agonists. None

were able to penetrate the blood brain barrier to a significant degree. Sulfoarecoline 5 itself acted as a full agonist suggesting that the isoxazole moiety reduces efficacy relative to the ester group. Along similar lines, examination of a series of isoxazoles 6 of arecoline and norarecoline showed that both 7 and 8 had reasonable efficacy but lower than that of arecoline. These two compounds have the advantage of readily penetrating into the CNS (58).

A series of oxadiazole derivatives 9 of arecoline having agonist properties has appeared in the patent literature (59,60). The direct analogue 10 proved to have higher receptor affinity but marginally lower efficacy than arecoline (42). Of a number of O-substituted tetrahydropyridine-3-carboxaldehyde oxime derivatives (61,62), the methyl derivative 11 was over 50 times more potent than arecoline in the hypothermia model. The acetyloxime 12 (CI-969) stimulated cortical PI turnover to 62% of the maximum and reversed the scopolamine-induced hyperactivity swimming activity in rats (62). A number of tetrahydro-3-pyridine-carboxylic acid derivatives having a bulky, preferably phenyl, substituent in the 4-position (13) reversed amnesia in rats produced by electroconvulsive shock treatment (67).



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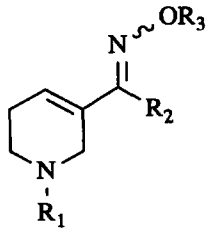
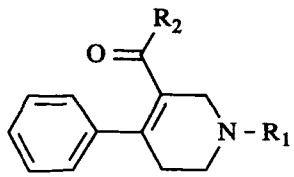


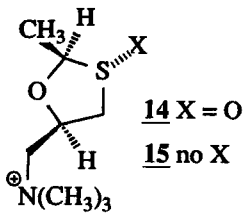


13

10 $R_1 = R_2 = CH_3$ 11 $R_1 = R_2 = H$; $R_3 = CH_3$

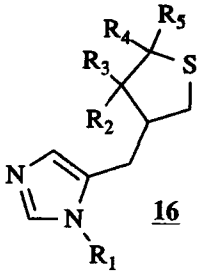
12 $R_1 = R_2 = CH_3$; $R_3 = COCH_3$

Muscarine-like molecules have been prepared and their biological profiles examined. The (-)-(2R,3R,5R) enantiomer of c-2-methyl-r-5-[(dimethylamino)methyl]-1,3-oxathiolane t-3-oxide methiodide 14 has been shown to be a selective and potent muscarinic agonist (64). The absolute configuration of this enantiomer is identical to that of L-(+)-muscarine (2S,3R,5S). The analogous 1,3-oxathiolane derivative 15 displayed comparable enantioselectivity (65). A series of thiopilocarpine derivatives 16 and a furanylimidazole 17 have been claimed to act on cholinergic receptors (66,67).



14 X = O

15 no X



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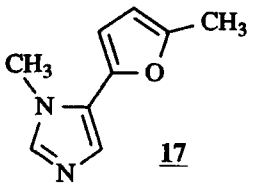
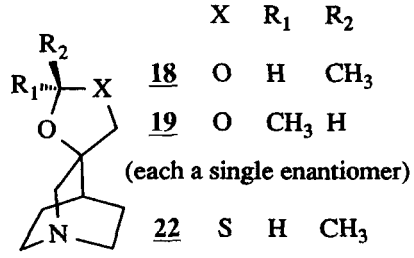
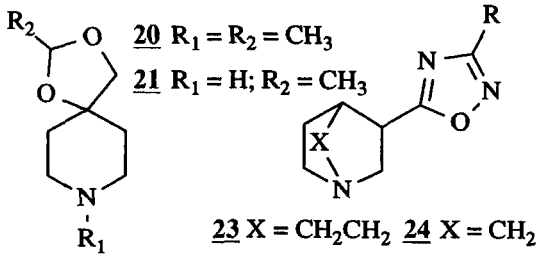


17

Rigid analogues of acetylcholine based on the spiro-dioxolane AF-30 have continued to be studied. The four isomers of this muscarinic agonist have been synthesised and their absolute stereochemistry

assigned (68). Biological assays indicated that whilst the (3R,2'S) isomer 18 was the most potent in binding studies, the (3R,2'R) isomer 19 displayed the largest functional selectivity between the ganglion (M_1) and the heart (M_2). Two azaspirodecanes 20 and 21 with greater conformational freedom than AF-30 have been prepared and shown to have greater predicted efficacy than this latter compound (53). AF102B 22 has been claimed to be more selective at the M_1 compared to M_2 receptors and to reverse cognitive deficits without peripheral side effects (69).



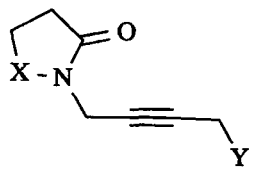


23 X = CH_2CH_2 24 X = CH_2

25 X = CH_2; R = NH_2

Oxadiazole derivatives have received particular attention (59,70,71) and this group has become clearly established as a replacement for a metabolically-labile ester functionality (42). Replacement of the tetrahydropyridine ring in arecoline by quinuclidine 23 and azabicyclo[2.2.1]heptane 24 gave potent muscarinic agonists (42). The aminooxadiazole 25 proved to be the most potent and most efficacious non quaternary muscarinic agonist known being 200-fold more potent than arecoline and displaying efficacy at cortical muscarinic receptors greater than acetylcholine itself.

Analogues of oxotremorine having differing levels of efficacy and selectivity have been discussed. The dimethylsulfonium 26 and 27 and thiolanium 28 and 29 analogues were found to be potent muscarinic agonists *in vitro* and *in vivo* (72). Studies on substituted 2-pyrrolidones 30 demonstrated that relatively small changes in structure can result in different intrinsic efficacy independent of affinity (73). Acyclic imide, sulfonimide, N-acetyl sulfonamide and trifluoroacetamide analogues of BM-5 had profiles ranging from agonists, partial agonists and antagonists in the guinea pig ileum (74). Evidence has been obtained that emesis induced by McN-A-343 is mediated by central M-1 receptors (75).



26 X = CH_2; Y = $\overset{\oplus}{S}(CH_3)_2$ 27 X = CO; Y = $\overset{\oplus}{S}(CH_3)_2$

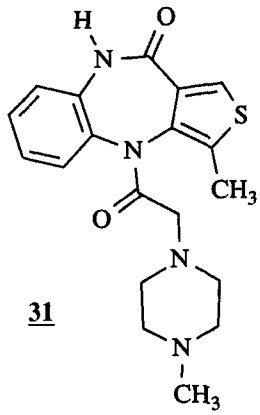
28 X = CH_2; Y = S^+ 29 X = CO; Y = S^+

30 X = $CHCH_3$; Y = $N(CH_3)_2$; $\overset{\oplus}{N}(CH_3)_3$; N

Selective Antagonists To Differentiate Muscarinic Receptor Subtypes - A number of selective antagonists are now available which clearly differentiate between the different subtypes found in high density in neuronal tissues, peripheral effector organs such as the heart and smooth muscle and glands. The M-1 receptor selectivity of PZ has been shown to be associated with semirigid side chains and only those

containing 6-membered ring systems showed M-1 selectivity (76). In contrast to methylatropine, PZ reversed oxotremorine-induced behavioural effects with a maximum antagonism of 50% when given to rats i.p. indicating that PZ can be given systemically to investigate the behavioural functions of M_1 receptors (77). The antagonist telenzepine, 31, has an M_1 selectivity comparable to PZ but is ca 10 times more potent (78). BTM-1086 32 has been shown to be more M_1-selective than PZ (79).

Detailed binding and pharmacological studies have established methoctramine 33 to be the most potent, selective antagonist of the cardiac M_2 muscarinic receptors (80-83). The compound is much less effective at glandular or ganglionic receptors. Mixed binding kinetics were displayed by the compound in cardiac membranes and a modification of the dissociation rate of [^{3}H]-NMS can be interpreted by the presence of both a competitive and allosteric interaction of the antagonist with the receptor. The antagonist profile of methoctramine is associated with a six-carbon chain separating the inner from the outer N-atoms and eight-carbon atoms between the inner nitrogen of the tetramine. Replacement of the 2-methoxybenzyl group by a furan-2-yl-methyl group also yielded a potent and selective antagonist. The pharmacological selectivity of methoctramine has been confirmed in binding experiments using [^{3}H]-PZ to label M_1 receptors of rat cerebral cortex and [^{3}H]-NMS to label muscarinic receptor populations of rat heart, cerebellum, ileum and salivary glands (84,85).



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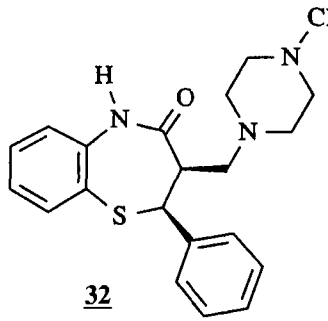

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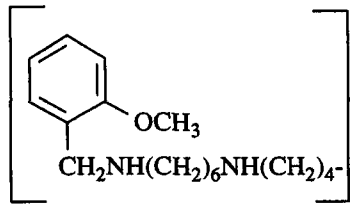
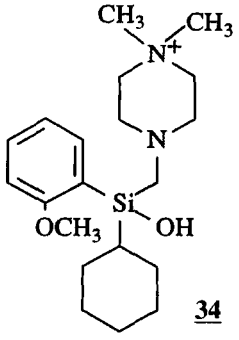


33

The cardioselective (M_2) antagonist AF-DX 116 has continued to feature in studies to probe the nature of muscarinic sub-types (86,87). A direct visualization of the rat brain M_2 receptors has been obtained (88) by autoradiographic studies using [^{3}H]-AF-DX 116. In contrast to the localisation of M_1 binding sites identified by PZ (concentrated in striatum, hippocampus and cortex), AF-DX 116 labelled sites were most densely populated in medulla and mid-brain. The ortho-methoxy analogue 34 of silahexocyclium can now be added to the group of selective antagonists related to hexocyclium, difenidol and their silicon analogs (89). In contrast to the selectivity of hexahydrosiladifenidol (HHSiD, $M_1 = M_2 >> M_3$) and silohexocyclium ($M_1 = M_3 > M_2$), 34 has high M_1 selectivity ($M_1 >> M_3 > M_2$). p-Fluoro-HHSiD 35 is the first M_3 selective antagonist having a selectivity for the M_3 receptor comparable with PZ for M_1 and methoctramine for M_2 (90). The M_1 selective antagonist behaviour of dicyclomine has been confirmed (91). The (R)-(-)-enantiomers of trihexylphenidyl 36 and

its methiodide have also been found to be potent M_1-selective antagonists (pA_2 = 10.1 and 10.6) with a 91- and 45-fold selectivity respectively for the M_1 receptor in rabbit vas deferens over the M_2 cardiac receptor (92). Some studies have already commenced using this range of selective antagonists to re-evaluate the distribution of the various subtypes of MR in both human (93) and animal brain (94-96). Using AF-DX 116 and PZ, the highest proportion of M_1 receptors in rat brain was found in the hippocampus whilst the cerebellum and the hypothalamus were the regions with the largest fraction of M_2 and M_3.



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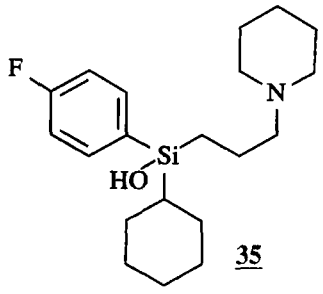

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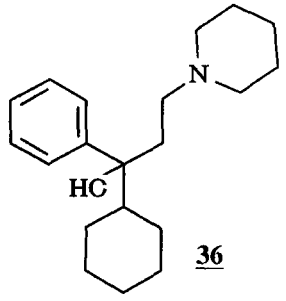
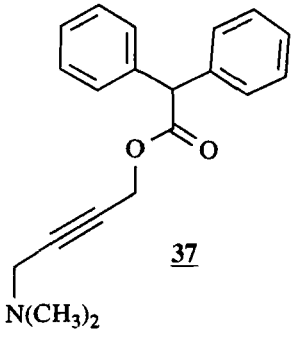


36

A number of other potentially selective antagonists have been studied. An acetylenic analogue of 4-DAMP 37, provided a much less polar compound; while comparably selective to the parent, it was 100-fold less active (97). The enantiomers of QNB, QNX and QNA in which the alcohol portion had the S absolute stereochemistry had lower affinity but were more selective for the M_1 receptor (98) in striatum than their corresponding R isomer. Muscarinic antagonists containing the 1,3-oxathiolane nucleus eg 38 and 39, have also pointed to differences between cardiac and ileal receptors and may be useful in defining brain MR heterogeneity (99,100).



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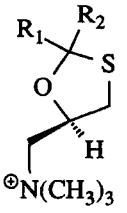

38 $R_1 = R_2 = C_6H_{11}$

39 $R_1 = C_6H_{11}$; $R_2 = C_6H_5$

Conclusions - Although much remains to be done to unravel the functional roles of the central MR subtypes, it is likely that a combination of molecular biology and the use of subtype selective ligands will help achieve this objective. Some surprises will undoubtedly arise from this work. Importantly, as far as the cholinergic hypothesis of Alzheimer's disease is concerned, the M_1 receptor may be joined (or dislodged completely) by another subtype, and this may redirect pharmaceutical research for the future.

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Chapter 5. Recent Advances In Excitatory Amino Acid Research

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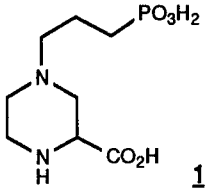
Introduction - The rapid growth and diversification of research in the area of excitatory amino acids (EAA) has continued unabated. The evolving understanding of the role of these compounds (glutamic, aspartic and other related diacidic amino acids) in disease, coupled with an increasing awareness of their unfulfilled therapeutic potential, has attracted both significant pharmaceutical research and popular media attention. The breadth of scientific interest in the excitatory amino acids is reflected in a wide range of recently published review articles and symposium proceedings (1-11). This report will seek to review recent advances across the spectrum of EAA research and highlight the structure and mechanism of action of new EAA based therapeutic agents.

Nomenclature - In keeping with efforts to harmonize the system for naming other neurotransmitter receptor systems, attempts have been made to rationalize the EAA receptor nomenclature (12). However, this has yet to be successful and the classification of receptors based on the names of selective ligands, N-methyl-D-aspartic acid (NMDA), kainic acid (KA), quisqualic acid (QUIS) and 2-amino-4-phosphonobutyric acid (AP4), remains dominant. Controversy regarding the nature of the binding site for PCP/sigma opiate ligands has been partially resolved with the definition of biochemically distinct PCP_1 (non-competitive NMDA antagonist, vide infra) and sigma binding sites (13); however, shortcomings of this nomenclature have been identified (14).

Physiology - Increasingly, EAAs have been shown to mediate a range of physiological functions including nociception (15,16), vision (11,17) cardiovascular reflex (18,19), respiration (20) and motor control (11). Competitive and non-competitive NMDA antagonists also have been shown to exert a protective action in animal models of anxiety (11). A glutamatergic hypothesis for schizophrenia has been described (21), and is supported an observed left hippocampal reduction in glutamate and kainate binding sites from post-mortem schizophrenic brains (22). However, studies of CSF glutamic acid levels in patients was not supportive of this hypothesis (23). NMDA receptors have been additionally implicated in learning and memory (24). Further evidence for this is derived from the observations that 2-amino-7-phosphono-heptanoic acid (APH), CPP (1), phencyclidine (PCP) and MK-801 (dizocilpine) (2) all produced memory impairing effects in rodents (25-27). EAAs have been suggested to play a role in the etiology of epilepsy (11,28). This is supported both from the observation that competitive and non-competitive NMDA antagonists suppress the generation of kindling (29,30), and that these compounds also demonstrate an antiepiletic action in a variety of animal seizure models (31,32). MK-801 also has shown limited anticonvulsant efficacy in the clinic (33).

The major pharmaceutical interest in the excitatory amino acids is focused currently on their role in the etiology and treatment of neurodegenerative disorders such as amyotrophic lateral sclerosis (34), Alzheimer's and Huntington's disease (35-37). A central role for EAAs in stroke has been firmly established (11). Together, these diseases have been suggested to have a common pathology, that is, chronic or acute cell death resulting from EAA-induced excitotoxicity (35,38-40). Further evidence for an excitotoxic action of glutamate acting at the NMDA receptor has been derived from numerous studies of cultured cortical neurons in vitro (39). In these models, an influx of calcium ions through the stimulated NMDA ionophore is suggested to be a prerequisite for cell death to occur (39,41,42). In addition to in vitro models, selective brain lesioning studies have also supported a role for glutamate in ischemic and hypoglycemic brain injury (43,44). The non-competitive NMDA antagonists, dextrorphan, MK-801 and PCP also protected against ischemia in vivo in a variety of species, including rabbit and cat (45-47). The competitive NMDA antagonists, kynurenic acid, APH, CPP and CGS 19755 (3) were similarly neuroprotective in the rat and gerbil (48-50).



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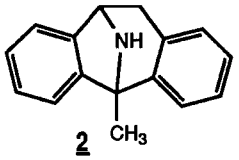

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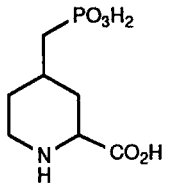

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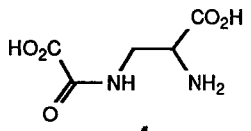
ß-N-Methylamino-L-alanine (BMAA) has been proposed as a plant derived excitotoxin responsible for a condition known as Guam amyotrophic lateral sclerosis-parkinsonism-dementia (GALSPD) (51). The related oxalate half amide of ß-amino-L-alanine (BOAA) (4) has been associated previously with lathyrism, a related neurodegenerative motorneuron disorder (52). Although the neurotoxic action of (4) is mediated through quisqualate and kainate receptors (53), BMAA's action is antagonized by MK-801, PCP, APH and CPP (51,54). However, unlike BOAA, the expression of BMAA neurotoxicity in cell culture requires the presence of bicarbonate ion, which may generate the glutamate-related transient carbamate ion (5) or a related structure (55).



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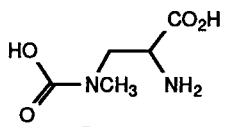

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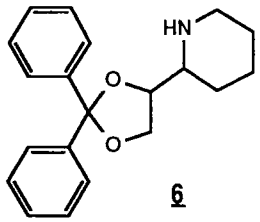

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Second Messengers - The second messenger pathways associated with EAA receptor activation have been summarized (56). NMDA receptor activation has been shown to stimulate the arachidonic acid cascade and induced proteolysis of brain spectrin (57,58). Both events may be implicated in long term potentiation. Kainate receptor activation induces the expression of the proto-oncogene, c-fos (59).

Receptor Isolation - Although not yet cloned and sequenced, both NMDA and quisqualate/kainate receptors have been expressed in Xenopus oocytes

(60-63). These receptors were activated by agonists and, in the case of the NMDA receptor, were also inhibited by APV, potentiated by glycine and noncompetively blocked by CNQX (**34**), a new quisqualate antagonist (vide infra) (60,63). Kainate responses were also blocked by CNQX (63). In a separate preparation, NMDA activated currents were blocked stereoselectively by dexoxadrol (**6**), and other PCP ligands (64). NMDA receptors have been solublized in an active form (65) and reconstituted in lipid bilayers (66).

New Receptor Ligands - The characterization of [^{3}H]APV and [^{3}H]CGS 19755 as new NMDA receptor ligands has been reported (67,68).

Receptor Characterization - The AP4 (or APB) receptor is the least well characterized of the EAA receptors. Once suggested to be a presynaptic autoreceptor, an AP4 binding site has also been located postsynaptically in hippocampus (69). The unequivocal characterization of distinct kainate and quisqualate receptors has not yet been achieved. Significant evidence suggests that the calcium insensitive low affinity component of kainate binding is identical to the quisqualate receptor (7). Confirmation of this relationship has also been derived from receptor radiation inactivation studies, where a common agonist binding site ([^{3}H]kainate and [^{3}H] AMPA) molecular weight of approximately 52,000 was derived (7). Electrophysiological studies however, are still supportive of separate QUIS and KA receptors (70). A non-depolarizing action of quisqualate to stimulate the mobilization of intracellular calcium in hippocampal neurons is suggested to represent the existance of a new glutamate receptor type (71).

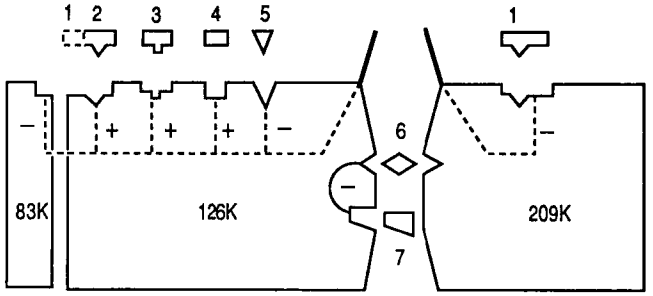
The NMDA receptor is the best characterized of the EAA receptors (72). Once thought to be restricted to the CNS, NMDA receptors have also been identified in guinea-pig myenteric plexus (73). Although considered a homogenous receptor population, discrepancies in the distribution of L-[^{3}H]glutamate (agonist) and [^{3}H]CPP (antagonist) binding may indicate the existence of two distinct receptor types or interconverting forms of the same receptor (74). Two agonist molecules appear necessary for receptor activation (75), with ligand recognition or ion channel activation being trypsin sensitive (76). Radiation inactivation studies suggest that the PCP, glutamate and glycine sites vide infra, are all located on one protein of 126,000 dalton molecular weight. The CPP antagonist site however, appears to be associated with a protein or proteins of 209,000 combined molecular weight (7).

NMDA Receptor Modulation - The nature of the interaction of the NMDA and PCP receptors has been defined further using both receptor binding and electrophysiology. Binding of PCP site ligands is reported to be dependent on both NMDA receptor activation, and membrane potential. Together, these results indicate that the binding site is located within the NMDA activated ion channel and that channel opening is needed for ligand access (77,78). It has been shown that subsequent application of APV can trap a non-competitive antagonist within the resulting closed channel (79). By acting to increase the probability of channel opening, glycine, at submicromolar concentrations, has been shown to potentiate the agonist activation of the NMDA receptor in a voltage independent manner (80). In the oocyte expressed NMDA receptor, it has further been shown that the presence of glycine is a prerequisite for receptor activation (81). Unlike magnesium, zinc has been shown to inhibit NMDA receptor activation in a voltage independent manner (82,83). The complex interrelationship of these modulatory sites has been clarified using

[^{3}H]MK-801 and [^{3}H]1-[1-(2-thienyl)cyclohexyl]piperidine (TCP) binding (84,85). Using this technique, a stimulatory action of the polyamines, spermine and spermidine, on NMDA receptor activation also has been observed (86).

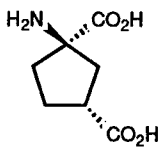
Receptor Structure - Combining those features summarized above and several reported diagramatic representations of the NMDA receptor (7,72,84), a composite picture of this receptor emerges (Figure 1).

Figure 1



NEW PHARMACOLOGICAL AGENTS

NMDA Agonists - All four stereoisomers of the conformationally constrained 1-amino-1,3-cyclopentanedicarboxylic acid have been prepared (87). One isomer, cis-1R,3R (**7**), completely mimicked the electrophysiological action of NMDA on rat CA_1 hippocampal neurons. The remaining isomers were kainate-like in action. The synthesis of cyclopropyl fused glutamate analogs has been reported (88). The 2S,3R,4S isomer (**8**) was a potent ligand in NMDA-specific [^{3}H]glutamate binding; however, its action, was not fully reversed by MK-801 or APV. Trans-2-carboxy-3-pyrrolidineacetic acid (CPAA)(**9**), has been reported to be an NMDA agonist (89). Inhibition of [^{3}H]kainate binding was also noted for (**9**). Weak, yet structurally dependent inhibition of glutamate binding, was noted for a series of glutamic acid dimers (90).



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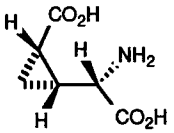

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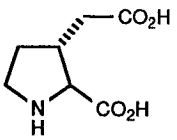

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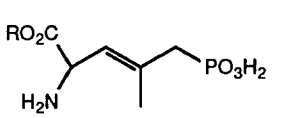
Competitive NMDA Antagonists - As expected from the high level of pharmaceutical interest in NMDA antagonists, increasing numbers of new antagonist structures have appeared in the literature. However, no deviations from the amino acid-spacer-phosphonic acid structural type have been reported. It seems unlikely that these new antagonists will penetrate the CNS significantly better than APH (91). Introducing a double bond into APV afforded CGP 37849 (**10**) which, in [^{3}H]CPP binding, had a K_i of 35nM and a long duration (>8hr) of anticonvulsant activity after oral dosing (92,93). Surprisingly, its ethyl ester, CGP 39551 (**11**), was also a potent antagonist in binding (K_i 350nM) and had a longer

duration of anticonvulsant action in vivo (>24hr). Introducing an alkenyl linkage into CPP to give **12**, as a single stereoisomer has been disclosed (94). Modifying the skeleton of CGS 19755 (**3**), the phosphonoalkenylpiperidines and pyrrolidines illustrated by **13**, **14** and **15** have been prepared (95). The synthesis and preliminary pharmacology of the phosphonoalkyl analogs, LY 278781 (**16**), 257883 (**17**) and 214600 (**18**) have been described (96-98). As expected, **17** was comparably active with **3** in [^{3}H]CPP binding. The introduction of a heteroatom into the phosphonoalkyl chain of **16-18** has been reported to result in NMDA receptor antagonism; however, no biological activity for these derivatives was disclosed (99). Although marginal as NMDA receptor antagonists, two series of 2-carboxy-3-phosphonalkylpyridines and piperidines have also been prepared (98).



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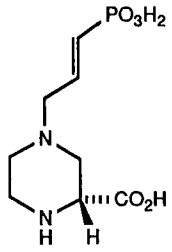

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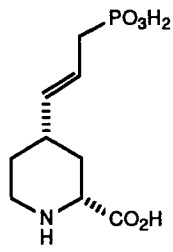

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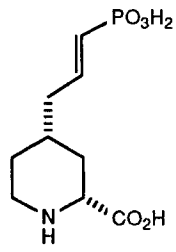

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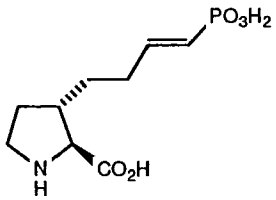

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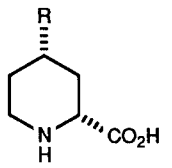
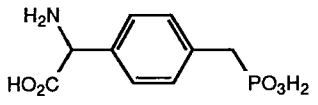


R

16 = $(CH_2)_4PO_3H_2$

17 = $(CH_2)_3PO_3H_2$

18 = $(CH_2)_2PO_3H_2$



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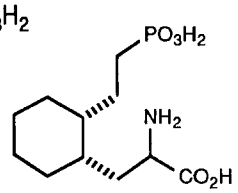


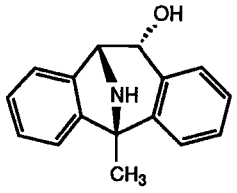
20

Further conformationally constrained meta and para phenyl-spaced derivatives of APH have been described (100-102). The 4-phosphonomethylphenylglycine derivative, PD 129635 (**19**), was reported to bind with similar potency to APH in a [^{3}H]CPP assay, but be more potent than APH against NMDA-induced death in mice (100,101). The synthesis and preliminary pharmacology of the new NMDA antagonist NPC 12626 (**20**) has been reported (103-105).

NMDA Receptor Modeling - To date, using the evaluation of known antagonists in a range of receptor binding paradigms to derive structure activity relationships, only crude impressions of the essential features of the NMDA receptor have emerged (72,106).

Non-Competitive NMDA Antagonists - MK-801 has been patented for the prevention or treatment of neurodegeneration (107). Fluoro and hydroxy MK-801 derivatives have been disclosed with the 11-hydroxy derivative **21** preferred (108). Dextromethorphan has been patented as an antineurotoxic

agent (109). In addition, other opioids and nonopioids including naloxone, have also been reported to protect against neurotoxicity in vitro (110,111). Tetrahydroaminoacridine (THA) (**22**), probably acting at the PCP receptor, also antagonizes NMDA neurotoxicity (112). Although inactive at the NMDA associated glycine binding site, strychnine is reported to antagonize NMDA channel stimulation in a voltage dependant manner (113).



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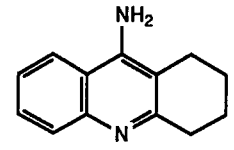
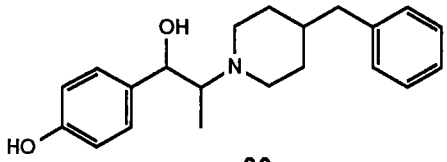


22

Based on receptor binding data for a range of PCP and sigma receptor ligands, PCP and sigma receptor models have been generated using computer assisted modeling techniques (114). From an analysis of resolved etoxadrol isomers and related compounds, an alternative receptor model suggesting greater nitrogen lone pair hydrogen bonding latitude was derived (115).



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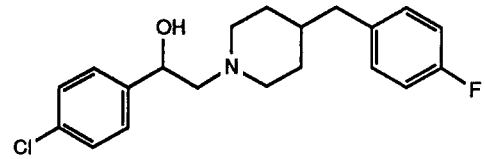


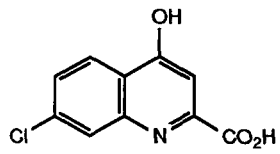
24

Ifenprodil (**23**) and its more orally bioavailable analog SL 820715 (**24**) represent new non-competitive NMDA antagonists which act at a site distinct from PCP and CPP. Although antagonistic to NMDA stimulated action in a range of paradigms, the site of action of these compounds has not been established (116). Significant protection against focal cerebral infarction was also noted for these compounds (117).

Glycine Agonists/Antagonists - The stoichiometry of the glycine modulation of the NMDA receptor has been determined (118). 7-Chlorokynurenic acid (**25**) has been shown to be a more potent and selective glycine antagonist than its parent, kynurenic acid (119). In addition, **25** has also been reported to protect cultured hippocampal cells against glutamate induced neurotoxicity in a glycine reversible manner (120). Differing in profile to **25**, HA-966 (**26**) was also a selective glycine antagonist (121,122). Potent glycine agonist action is shown by D-alanine, D-serine and **27** (123,124). Cycloleucine (**28**) is a selective, but weak antagonist (125). Although selective quisqualate antagonists, DNQX (**33**)and CNQX (**34**) are also antagonists at the strychnine insensitive glycine receptor (126). Cycloserine acts as a partial agonist at the glycine site (127).

Zinc Ligands - Tricyclic antidepressants and phenothiazines, including desmethylimipramine and ethopropazine have been suggested to act as zinc site ligands (128,129). The relationship of the zinc site to the magnesium site has also been explored (129).

Glutamate Release Antagonists - Reported to be effective in a model of global ischemia, E 2001 (**29**), has been shown to inhibit the stimulated release and accumulation of extracellular glutamate (130,131).



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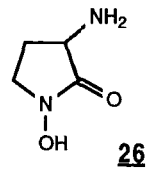

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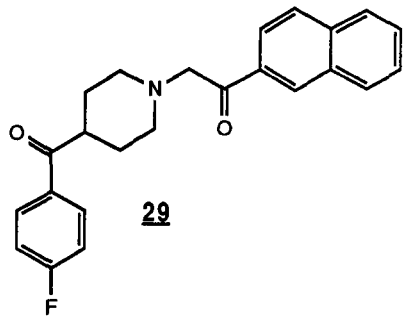

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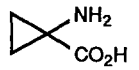

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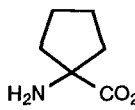
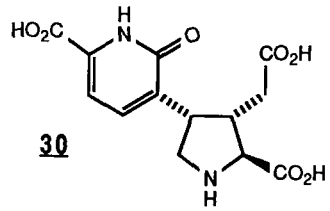


28

Kainate/Quisqualate Agonists - In the rat spinal cord, acromelic acid (30), is a more potent kainate agonist than kainate itself (132). Stizolobic acid (31), was also a kainate-like agonist in mammals, yet an antagonist at the crayfish neuromuscular junction (133). Although uncertain as to its agonist or antagonist profile, the conjugate, kainyl-bovine serum albumin (32), binds with high affinity to a population of kainate receptors in chick cerebellum (134).



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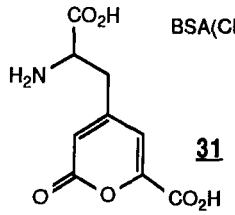

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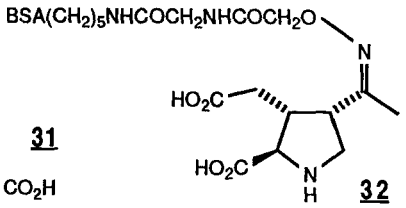

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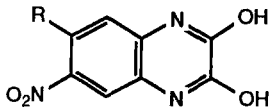
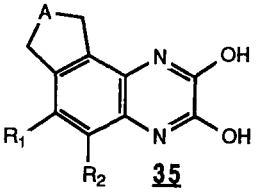


33 R = NO_2

34 R = CN



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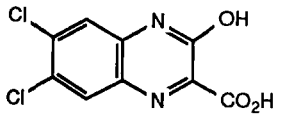

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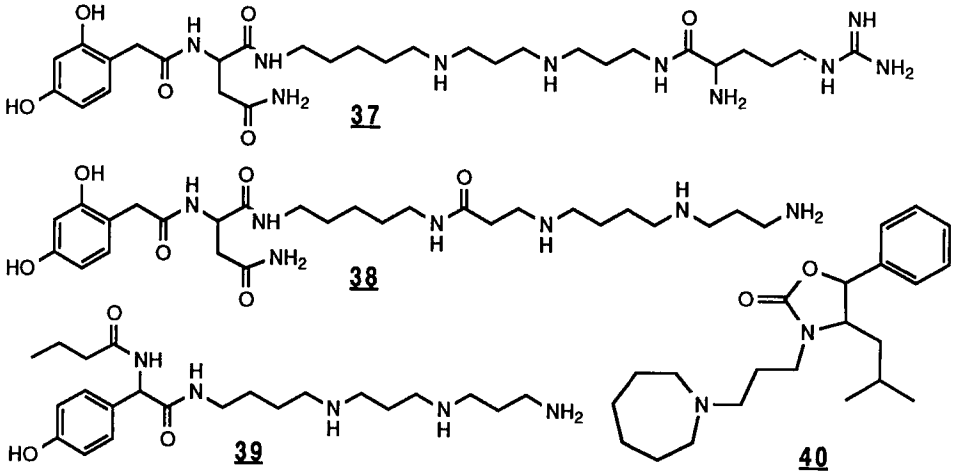
Kainate/Quisqualate Antagonists - The 6,7-dinitro and 6-cyano-7-nitroquinoxalinediones (DNQX or FG9041)(33) and (CNQX or FG9065)(34) are the first examples of potent and selective competitive quisqualate and kainate antagonists (135,136). In a range of electrophysiological preparations, the quisqualate response was antagonized slightly more potently than the kainate (137). Additional heterocyclic and carbocyclic ring-fused analogs of CNQX and DNQX (35), have been disclosed (138). Structurally related to these antagonists, 6,7-dichloro-3-hydroxy-2-quinoxalinecarboxylic acid (diCL-HQX)(36) is a kainate antagonist (139). Unlike CNQX and DNQX, (36) is a weak quisqualate antagonist. This compound equipotently antagonizes NMDA responses, probably through an interaction at the glycine site.

Invertebrate Glutamate Antagonists - Glutamate is an established neurotransmitter in the invertebrate nervous system. Here, unlike the vertebrate nervous system, postsynaptic responses are mediated predominantly by a quisqualate sensitive receptor (140). The existence of NMDA-sensitive receptors has not been demonstrated in invertebrates. Using a variety of arthropod neuromuscular junction preparations, potent

inhibitors of glutamate stimulation have been identified from the venoms of several species of spiders and wasps (140-143). These antagonists, which include, argeriotoxin (**37**), joro spider toxin (**38**) and philanthotoxin (**39**) display common structural elements. Several syntheses of these compounds have appeared (143,144). In addition, the minimum structural elements necessary for receptor antagonism have been determined (145). An open channel blocking mechanism of action has been suggested (146). The inhibition of synaptosomal glutamate uptake as well as potent antagonism of the vertebrate quisqualate/kainate receptors has also been demonstrated for these antagonists (147,148) (See also Chap.30).



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39 **40**

Using the crayfish neuromuscular junction, a series of heterocyclic amines has been identified as potent glutamate antagonists (149). Illustrative of these compounds, MLV-6976 (**40**), has been shown to antagonize vertebrate cortical and brain stem glutamate receptors in a use dependent, but non-selective manner (8,149). Although, (**40**) did not antagonize NMDA or kainate responses in the cortical wedge, it was neuroprotective in a model of global cerebral ischemia (150,151). Further examples of this compound class have been disclosed (152).

Conclusion - It is clear that the foundation now exists to support rapid future progress in the area of excitatory amino acid research. However, the transformation of basic research into a marketable product will likely be dependent on minimizing the probable neuropsychological and cognitive liabilities of these potential therapeutic agents.

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Section II - Cardiopulmonary and Vascular Agents

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Chapter 6. Antihypertensive Agents

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Introduction - Hypertension is a major health risk factor and its control by drugs has reduced the incidence of stroke, heart failure and renal damage. The importance of the central nervous system (1) and the kidney (2) have been critically reviewed, however, antihypertensive therapy has failed to significantly reduce the incidence of coronary artery disease (3). Considering these results, recent reports have advocated the selection of antihypertensive agents based not only on their efficacy and tolerance, but also on their potential to have positive or at least neutral effects on other factors such as serum lipids, glucose tolerance, or potassium excretion (4,5). In light of their favorable side effect profile and their neutral effect on serum lipids and other risk factors, angiotensin converting enzyme (ACE) inhibitors, α-adrenoceptor antagonists and calcium channel antagonists are preferred as monotherapy or combination therapy (3-7). Furthermore, an emphasis is being placed on individual patient therapy rather than drug selection based on the traditional stepped-care approach (8-10).

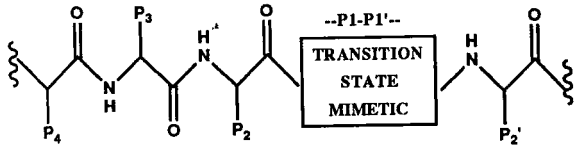
Renin Inhibitors - Two approaches to renin inhibition, renin antibodies and pseudo-renin substrates, continue to be areas of vigorous research. Although both types of agents lower blood pressure in high renin animal models, the pseudo-substrates have dominated the search for novel drugs (11-13). Major developments include clinical reports on the effects of intravenous administration of renin inhibitors (14-18) and reports of orally active renin inhibitors in primate models (19-24). In both humans and primates, many investigators report the lack of a linear correlation between plasma renin activity (PRA) reduction and blood pressure effects, suggesting the possibility that non-plasma renin may also be involved in blood pressure control (14-18,25).

The isolation of recombinant human prorenin and its conversion to active renin have been reported (26), but X-ray crystallographic data on human renin remain unpublished. In the absence of X-ray data, many research groups have relied on calculated models of the renin enzyme which have been derived from highly homologous aspartic proteinases, such as endothiapepsin or penicillopepsin, for molecular modeling studies (21,27-37). In addition, the X-ray structures of renin inhibitor complexes with endothiapepsin and penicillopepsin have been published, affording the details of binding interactions as a model for renin inhibition (32-34). Molecular modeling has suggested cyclic

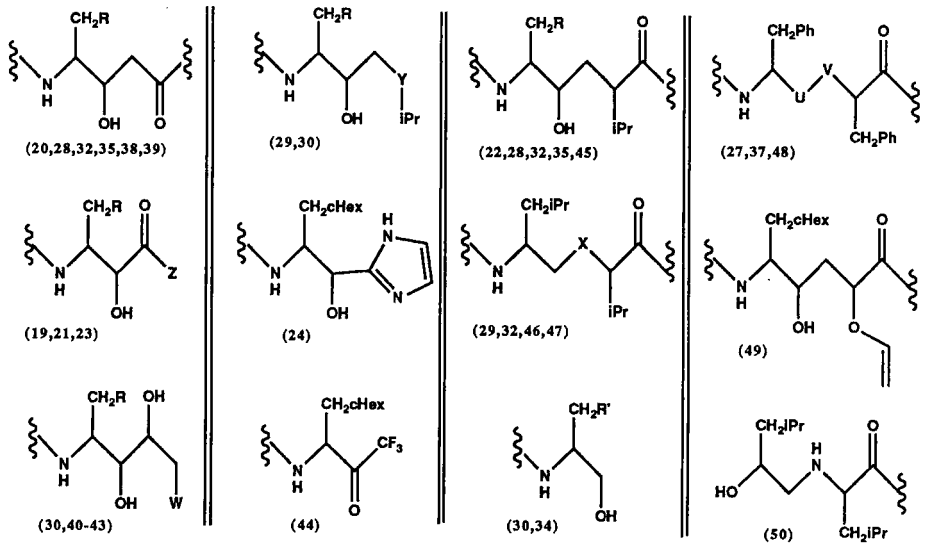
conformational constraints that are compatible with in vitro renin inhibition (35-37).

Emerging structure-activity relationships for renin inhibitors indicate that potent agents include a non-cleavable transition state mimetic of the angiotensinogen sissile bond, plus recognition functionality that spans a minimum of the P_4 to P_2' positions of renin substrate. The many transition state mimetics (P_1-P_1') that have been published recently are listed in Table 1.

Table 1



P1 - P1' TRANSITION STATE MIMETIC (ref)

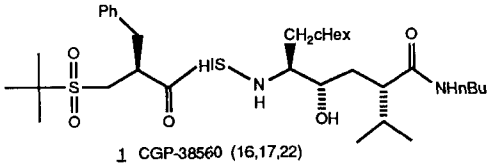


R = iPr, cHex U-V = CH2-NH, NHC(O) W = iPr, N3 Z = OMe, OiPr, NHiPr, NHBu

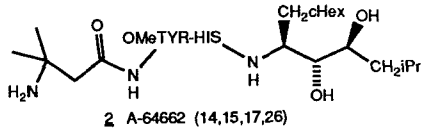
R' = iPr, cHex, CH(CF3)CH3 X = S, S(O), NH, O Y = CH2, S

Selectivity for renin vs. other aspartic proteinases such as cathepsin D and pepsin is attributable to the P_2 residue where histidine, alanine and 4-thiazolylalanine offer the greatest renin selectivity compared to other amino acids (20,22,30,31,51). Numerous chemical modifications of the P_3 and P_2 residues that enhance stability toward degradative enzymes such as chymotrypsin have been described (16,19,20, 31,35,43). Lipophilic substituents are required at P_1 and P_3, but highly polar or basic substituents may be installed at P_4, P_1', P_2' and P_3' to afford active renin inhibitors with good water solubility (20,24,31, 38,41).

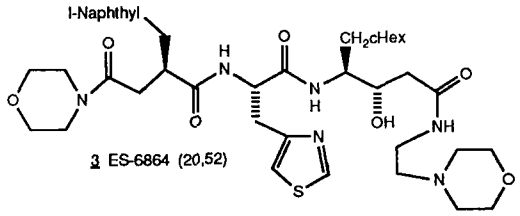
Compounds 1-4 are the leading renin inhibitors currently under active evaluation.



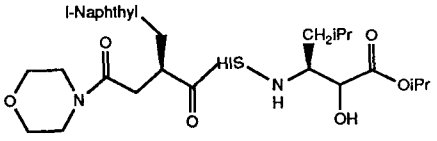
1 CGP-38560 (16,17,22)



2 A-64662 (14,15,17,26)

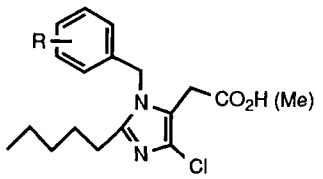


3 ES-6864 (20,52)



4 KRI-1230 (19,21)

Angiotensin II Receptor Antagonists - The identification of a putative angiotensin II receptor gene encoded by the human mas oncogene (53), the preparation of new probes for angiotensin II receptors (54,55), the study of angiotensin II conformation in aqueous and lipid environments (56), and the continued investigation of receptor properties (57,58) indicate progress toward the development of biochemical tools and molecular models that will facilitate the development of angiotensin II antagonists. Additional examples of three types of angiotensin II antagonists have been reported, including peptide antagonists (analogs of saralasin and sarmesin) (59-62), monoclonal antibodies to angiotensin II (63), and non-peptide antagonists 5 (64-66).



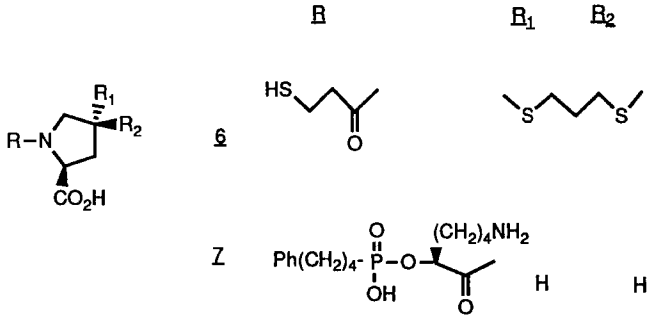
5

R = 2-Cl
4-NO_2
4-CO_2H
4-NH-CO-(2-CO_2H)Ph

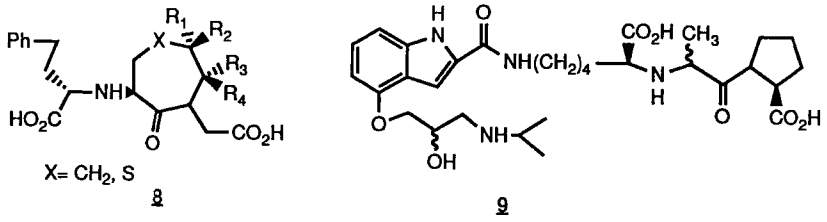
ACE Inhibitors - Molecular biology techniques are being used to provide a more detailed analysis of the angiotensin converting enzyme (ACE). Bovine and mouse kidney ACE have a high degree of structural homology to that of rabbit lung (67), and ACE from mouse epithelial and endothelial cells are believed to be identical based on expression of cDNA ACE.31 (68). Cloning and mapping has also revealed two homologous sequences within human ACE, corresponding to two putative active centers for a single protein molecule (69). A neutron scattering study has provided further information about the molecular weight and elongated shape of the enzyme in aqueous solution (70).

The importance of tissue ACE over plasma enzyme in the long-term regulation of blood pressure has been emphasized (71), and the presence of ACE in vascular tissue and its inhibition has been demonstrated in rats (72,73). Studies of structural relationships of ACE inhibitors to their tissue distribution and elimination have been reported (74,75), and these physiochemical parameters related to their efficacy and side-effect profiles.

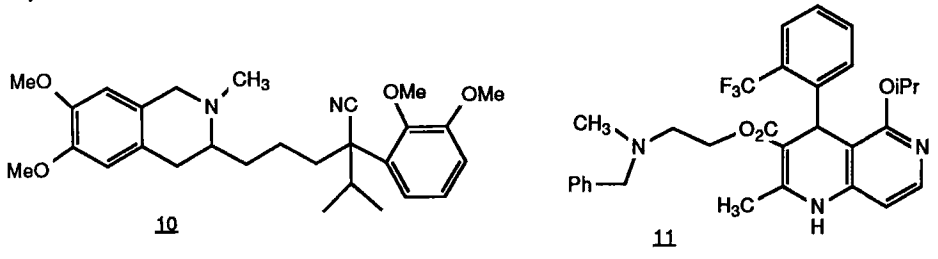
Structure-activity investigations around the proline-containing ACE inhibitors have appeared (76,77). Hydrophobic substitution in the 4-position generally increased potency. The thioketal **6** has also been described as being potent *in vitro* and *in vivo* (77). SQ-29,852 **7** is a proline derivative where oral activity was dependent on the presence of an aminobutyl side chain (78). Ring stereochemistry has also been investigated in two series of perhydroazepin-2-ones, **8**; in each case mono-phenyl R_1 or R_4 was potent *in vitro*, with the latter series demonstrating longer duration of action *in vivo* (as the monoester) (79,80).



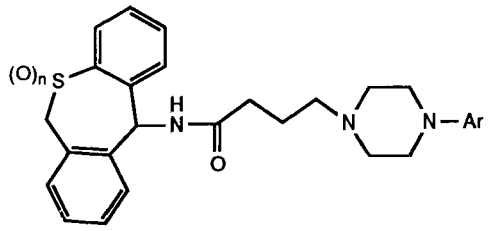
A combination ACE inhibitor-β-blocker BW A575C **9**, was reported to lower diastolic blood pressure with efficacy equivalent to enalapril and similar renovascular effects (81). It also decreased cardiac contractility and rate.

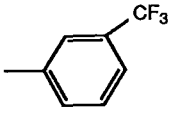


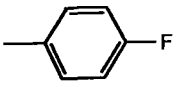
Calcium Channel Antagonists - Recent emphasis has been placed on an extended duration of action. The plasma half-life of RS-93007 **10** is 11.2 hours following oral dosing to humans (82). GOE 6070, **11**, binds to the dihydropyridine receptor with a Ki of 0.43 nM (83), and exhibits a duration of action of at least 8 hours in spontaneously hypertensive rats (84).



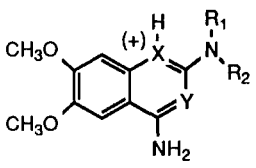
A series of novel tricyclic piperazine derivatives shows potent blockade of K^+-induced contractions of rat aorta and antihypertensive activity in spontaneous hypertensive rats (85). The thiepin derivative **12** was the most potent inhibitor of K^+-induced contractions, with a potency similar to nifedipine. Similar results were obtained with the sulfoxide and sulfone analogs **13**, which had an oral duration of action greater than 5 hours.



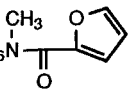
12 Ar =  n = 0

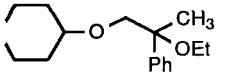
13 Ar =  n = 1,2

Alpha Agonists and Antagonists - The α-1 adrenoceptor (86) and α antagonists for the treatment of hypertension (87) have been reviewed. Structural studies with isoquinoline and quinoline analogs **14**, have illustrated the importance of protonation of N-1 in the quinazoline (or quinoline) ring at physiological pH to provide a key pharmacophore for α-1 adrenoceptor recognition (88,89). Replacement of the piperazine ring of prazosin with alkanediamines **15** (90) and substituted oxyethoxy-piperidines **16** (91) affords compounds with good receptor affinity and moderate to good activity in vivo.

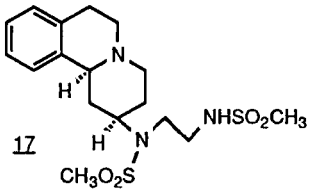


14 X=N and Y=CH , or X=CH and Y=N

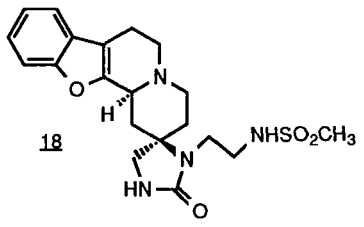
15 X=Y=N R1=CH3 R2= 

16 X=Y=N R1,R2= 

Animal models for the detection of post-junctional α-2 selective antagonists have been developed (92). Increased α-2 selectivity has been achieved with the bis-sulfonamide **17** (93), and a spirocyclic compound **18** (94).



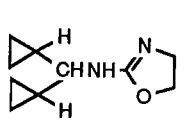
17



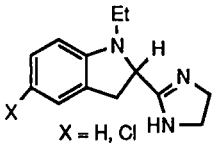
18

Rilmenidine (S 3341) **19**, a novel α-2 agonist has been the subject of a symposium (95). It is reported to be less sedative than clonidine, not because of separation of α-2 activity, but rather due to an unknown central component which counteracts sedation (96). A series of

substituted 2-imidazolinylindolines, 20, was investigated and found to be potent mixed α-2 antagonists and α-1 agonists.

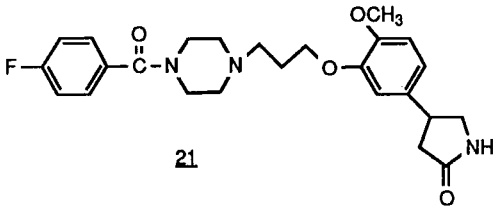


19



X = H, Cl

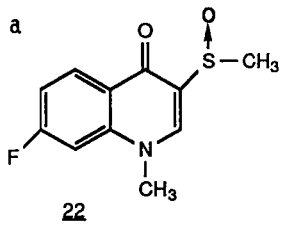
20



21

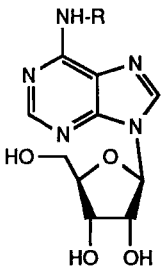
The relationship between the α-adrenergic and serotonergic systems was the subject of a recent symposium (97). Blockade of 5-HT_2 receptors by various drugs does not affect blood pressure (98,99); however interactions between the α-adrenergic and serotonergic systems are proposed to exist at the level of signal transduction beyond the individual receptors (97). ZK 33,839 21, combines high affinity for 5-HT_2 and alpha-1 adrenoceptors. Its vasodilatory properties are ascribed to selective alpha antagonism, while platelet antiaggregatory, antivasospastic and vasoprotective properties are suggested to be due to selective 5-HT_2 receptor blockade (100).

Other Compounds - Flosequinan (BTS-49465) 22 is a direct-acting vasodilator with a mechanism of action that is poorly defined, but may involve elevations in cGMP (101,102). Initial clinical studies indicate that flosequinan is effective in essential hypertension (103) as well as in severe congestive heart failure (104,105).

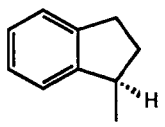


22

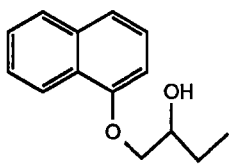
Two adenosine receptor agonists, PD 117519 23 and SC 32796 24, are relatively selective for adenosine A1 vs. A2 receptors and lower blood pressure without tachycardia in several hypertensive animal models (106-108). The antihypertensive effect may be mediated by direct vasorelaxation (A2 receptor mediated), as well as indirect actions through inhibition of neurotransmitter release (A1 receptor mediated) (109). Both agents show greater activity when plasma renin activity is elevated and SC 32796 has been shown to inhibit renin release (110,111).



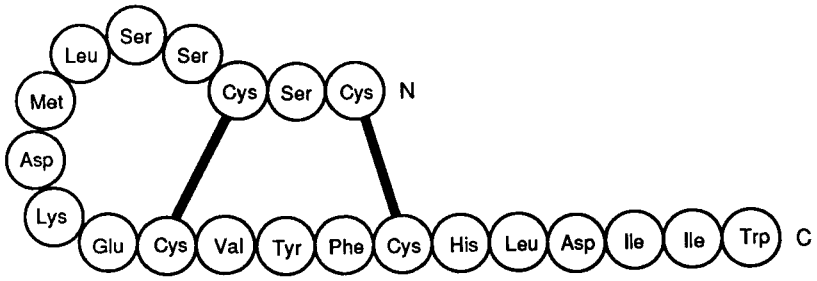
23 R =



24 R =



Future Directions - A contractile peptide substance termed "endothelin" was isolated from porcine aortic endothelial cells (112). Porcine endothelin is a 21-amino acid peptide with 2 disulfide bonds. The amino acid sequence is identical to human endothelin (113), but differs from rat endothelin by 6 residues (114). Endothelin appears to be synthesized de novo as a 203 amino acid peptide termed preproendothelin, cleaved by endopeptidases to a 39 amino acid residue termed proendothelin or big endothelin, and then converted to active endothelin by a converting enzyme (112). In isolated vascular strips, endothelin is a potent (EC_{50} = 4 x 10^{-10} M) and slow-acting contractile agent. In vivo a single dose elevates blood pressure for 20-30 minutes (112). Endothelin binds to a single class of receptor sites which are distinct from other autonomic receptors and voltage- dependent calcium channels (115,116). Other properties of endothelin include inhibition of renin release (117), stimulation of atrial natiuretic factor release in rat atrial myocytes (118), and a positive inotropic action in guinea pig atria (119). Research on endothelin is progressing rapidly (120) and opportunities for new antihypertensive drug discovery should become evident in the not too distant future.



Endothelin

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Chapter 7. Pulmonary and Antiallergy Agents

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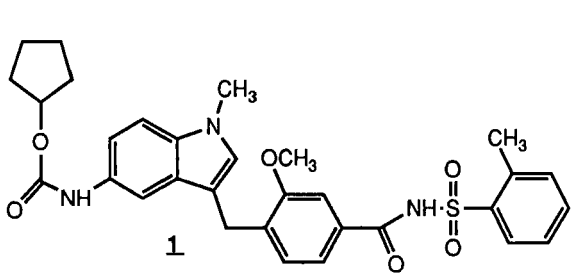
Introduction-The role of leukotrienes (LTs) and platelet activating factor (PAF) as mediators of asthma still remains to be proven. Significant progress has been made, however, in the introduction of orally-active LT antagonists, 5-lipoxygenase inhibitors and PAF antagonists into the clinic. In addition, interest continues in the development of phospholipase A_2 inhibitors, thromboxane antagonists, novel bronchodilating substances, peptide agonists and antagonists, mast cell mediator release inhibitors, anticholinergics and elastase inhibitors for use as antiasthmatics/antiallergics and for the treatment of emphysema. This chapter reviews the progress made during the past year in several of these areas. The reader is referred to Chapter 9 on PAF antagonists and Chapter 17 on inhibitors of phospholipase A_2 for more detailed information on each of these topics .

REGULATORS OF LIPID MEDIATORS

LTD_4 Antagonists - Although several LTD_4 antagonists have been tested clinically, these "first generation" compounds were not potent enough to fully test their role in treating asthmatics (1-3). More potent, orally-active compounds have recently been reported. ICI 204,219 (**1**) is a selective, competitive antagonist of LTD_4-induced contractions of guinea pig trachea (pK_b = 9.2) and isolated human bronchi (pK_b = 8.5). In guinea pigs, it produced a dose-dependent antagonism of LTD_4-induced bronchoconstriction by the oral, intravenous and aerosol routes and reversed LTD_4-induced bronchoconstriction (4). Chemistry leading to the styrylquinoline, L-660,711 (**2**), has also been described (5). This antagonist competed for ^3H-LTD_4 binding in guinea pig (IC_{50} = 1 nM) and human (IC_{50} = 10 nM) lung homogenates (6). It antagonized the action of LTD_4 in guinea pig ileum (pA_2 = 10.5), guinea pig trachea (pA_2 = 9.4) and human bronchi (pA_2 = 8.5). It was orally active in antagonizing LTD_4-induced bronchoconstriction in squirrel monkeys and antigen-induced bronchoconstriction in an "asthmatic" rat model (7). The (+)-enantiomer, L-668,018, was more active than the (-)-enantiomer *in vitro* but the potency was reversed *in vivo*, presumably due to a more facile metabolism of the (+)-enantiomer (8). Development of WY 45,911 (**3**) has been discontinued due to instability and mutagenicity but these problems were overcome with a close analog, WY



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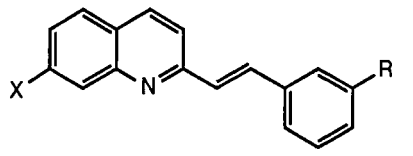
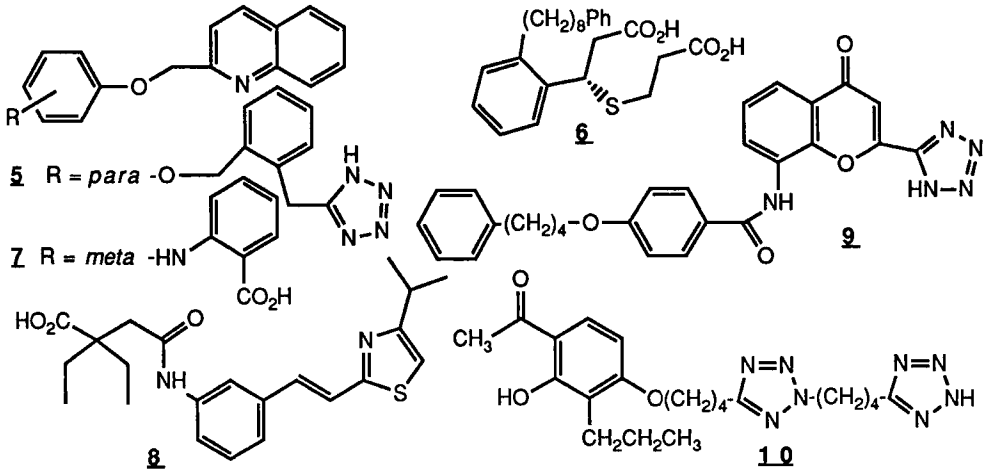


2 X = Cl, R = CH($S(CH_2)_2CO_2H$ (Na^+))($S(CH_2)_2CON(CH_3)_2$)

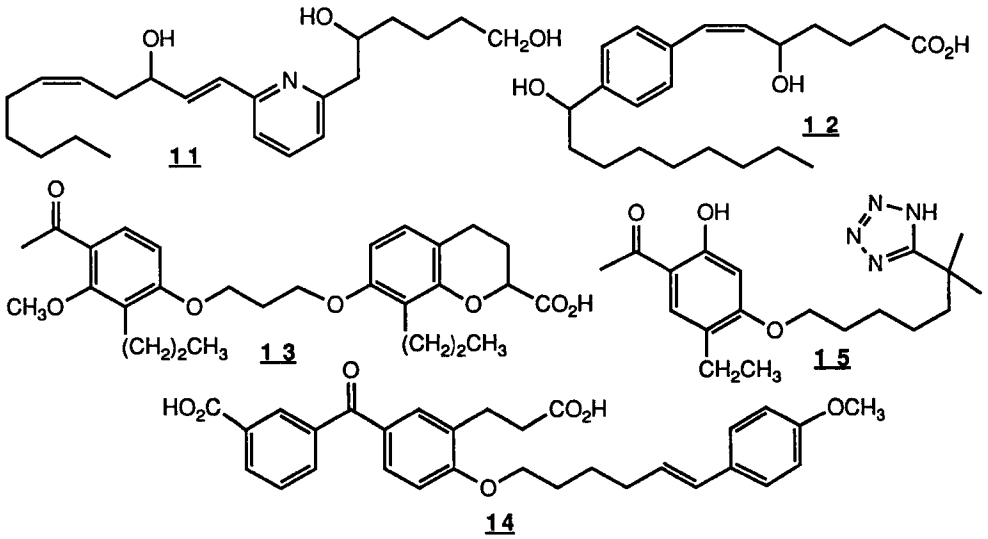
3 X = H, R = $N(OH)COCH_2CH_2CO_2CH_3$

4 X = H, R = $NHSO_2CF_3$

48,252 (4), in which a trifluoromethanesulfonamide group is substituted for the hydroxamic acid group (9). WY 48,252 was orally active against LTD_4-induced bronchoconstriction in guinea pigs (9) and sheep (10) and LTD_4-induced hypotension in rhesus monkeys (11). RG-12525 (5) evolved from a continuing synthetic effort with quinoline-based antagonists (12). It showed potent binding (Ki = 2.5 nM) in guinea pig lung homogenate and was active *in vivo* intravenously, orally and by aerosol. SKF 106,203 (6) antagonized LTD_4-induced contractions in guinea pig trachea (pK_b = 7.6) and was active *in vivo* following intravenous (ID_{50} = 1.1 mg/kg), intradermal, oral and aerosol administration (13). SR-2640 (7) has entered phase II studies (14). MCI 826 (8) was reported to dose-dependently inhibit antigen-induced late phase constriction in passively-sensitized, isolated human bronchi, *in vitro* (15). The synthesis of analogues leading to ONO-1078 (9) has been reported (16). A novel tetrazole compound, LY203647 (10), was described (17).

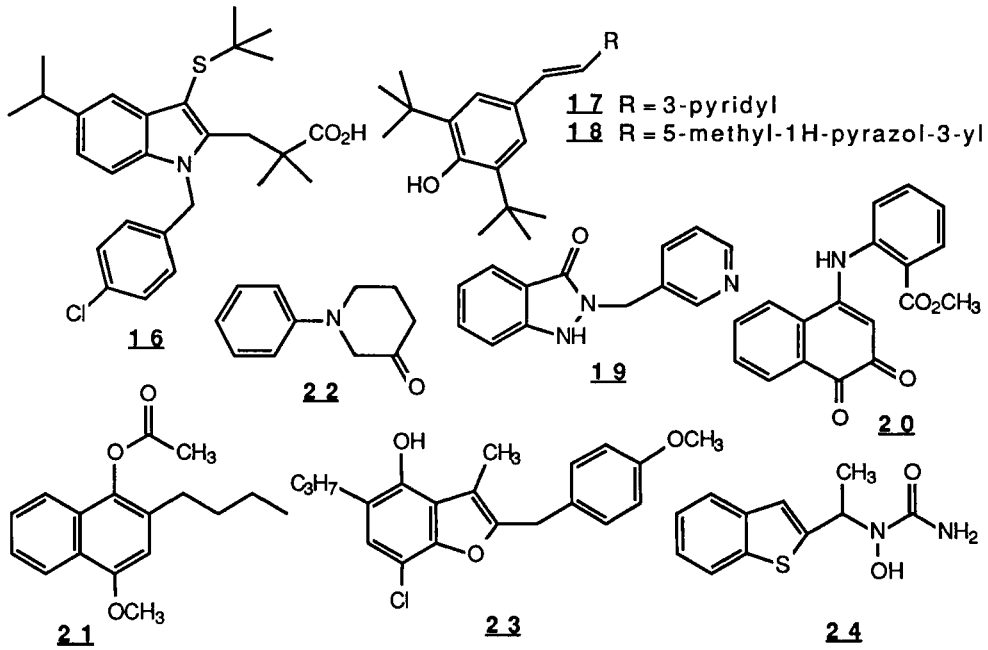


LTB4 Antagonists - LTB_4 is hypothesized to be a potential mediator of lung eosinophilia in asthmatics, a phenomenon believed to lead to airway hyperreactivity. For this reason,



LTB_4 antagonists may be useful in treating asthma. Several antagonists have recently been described, including U-75302 (11) and (12), in which the conformational freedom of the LTB_4 molecule has been restricted by incorporation of aromatic rings (18,19). U-75302 bound to human neutrophils (Ki = 1.3 μM) and prevented antigen-induced eosinophilia in guinea pigs at an oral dose of 1-30 mg/kg (20). SC-41930 (13) emerged from a series of chroman carboxylic acids after it was found that methylation of the hydroxy moiety of the acetophenone ring imparted selective LTB_4 antagonist activity (21). It competed with ^3H-LTB_4 binding to human neutrophils (IC_{50} = 0.3 μM) and was active in a number of chemotactic assay systems (22). LTB_4 antagonism has been reported (23,24) for LY223982 (14) and LY255283 (15) against 0.1 nM ^3H-LTB_4 in human neutrophils (IC_{50}'s = 12 nM and 87 nM, respectively).

5-Lipoxygenase (5-LO) Inhibitors - The initial steps involved in 5-LO activation in ionophore A23187-activated leukocytes (25) and IgE-stimulated RBL-2H3 cells (26) have been elucidated. During cellular activation, there is a Ca^{+2} dependent translocation of 5-LO from the cytosol to a membrane-bound site. The membrane-associated enzyme is preferentially utilized for LT synthesis, and this enzyme is inactivated as a consequence of LT metabolism (25). L-663,536 (16) is a unique compound which has been reported to inhibit LT synthesis in leukocytes (IC_{50} = 0.1 μM) by specifically inhibiting the translocation of 5-LO to the membrane (27).

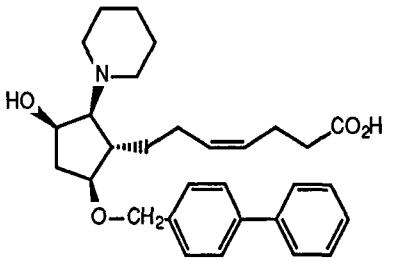


Several new dual 5-lipoxygenase/cyclooxygenase (5-LO/CO) inhibitors were also described. BI-L-93BS (17) is a t-butylphenol which exhibits dual 5-LO/CO inhibition *in vitro* and is orally active in animal models (28). A series of related styrylheterocyclic compounds was also reported and an orally-active compound, PD 127443 (18), was identified (29).

Of special interest with regard to future antiasthmatic therapy are reports of new, orally-active, selective 5-LO inhibitors. ICI 207,968 (19) was reported to be 200-times more potent an inhibitor of 5-LO than CO (30). This drug has been shown to be orally active in an *ex vivo* LT synthesis model in rats and rabbits. Similarly, CGS 8515 (20) is a potent selective 5-LO inhibitor *in vitro* and is orally-active (2 to 50

mg/kg) in *ex vivo* studies in rats (31). U-66,858 (**21**) is orally active (5 to 10 mg/kg) in rhesus monkeys challenged with *Ascaris* antigen (32). Finally A-53612 (**22**), L-656,224 (**23**) and A-64077 (**24**) also have been reported to be promising, orally-active, selective 5-LO inhibitors (33-35). A-64077 is currently in clinical evaluation and single doses of 25 to 800 mg/kg have been well-tolerated and have inhibited ionophore-induced LTB_4 production in *ex vivo* evaluations in man (35).

Thromboxane (TXA_2) Antagonists - The TXA_2 antagonist, GR 32191 (**25**), was reported to decrease allergen-induced bronchoconstriction in man (36). S-145 (**26**) and SQ-30741 (**27**), two new TXA_2 antagonists which contain structural elements of 13-azaprostanoic acid and the sulfonamide BM-13177, should prove useful in further defining the role of TXA_2 in human bronchopulmonary disease (37,38). In washed human platelets, (**26**) competed for binding with several TXA_2/PGH_2 mimics (IC_{50} = 3 nM). Studies with a previously reported series of compounds containing a 1,3-dioxane ring system has led to ICI 192,605 (**28**) which was orally active (0.01 mg/kg) in guinea pigs (39). A new structural series of antagonists, as exemplified by the indole -2-propanoic acid, L-655,240 (**29**), has been described (40). This series appears to exhibit a dual mode of action: antagonism of TXA_2 and inhibition of LT biosynthesis (e.g.**16**). Studies aimed at separating these activities yielded the specific TXA_2 antagonist L-670,596 (**30**). This compound has an IC_{50} = 2.5 nM and a pA_2 = 9 against U-44069-induced platelet aggregation and guinea pig tracheal contraction (41).



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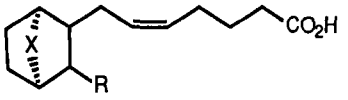
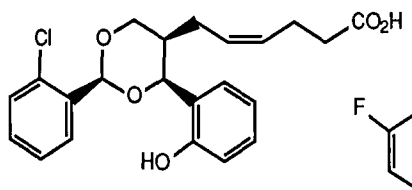


26 X = CH_2, R = NH-SO_2-C_6H_5

27 X = O, R = $CH_2NHCOCH_2NHCO(CH_2)_5CH_3$



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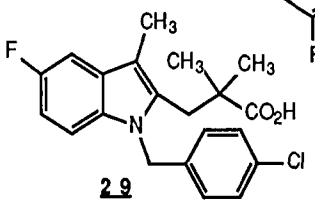

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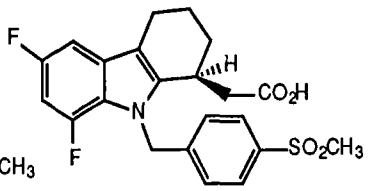

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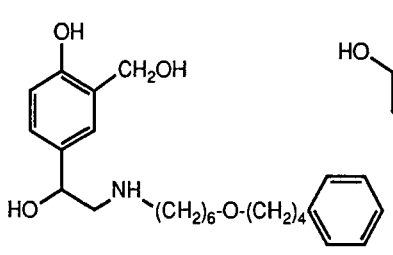
Phospholipase Inhibition and Steroids - Phospholipases constitute important families of enzymes which are now recognized to be major regulators of lipid mediator biosynthesis and are important enzymes in the signal transduction mechanisms found in key cells associated with the initiation of asthmatic disease. An important observation reported recently was the finding that in many cells, receptor-stimulated activation of phospholipase A_2 (PLA_2) occurs through the synthesis of a 28 kDa phospholipase activating protein, PLAP (42). Another important discovery was that phospholipase D activation may also be a key event in the triggering of inflammatory cells (43). Much of the past interest in phospholipases has centered on PLA_2 because of the hypothesis that steroids act partly through the synthesis of lipocortins which inhibit PLA_2 (44). Several recent studies have questioned the physiological significance of PLA_2 inhibition by lipocortins (45-47). In particular, two studies suggested that lipocortins interact non-specifically with the substrates in enzymatic reactions (46) and not directly with

the enzyme itself (47). Regardless of the above findings, the rationale remains strong that other types of inhibitors of PLA_2 may be useful antiasthmatic/antiinflammatory agents. This area of research continues to be active in the pharmaceutical industry and many new leads have been reported (see Chapter 17 of this volume).

BRONCHODILATORS

β_2-Agonists - Two β_2-adrenoceptor agonists, salmeterol (**31**) and formoterol (**32**), have been evaluated clinically by inhalation. In asthmatics, salmeterol (50, 100, 200 µg) produced sustained bronchodilation over a 12 hour period with no tachyphylaxis after 9 days of treatment (48). Inhaled formoterol, which is 10-times more potent than salbutamol, produced clinically significant bronchodilation for 8 hours when administered at a dose of 6 µg (49). The increased durations of effect of these two new β_2-agonists may make them particularly useful in patients with nocturnal asthma or persistent wheeze.



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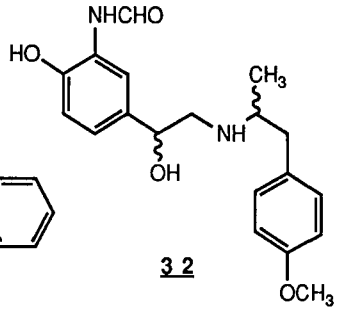

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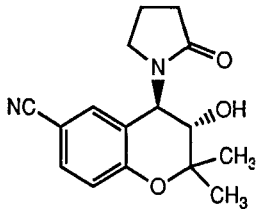

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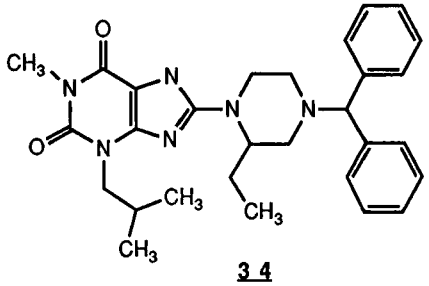
K^+ Channel Openers - Cromakalim (BRL 34915) (**33**), a novel potassium channel opener, protected both anesthetized and conscious guinea pigs from the bronchoconstrictor effects of serotonin and histamine (50). This drug was also reported to inhibit histamine-induced bronchonstriction in man (51). These observations suggest (**33**) may find clinical use as a bronchodilator. For a more complete review of compounds which modulate membrane K^+ channels see Chapter 10 of this volume.

Phosphodiesterase (PDE) Inhibitors - Interest in this area relates to the hypothesis that PDE inhibition is associated with the bronchodilatory activity of theophylline. A major goal in development of new theophylline (xanthine) derivatives has been the elimination of the CNS and cardiovascular side effects associated with theophylline. The orally active 8-amino alkyl substituted xanthine, S9795 (**34**), had no CNS or cardiovascular activity, was a more potent PDE inhibitor and had a longer duration of action than



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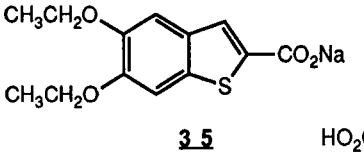

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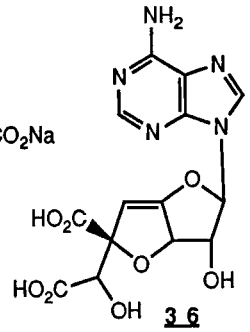

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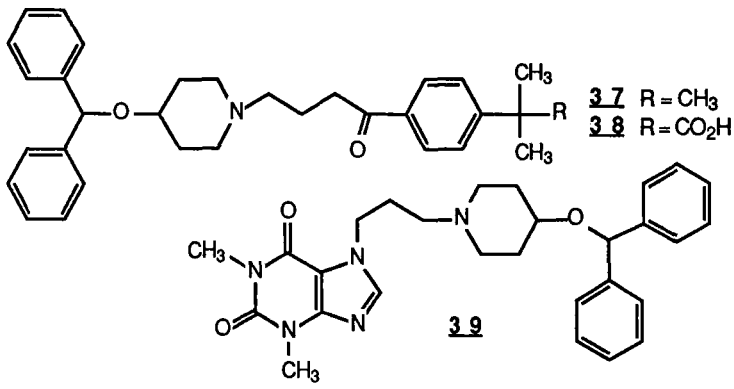
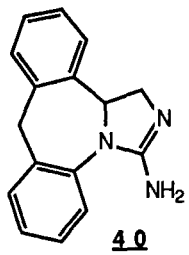
theophylline (52). S9795 also inhibited mediator release from mast cells (52). Tibenelast, LY186655 (35), an inhibitor of superoxide anion release and of leukocyte PDE, prevented spontaneous bronchoconstriction in asthmatics (53). New, potent and selective PDE inhibitors (54) have been derived from the nucleoside, griseolic acid (36). The search for airway-specific PDE inhibitors should be aided by progress in characterization of tissue-specific PDE isozymes (55) displaying distinct, isozyme-specific pharmacologic properties (56,57). Evidence for 5 isozymes in tracheal tissue has been presented (58).

ANTIALLERGICS

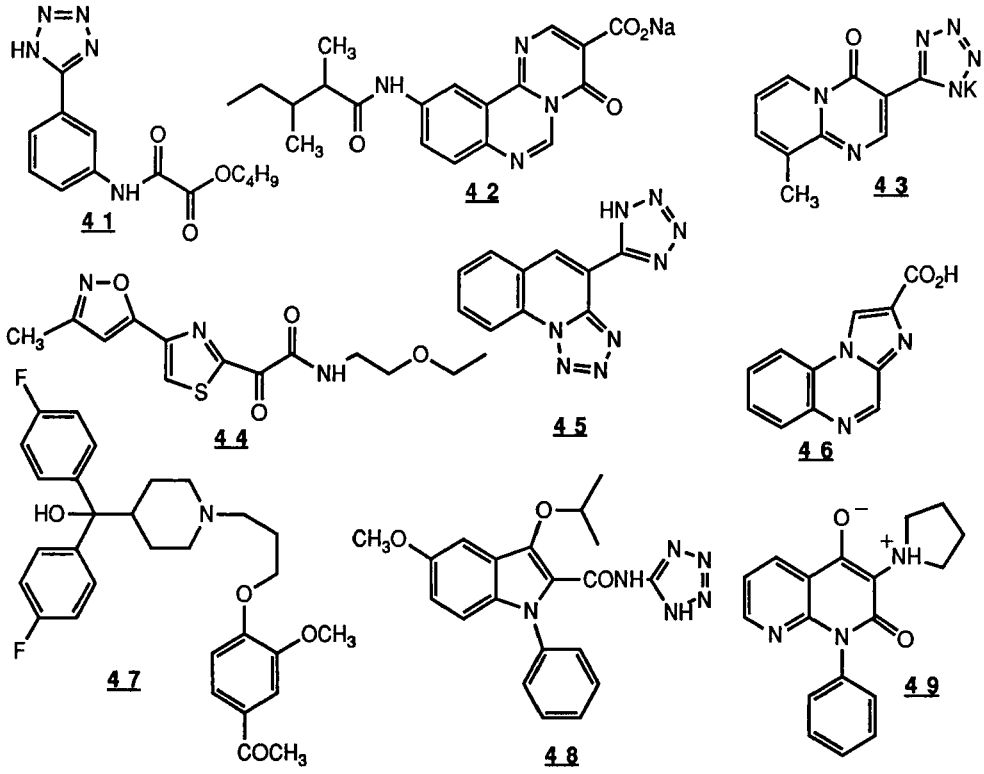
Non-sedating Antihistamines - The recent approval of the NDA for astemizole will allow this substance to join terfenadine as the only compounds of this class marketed in the US. Approval of NDAs for loratidine and cetirizine are expected in the near future. Ebastine (37) has been reported to be a potent, long-lasting antihistamine with no apparent sedation at an oral dose of 10 mg (59,60). Extensive first pass metabolism results in a carboxylic acid derivative, carebastine (38), which is responsible for most of the antihistaminic activity of (37). Once-a-day dosing may be possible with ebastine since it exhibited a $t_{1/2}$ of 10.6 hours in man at a dose of 10 mg (61). A complete pharmacological profile was reported for Wy-49,051 (39). As an antagonist of histamine-induced contractile responses in the isolated guinea pig ileum, Wy-49,051 is approximately 360-times more potent than astemizole (62). Wy-49,051 had a long duration of action in guinea pigs and low CNS penetration. A preliminary report also appeared recently on the *in vitro* and *in vivo* safety and efficacy of epinastine (40), a new, non-sedating antihistamine (63).





Mediator Release Inhibitors (MRI) - Five new compounds, including WP-833 (41), FR 50948 (42), TBX (43), Z1819 (44) and MDL 26,024G0 (45) have been described which were orally-active in the rat passive cutaneous anaphylaxis (PCA) test and inhibited IgE-induced mediator release from mast cells *in vitro* (64-70). A series of tricyclic-2-carboxylic acids active in the PCA test was also described of which (46) has been chosen for further development (71). AHR-5333 (47) was reported to inhibit immediate hypersensitivity reactions in 4 species by both the oral and aerosol routes and to exhibit a long duration of action. Its mechanism of action appears to result from both MRI and antagonism of H_1 and 5-HT_2 receptors (72-74). CI 949 (48) is an orally active MRI which protects against allergen-induced anaphylaxis in guinea pigs and dogs (75). *In vitro* (48), inhibits histamine release induced by a variety of stimuli in human basophils, and histamine, LT, and TXA_2 release from human and guinea pig lung

fragments (76,77). This compound also inhibits superoxide production by eosinophils and primary granule release from neutrophils (78,79), possibly by calmodulin antagonism (80). SCH 37224 (49) is now being studied clinically. It was reported to be active in rodent models and to inhibit allergen-induced early and late phase responses in allergic sheep (81,82). *In vitro*, (49) inhibits LTD_4 and TXA_2 release from allergen-stimulated guinea pig lung, but has little effect on histamine release (81).



It has been suggested that IgE receptor antagonists may be pursued in the future as MRI (83). A 76 amino acid fragment of the Fc portion of IgE is a lead for this approach since it was active in a ragweed PK reaction in human skin (84). Recent progress in the cloning of the subunits of the human IgE receptor may allow the future development of high flux *in vitro* screens for non-peptide antagonists (83). In addition, histamine release inhibitory factors (85) have been identified and may be novel tools in the development of MRI.

OTHER TOPICS

Peptides - Vasoactive intestinal peptide (VIP) has been postulated to be the peptide mediator of the non-adrenergic, non-cholinergic inhibitory pathway (86). Thus, VIP may be an important endogenous bronchodilator in man. For this reason, analogs of VIP may be a new class of bronchodilators. A novel precursor analog of VIP containing a C-terminal extension was reported to be 4-times more potent than native VIP as a relaxant of guinea pig airway smooth muscle (87). This preVIP (50) could be expressed genetically. Inhalation of (50) protected against *Ascaris*-induced bronchoconstriction in dogs (88). Helodermin (51), a 35 residue peptide structurally related to VIP, was

approximately equipotent to VIP as a relaxant of guinea pig tracheal smooth muscle; however, the relaxant action was 4- to 10-times more sustained (89).

50 Leu^{17} VIP-$Gly^{29}Lys^{30}$-OH

51 Ile^5 $Gln^{9,10}$ Ser^{11} Lys^{12} Leu^{14} Ala^{15} Lys^{16} $Leu^{17,19}$ Gln^{20} Ala^{24} Gly^{28} VIP-Ser^{29} Arg^{30} Thr^{31} Ser^{32} $Pro^{33,34,35}$-OH

52 D-Arg [Hyp^3 Thi^5 D-Phe^7 Thi^8]BK

53 D-Arg [Hyp^3 D-Phe^7]BK

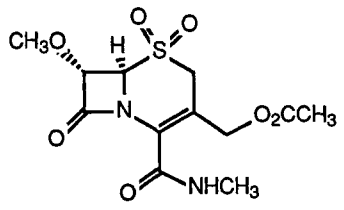
Thi = β-(2-thienyl)-L-alanine

Hyp = L-4-hydroxyproline

Antagonists of airway smooth muscle bradykinin (BK) receptors (B2) or neurokinin A (NK_2) receptors may represent novel anti-asthmatic therapies because both BK and neurokinin A are potent bronchoconstrictor agents in asthmatics, but not normal subjects (90,91). Two specific B2 antagonists have been reported. Peptide B-3824 (**52**) was an antagonist of BK-induced contraction of guinea pig isolated ileum (pA_2 = 6.4) and was also effective in equimolar intradermal concentrations against BK-induced vascular permeability in blood vessels of rabbit skin (92). Peptide NPC 567 (**53**) partially reversed BK-induced bronchoconstriction in guinea pigs when given intravenously (100 μg/kg), but it was not effective following inhalation (93). No specific NK_2-receptor antagonists have been reported.

Anticholinergics - Pirenzepine, an orally-active, specific M1 antagonist, increased lung volume in both large and small airways in asthmatics (94). These results indicated the functional role of M1 receptors in human airways and suggest the potential for development of anticholinergic drugs based on pirenzepine.

Elastase Inhibitors - Emphysema is hypothesized to result partly from an increase in human neutrophil elastase (HNE)load in the lungs. The β-lactam, L-657,183 (**54**), was reported to inhibit HNE *in vitro* and to inhibit elastase-induced hemorrhage in hamsters when instilled into the lung (95). ICI 200,880 (**55**) is a selective, "slow-binding", competitive inhibitor of HNE with a Ki = 0.5 nM (96). After aerosol administration, (**55**) demonstrated a long duration of action (inhibiting HNE-induced inflammation) in hamsters and was also active when administered under a "therapeutic" protocol, e.g. when given 24 hours after the elastase (97).



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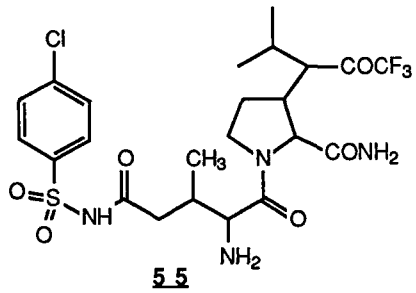

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Chapter 8. Novel Applications of Leukotriene Intervention

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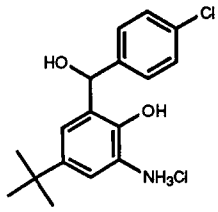
Introduction — Leukotrienes (LTs) are endogenous mediators with potent biological activities. The peptidoleukotrienes (PLTs) have profound effects on bronchial and vascular smooth muscle contractility and also promote extensive plasma extravasation by increasing the permeability of the postcapillary venules (1-4). Furthermore, LTB_4 is a potent chemotactic agent, enhancing the infiltration of leukocytes and their subsequent degranulation (5). As a class, LTs are intensively studied, especially in the areas of pulmonary and allergic diseases (Chapter 7), inflammation (6), inflammatory bowel disease (Chapter 18), and psoriasis (Chapter 19). In recent years, reports have appeared suggesting that LTs play a major role in many other diseases, and in this chapter evidence is reviewed that indicates a pathogenic role for LTs in the best studied of these lesser explored areas of disease. This perspective will be limited to those LTs (5-HETE, LTB_4, LTC_4, LTD_4, and LTE_4) that are synthesized from arachidonic acid (AA) *via* the 5-lipoxygenase (5-LO) pathway.

Cerebral Diseases

Stroke — Stroke is the third leading cause of death in the United States today (7) and despite tremendous advances in the understanding of the pathophysiology of cerebral ischemia, no drug therapy has yet proven beneficial for the treatment of stroke (8). It has been demonstrated that brief periods of cerebral ischemia do not necessarily lead to irreversible brain damage (9). As cells become ischemic, a cascade of events occurs which results in the release of AA from membrane phospholipids (7,9). Under normal conditions, the membrane fatty acids and phospholipids are recycled back into the membrane, resulting in a very low concentration of free AA. However, these processes require energy and in energy-spent ischemic tissue, membranes remain depleted of critical phospholipids and there is a rise in the intracellular concentration of free fatty acids (10). In general, cyclooxygenase (CO) and thromboxane synthetase inhibitors have not been efficacious in alleviating ischemic brain injury, and in some cases, this administration even seems to aggravate damage (11). By implication, if the eicosanoids are important mediators in ischemic injury, products of 5-LO rather than CO may be the injurious mediators.

In support of this contention, a study on selective 5-LO inhibitors (5-LOIs) in a spontaneously hypertensive rat cerebral ischemia model reported that ONO-LP-016, **1**, and AA-861, **2**, significantly inhibited LTC_4 levels (12). There did not appear to be any reduction of brain edema as measured by tissue water content. More recently, the 5-LO/CO inhibitors LY-178,002, **3**, and LY-256,548, **4**, have been reported to ameliorate neuronal necrosis in the hippocampus of gerbils subjected to carotid artery occlusion (13).



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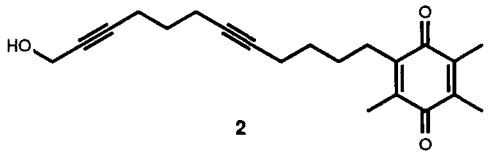

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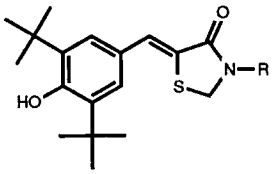
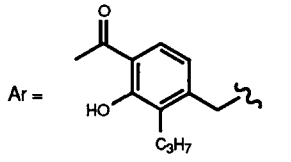


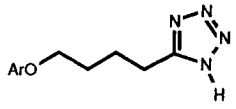
3 R = H
4 R = Me

Headache — The evidence for LT involvement in headaches is limited. In a study of 8 cluster headache patients, plasma levels of LTB_4 were found to be significantly elevated during an attack compared to symptom-free periods (14). Although the plasma levels of LTB_4 were not elevated in migraine headache patients, it was noted that A23187-induced release of LTB_4 from isolated polymorphonuclear leukocytes (PMNs) was approximately 50% greater during an attack than during symptom-free periods (14). More recently, it was reported that LTC_4 was detected in the plasma of migraine patients during the prodromal phase and in greater amounts during the beginning of the attack phase (15). This PLT was gradually replaced by LTB_4 as progression occured through the post-attack phase. The symptom-free period was characterized by nondetectable levels of both LTs.

Cardiovascular Diseases

Shock and Trauma — Although not conclusive, recent evidence strongly implicates LTs as significant factors in the pathophysiology of anaphylactic, endotoxic, and traumatic shock (1,16-19). Products of 5-LO have been detected in several, although not all (20), animal models of shock. LY-171,883, **5**, a LTD_4 antagonist and phosphodiesterase inhibitor, has been shown to reduce endotoxin-induced leukopenia and hemoconcentration in the rat (21), as well as increasing survival time (22). Pretreatment with **5** also significantly blunted the endotoxin-induced reduction in cardiac output, mean arterial pressure, and multiple organ blood flows (23). In a canine model of septic shock, **5** appeared to increase survival time, but did not improve overall mortality or erythrocyte deformability (24). Endotoxemia was also induced in conscious sheep and in this model, **5** increased cardiac output, which was accompanied by a drop in pulmonary arterial pressure (25). Similarly, when isolated guinea pig hearts were challenged with ovalbumin in the presence of antihistamines, **5** was effective in blocking the reduction in coronary flow (26). Other LTD_4 antagonists have also been examined. Hemorrhaged rats treated with L-649,923, **6**, maintained a higher post-reinfusion mean arterial blood pressure and demonstrated a significantly increased survival rate (27). Comparable results were noted upon pre-treatment with LY-163,443, **7**, in a murine model of splanchnic artery occlusion shock (28); however, no improvement was observed when **7** was administered 15 min after occlusion (post-treatment). SKF-104,353, **8**, significantly improved mean survival time and survival rate at 48 h over endotoxin alone in conscious rats (29). Hemoconcentration was significantly attenuated as well.





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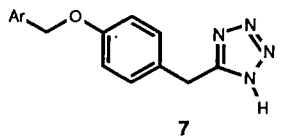

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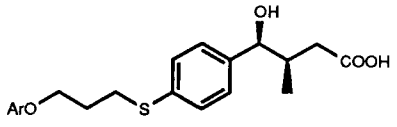

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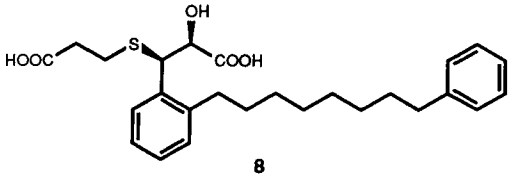

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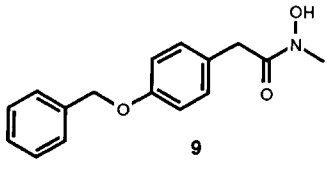
In the presence of antihistamines, the selective 5-LOIs, AA-861, **2**, and RG-6866, **9**, were found to effectively block the ovalbumin-induced drop of coronary flow in isolated guinea pig hearts (26). Infusion of CGS-8515, **10**, into isolated, sensitized, and indomethacin-treated guinea pig hearts markedly inhibited both the ovalbumin-induced rise in coronary perfusion pressure and LTC_4 release in a dose dependent fashion (30). Compound **10** also reduced the endotoxin-induced hypotension and leukopenia in shocked rats (31). Histologic examination revealed that **10** prevented the pulmonary sequestration of neutrophils.

Dual 5-LO/CO inhibitors have also shown some efficacy. BW755C was found to be effective in ameliorating many of the changes observed with endotoxin or ovalbumin adminstration (32,33). In two murine models of endotoxic shock, SKF-86,002, **11**, protected the animals from mortality (34). Protection was also observed when **11** was administered after endotoxin injection, albeit only at early time points. A combination thromboxane receptor antagonist and LO inhibitor, L-655,240, **12**, demonstrated significant protection in the rat traumatic shock model, increasing survival time from 82 min to 206 min (35). In the same model, dual 5-LOI and phospholipase A_2 inhibitors, CGP-33,304, **13**, and CGP-35,949, **14**, were found to be more effective than just the single mediator agent, selective 5-LO inhibitor CGS-5677 (36).



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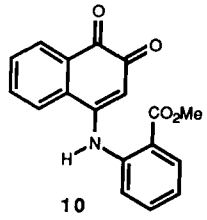

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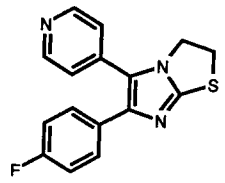

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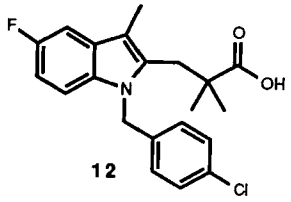

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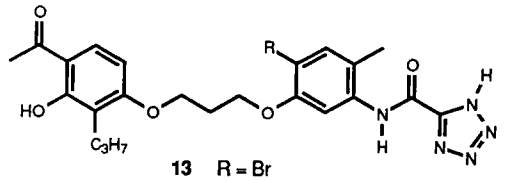


13 R = Br
14 R = Cl

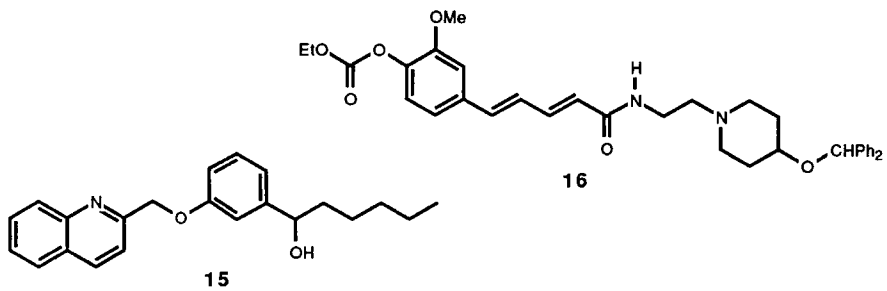
Myocardial Infarction — The principal focus of LT research in heart diseases has been on cardiac ischemia, as it relates to myocardial infarction (MI) (37-39). As in cerebral ischemia, LTs are not thought to precipitate the initial occlusion, but rather to be important factors in the expansion of the local ischemic event, ultimately leading to cellular necrosis (cf. Stroke section). There is some evidence that the PLTs may not play a major direct vasoconstrictive role in cardiac ischemia (40,41); that in fact, the cardioprotection afforded by 5-LOIs is due only to the suppression of neutrophil accumulation (38,41).

Early canine studies involving coronary artery occlusion demonstrated approximately a 50% reduction in infarct size upon pre-treatment with nafazatrom, a 5-LOI and antithrombic agent (42). More recently, a single, oral dose of AA-861, **2**, was shown to decrease LTB_4 levels to that of the controls and suppress both PMN counts and infarct size in a rat coronary artery occlusion model (43). REV-5901, **15**, a combined 5-LOI and LTD_4 antagonist, was tested in a rabbit myocardial occlusion/reperfusion model. Infarct size was reduced and this myocardial protection was accompanied by reduced neutrophil accumulation in the ischemic regions of the heart (44).

Renal Diseases

Evidence implicating the involvement of LTs in diseases of the kidney has been reviewed (45-49). The most frequently mentioned renal disease suspected of being at least partly LT mediated is human glomerulonephritis, a principal cause of renal failure. In two models of rat nephrotoxic serum glomerulonephritis (NSGN), production of LTs by isolated glomeruli was elevated (50-53). Glomerular tissue is the indicated source of LTs in NSGN, perhaps with initial surface activation by C_{5a}. Platelet activating factor (PAF), synthesized by kidney, was also noted as a possible mediator of renal disease, an action partially attributable to stimulation of LT synthesis (54).

In normal isolated perfused rat kidneys, i.v. infusion of LTC_4 produced a dose dependent increase in vascular resistance. This action was blocked by FPL-55,712 (55). Rats with NSGN were treated with a bolus i.v. dose and an infusion of the LTD_4 receptor antagonist SKF-104,353, **8**. The single nephron glomerular filtration rate was maintained, as was the the glomerular capillary ultrafiltration coefficient. In these experiments, **8** also blocked proteinuria. This latter effect is consistent with the ability of LTC_4 and LTD_4 to increase microvascular permeability to plasma proteins (56). In a preliminary report, the 5-LO inhibitor TMK-688, **16**, also inhibited proteinuria associated with NSGN in rats (57).



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15

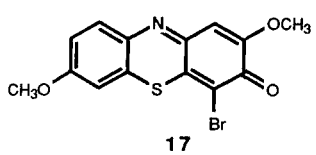
Gastrointestinal Diseases

The upper gastrointestinal effects of LTs have been briefly reviewed (58,59). The effects of i.a. infusions of LTB_4, LTC_4 and LTD_4 were studied in isolated fundic portions of dog stomach (60). Infusions of LTC_4 or LTD_4 significantly and dose-proportionally reduced gastric blood flow, gastric mucosal blood flow, gastric oxygen consumption and acid output; LTB_4 had no effect. LTC_4 or LTD_4 also gave significant dose-dependent inhibition of histamine-stimulated elevations in the gastric acid output, gastric blood flow and gastric mucosal blood flow (60). These results suggested that LTC_4 and LTD_4 inhibited gastric secretion as a result of direct inhibition of oxyntic glands with a possible contribution from induced ischemia of the gastric mucosa (60). Vasoconstrictor effects of LTC_4 and LTD_4 have been shown in segments of dog distal ileum (61). LTC_4 infused into conscious dogs with Thomas gastric fistulas or Heidenhain pouches gave significant dose-dependent inhibition of histamine-induced acid secretion. Similar results were obtained with pentagastrin and meal-induced acid secretion (62).

In a model for gastric cytoprotection utilizing concentrated ethanol as an irritant to induce hyperemia and gastric lesioning in rats, the ethanol-induced lesions were blocked by BW755C or nordihydroguaiaretic acid (NDGA). The hyperemia was proposed to be initiated by a rapid LTC_4-induced venous constriction followed by a contribution of LTC_4 to severe mucosal edema formation (63). Pretreatment in this model with the 5-LO inhibitor L-651,392, **17**, tended to inhibit lesions. Eicosapentaenoic acid produced a significant reduction in gastric lesioning (58). Administration of either LY-171,883, **5**, or REV-5901, **15**, gave dose-dependent inhibition of gastric lesioning. The effect of **15** was not reversed by indomethacin pretreatment, but that of **5** was reduced, implying that at least **15** was working through a 5-LO rather than a prostaglandin mechanism (64).

The proposal that LTC_4 has importance in causing ethanol lesions was recently challenged (65). Compound **17** significantly and dose-proportionally inhibited LTC_4 production (74% maximum inhibition) as did BW755C. At a dose of 20 mg/kg i.v., L-649,923, **6**, also significantly inhibited gastric lesions. BW755C and **17** did not increase prostaglandin release; however, **6** approximately tripled the output of prostaglandins measured as 6-keto-$PGF_{1\alpha}$. There was not a correlation between LTC_4 released from hemorrhagic tissue and the degree of lesioning (65).

While gastrointestinal irritation from NSAID therapy could arise from the inhibition of cytoprotective prostaglandins, mediation by enhanced LT synthesis *via* AA shunting has also been suggested. It was shown that 5-LOIs, **15** and **17**, can block NSAID-induced lesions in mouse stomachs (66). Several antagonists of LTD_4 also gave significant decreases in lesioning, such as **5**, **6**, FPL-55,712, and WY-44,329, **18**. The LTD_4 antagonists, but not the 5-LOIs, were effective against aspirin induced lesions (66).



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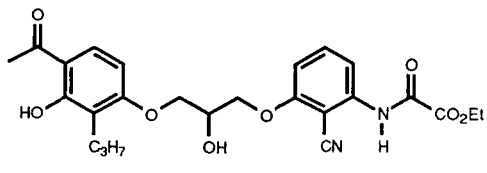
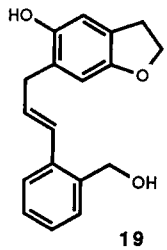


18

Ocular Diseases

A number of 5-LOIs have been studied in experimental models of ocular inflammation. Antigen-induced vascular permeability changes in guinea pig conjunctiva were significantly inhibited by topical applications of L-651,392, **17**, and L-651,896, **19**, (67). Nafazatrom and NDGA were examined in a lens protein induced granulomatous uveitis model in rats (68). Both 5-LOIs were found to inhibit the increase in choroidal thickness. This attenuation of granulomatous inflammation was accompanied by a reduction in the infiltration of pigment containing giant cells. In both the allergic conjunctivitis model and the granulomatous uveitis model, indomethacin potentiated the effects of the antigen challenge, presumably by AA shunting to 5-LO.

No significant inhibition was found with L-649,923, **6**, and L-648,051, **20** (selective LTD_4 antagonists), after a single challenge of ovalbumin in the guinea pig allergic conjunctivitis model (69). The increase in conjunctival microvascular permeability was blunted by mepyramine (H_1 receptor antagonist) and this inhibition was not increased by combining mepyramine with **20**. However, significant inhibition was observed after a second challenge 24 h later. In actively sensitized guinea pigs, topical ocular administration of antigen produced an increase in extravascular albumin content (EAC) in the bulbar conjunctiva (70). This response was unaffected by pre-challenge doses of FPL-55,712, SKF-102,922 (**21**, LTD_4 antagonist), and NDGA, whereas a pyrilamine/cimetidine pretreatment reduced the increase in EAC by 50%. However, there was a greater reduction in EAC when the LTD_4 antagonists or 5-LOI were used in conjunction with the histaminergic agents.



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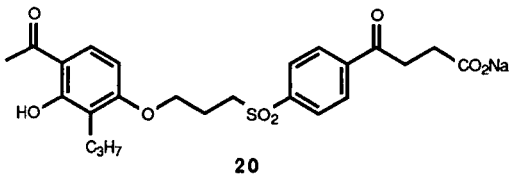

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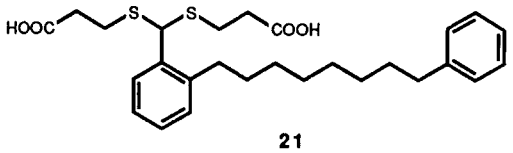

21

Burn Injury

Depending on the extent of injury, thermal damage as a result of burn can lead to severe alterations in immune functions. Included are PMN dysfunctions of chemotaxis, phagocytosis, bacterial killing and the mounting of inflammatory reactions (71-73). In severely burned human patients there is a rapid onset and doubling of *in vitro*, A23187 stimulated, LT synthesis capacity (5-HETE and LTB_4) by circulating PMNs (71). This effect peaks at 3 h, returns to below control levels by 24 h, and

remains significantly depressed for approximately 20 days during the post-burn anergic phase. Patient recovery is associated with a return to normal capacity of PMN LT synthesis. The early burst of LTB_4 production from PMNs in burn patients has been proposed as a possible contributor to the cause of post-burn immune suppression (71-73). LTC_4 levels were shown to roughly parallel the LTB_4 trend and were elevated after 20 days in association with an increase in eosinophil content of granulocytes (71). The observed decreased synthesis of LTB_4 by PMNs may be partially attributable to increased metabolism of LTB_4. PMNs from severe burn patients were shown to have a significantly decreased expression of LTB_4-receptors. Explanations for reduced LTB_4-receptor expression include a shift to an immature PMN population and receptor desensitization due to high circulating, tissue derived, LTB_4 levels found in severe burn patients (74). Post-burn high plasma LTB_4 levels and the initial LTB_4 synthesis burst, the accompanying state of immune suppression and lack of neutrophil responsiveness to LTB_4, suggest that rapid intervention with a 5-LOI or LTB_4 antagonist, sufficient to return LT activity to normal levels, could be of therapeutic value.

Allograft Rejection

Although kidney transplantation is common, rejection of the graft remains a serious problem. Renal allografts which are undergoing rejection, are involved in complex immunologic and inflammatory responses with production of high levels of a host of potential mediators including LTs. Renal cortical tissue from dog renal allografts undergoing rejection produces significant quantities of 5-HETE (75) and a 47-fold increase in LTB_4 (76) upon stimulation with A23187. LTC_4 production is elevated in tissue from rejecting renal allografts in rats (77). There was not a significant difference in LT production from normal and rejecting renal medullary tissue. Dogs with renal allografts were treated orally from the time of transplantation with 10 mg/kg b.i.d. of BW755C. Decrements in blood flow, filtration rate, and urine production were significantly inhibited versus non-drug treated dogs. Drug treated animals also experienced significantly less cellular infiltration and tissue damage (78).

Conclusion

The 5-lipoxygenase products from arachidonic acid have been implicated as mediators in a diversity of diseases. Although these leukotrienes may not be involved in the initiating events, they appear to play important roles in the propagation of the disease states, extending and in fact exacerbating the local events, ultimately leading to tissue damage. A contribution of leukotrienes to homeostasis is also frequently implied. However, the exact nature of these roles remains to be defined. The development of second generation 5-lipoxygenase inhibitors and antagonists of LTB_4, LTC_4, and LTD_4 with extremely enhanced potency and selectivity offers the promise of new insights into the contributions of leukotrienes to the pathophysiology of these diseases.

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Chapter 9. PAF Antagonists

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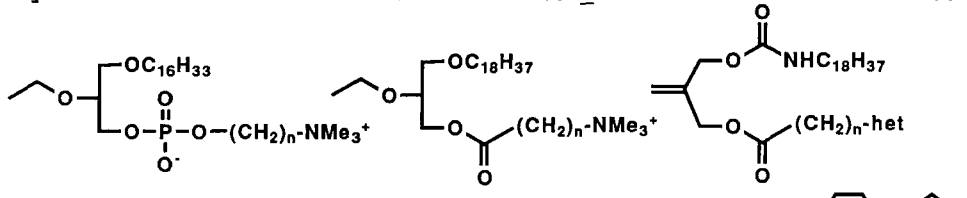
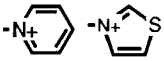
Introduction - Since the discovery of platelet activating factor (PAF) in 1972 and its first synthesis in 1979 a wide variety of structural types exhibiting potent PAF antagonist activity have been discovered. Evaluation of these antagonists has facilitated an assessment of the physiological and pathophysiological roles of PAF, not only using animal models, but also in man. Reviews on the role of PAF as a mediator of allergic disease (1) and in cellular responses (2) have appeared, as have general reviews on PAF research (3,4,5,6). The use of antagonists in determining the biological significance of PAF has also been reviewed (7,8), with particular emphasis on asthma (9) and ischaemic states (10).

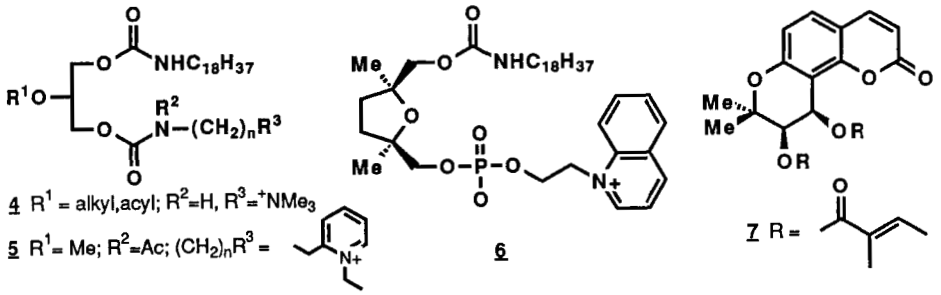
SAR OF PAF ANTAGONISTS

PAF Analogues and Natural Products - The proaggregatory agonist activity of the O-ethyl PAF analogues **1** falls dramatically as the length of the alkyl chain separating the phosphate and trimethyl ammonium moieties is increased. Compounds **1**, where n = 5 or 6, are pure antagonists with IC_{50}'s of 4 and 10 μM respectively in rabbit platelets (11). Substitution of the phosphate group with a carboxyl isostere in both **2** and **3** abolishes agonist activity, giving full antagonists (12,13). N-Acylation of the carbamate isostere series **4** resulted in more than a ten-



1 n = 2 to 6 **2** n = 2 to 4 **3** n = 5 to 6, het = 

fold increase in antagonist potency (14). Subsequent replacement of the trimethyl ammonium group with either N-alkyl pyridinium or N-alkyl thiazolium also enhanced potency, leading to CV 6209 (**5**) (IC_{50} = 75 nM), where the enantiomers are equipotent (15). In contrast, the N-quinolinium tetrahydrofuran SRI 63-675 (**6**) is reported to show differences in the potency of its enantiomers (16), while the racemic SRI 63-675 inhibits rabbit platelet aggregation with a pA_2 of 7.18 using a standardised method which has been used to compare several distinct PAF antagonists (17, 18). The chemistry, pharmacology and clinical applications of the ginkgolide antagonists have been reviewed (19, 20), as have the chemistry and pharmacology of the diketopiperazine derivatives (21). A series of khellactone derivatives with weak antagonist activity has been described (22) where optimum activity occurs with cis stereochemistry and acylated derivatives. Optimal activity resides in **7** which has an IC_{50} of 40 μM.



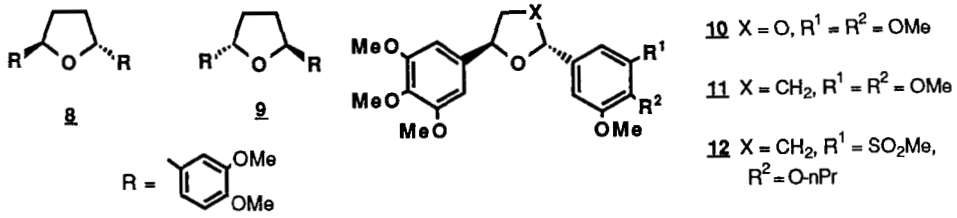
4 R^1 = alkyl,acyl; R^2=H, R^3=$^+NMe_3$

5 R^1= Me; R^2=Ac; $(CH_2)_nR^3$ =

6

7 R =

Synthetic PAF Antagonists - The two enantiomers **8** and **9** of 2,5-bis-(3,4-dimethoxyphenyl)tetrahydrofuran were essentially equipotent with Ki's of 0.43 and 0.37 μM, respectively (23), suggesting the oxygen and $-CH_2-CH_2-$ units of the tetrahydrofuran are interchangeable on the receptor. The 1,3-dioxolane **10**, an analogue of L 652731 (**11**), was also synthesised (23) and showed antagonist activity (Ki = 0.3 μM). The novel nonsymmetrical diaryl tetrahydrofuran, L 659989 (**12**) is approximately 8 times more potent than L 652731 with a K_B of 1.1 nM against PAF-induced rabbit platelet aggregation (24). The *cis* isomer is 100 to 200 times less potent and, interestingly, the (+)-isomer of **12** is 20 to 30 fold more potent than the (-)-isomer (25).

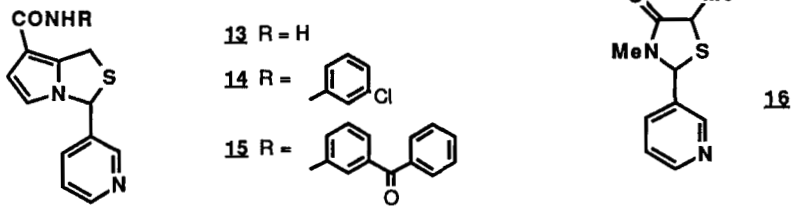


10 X = O, R^1 = R^2 = OMe

11 X = CH_2, R^1 = R^2 = OMe

12 X = CH_2, R^1 = SO_2Me, R^2 = O-nPr

Modification of the pyrrolodihydrothiazole RP 48740 (**13**) led to the more potent series of N-aryl amides. RP 52770 (**14**), the N-(3-chlorophenyl) derivative, displayed highly potent and specific PAF antagonist activity some 26 times greater than L 652731 (26). Radiolabelled RP 52770 has been used to characterize the PAF binding sites on rabbit platelets and human polymorphonuclear leukocytes (PMNL) (26, 27). In both preparations the (+)-enantiomer of RP 52770 was 300 to 600 fold more potent than the (-)-enantiomer. Further optimisation of the pyrrolodihydrothiazole series led to RP 59227 (**15**), which is a single enantiomer and highly potent with an IC_{50} of 0.16 nM against rabbit platelet aggregation (28). In contrast, a related thiazolidine derivative, SM 10661 (**16**), was much weaker (IC_{50} = 3.9 μM) in a similar assay (29).



13 R = H

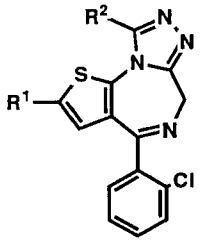
14 R =

15 R =

16

The methyl substituent of the triazole group in brotizolam (**17**) is the optimum for PAF antagonist activity. Replacement with hydrogen **18**, cyclohexyl **19**, or hydroxyl **20** reduces the affinity for the PAF receptor such that **19** and **20** are inactive (30). Similarly, the cyclohexyl analogue **21** is 400 times weaker than WEB 2086 (**22**) as an inhibitor of whole blood aggregation (31). Modification of WEB 2086 led to two new

antagonists, WEB 2170 (**23**) and STY 2108 (**24**), inhibitors of human platelet aggregation with IC_{50}'s of 0.3 μM and 0.04 μM respectively (32). The (-)-isomer of WEB 2170 is the more potent enantiomer, the (+)-isomer being approximately 20 fold less potent (33).



17 R^1 = Br, R^2 = Me

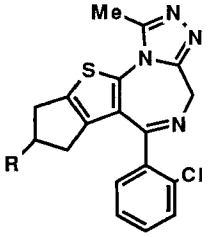
18 R^1 = Br, R^2 = H

19 R^1 = Br, R^2 = cyclohexyl

20 R^1 = Br, R^2 = OH

21 R^1 = CH_2CH_2CON(morpholine), R^2 = cyclohexyl

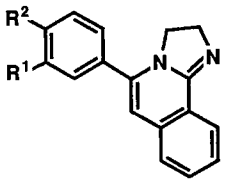
22 R^1 = CH_2CH_2CON(morpholine), R^2 = Me

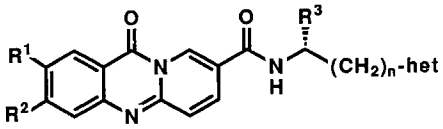


23 R = CON(morpholine)

24 R = CH_2N(morpholine)

The dihydroimidazoisoquinolines were derived from consideration of the structure of PAF and one example, SDZ 64-412 (**25**), exhibited potent antagonist activity with an IC_{50} of 60 nM against human platelet aggregation (34, 35). A group of pyridoquinazoline carboxamide antagonists have been synthesized and the SAR discussed (36). A 3-pyridyl or 5-pyrimidyl group coupled with a hexyl or butyl chain were the optimum amide substituents with **26** being the most active *in vitro* (IC_{50} PAF binding = 0.1 μM). A methyl group was introduced α to the amide nitrogen to inhibit *in vivo* degradation, where the R isomer **27** was the more potent and stable isomer.



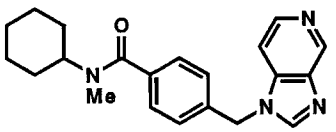


25 R1 = H, R2 = CH_2CH_2-(2,3,4-trimethoxyphenyl)

26 R^1 = R^2 = Me, R^3 = H, n = 3, het = 4-methylpyrimidin-5-yl

27 R^1 = iPr, R^2 = H, R^3 = Me, n = 3, het = 3-methylpyridin-4-yl

The imidazopyridine derivative **28** and the antithrombotic dihydropyridine PCA-4230 (**29**) were both reported to have weak antagonist activity with IC_{50}'s of 5 and 38 μM, respectively, against human platelet aggregation (37, 38).



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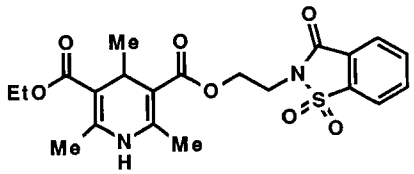

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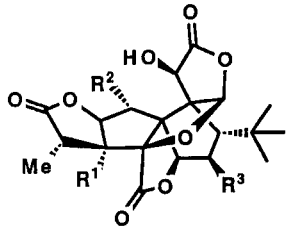
Receptor Studies - Specific receptor sites for PAF have been characterised in rat liver (39) and gerbil brain (40). The former displayed a single population of receptors (Kd = 0.51 nM, Bmax = 141 fmol.mg $protein^{-1}$), whereas the latter was reported to show two binding sites (Kd_1 = 3.66 nM, $Bmax_1$ = 0.83 pmol.mg $protein^{-1}$; Kd_2 = 20.4 nM, $Bmax_2$ = 1.1 pmol.mg $protein^{-1}$). The antagonists BN 52021 (**31**) and L 652731 produced only partial displacement of ^{3}H-PAF from the brain preparation, suggesting that they interact with only one site. A second

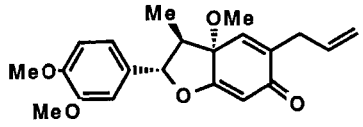
receptor type has been postulated in human PMNL, distinct from the platelet receptor, based on differences in antagonist potencies and G-protein coupling (41). A range of antagonists have been profiled for displacement of ^{3}H-PAF from canine (42) and human (43) platelet membranes and two studies attempting a correlation of ^{3}H-PAF displacement from human platelets with in vivo activity have been reported (44, 45). Poor correlations were noted across the various structural types, but within related series, such as PAF analogues, the correlation was good. In a comparative study using human platelets ^{3}H-WEB 2086 appeared to be a more suitable antagonist for labelling PAF receptors than ^{3}H-RP 52770, since ^{3}H-WEB 2086 exhibited classical competitive antagonism (46).

PHARMACOLOGICAL EVALUATION OF PAF ANTAGONISTS

The functional consequences of PAF antagonist interaction with PAF receptors has been evaluated in various cell types and isolated tissues in vitro and in several organ systems in vivo. In many cases, PAF antagonist efficacy has been evaluated against responses to exogenous and endogenous PAF and where possible these studies are compared directly in the following sections.



30 R^1 = OH, R^2 = R^3 = H
31 R^1 = R^2 = OH, R^3 = H
32 R1 = R2 = R3 = OH
33 R1 = R3 = OH, R2 = H
34 = **30** : **31** : **32** - 2 : 2 : 1



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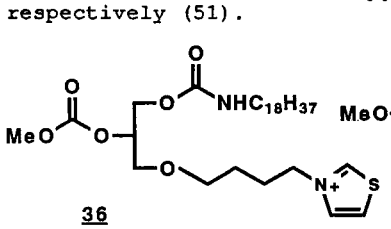
Inflammation - PAF can induce several functional responses in a variety of inflammatory cells. Thus, BN 52021, L 652731 and kadsurenone (**35**) inhibit the adhesion of PMNL to endothelial cells and the release of inflammatory factors induced directly or indirectly by PAF (47). L 659989 is a potent antagonist of PAF-activated degranulation of rat (ED_{50} = 4.5 nM) and human (ED_{50} = 10 nM) PMNL (24). BN 52021 inhibits chemotaxis induced by PAF (IC_{50} = 490 nM) (48) and WEB 2086 antagonises the degranulation of human eosinophils (IC_{50} = 4-6 nM) (49) and the rise in intracellular calcium in cells from guinea pig (50). PAF, LTB_4 and ionomycin increase intracellular calcium levels in cultured U937 cells, which possess many characteristics of macrophages. Only the PAF-induced response was inhibited by WEB 2086, RO 19-3704 (**36**), L 652731, BN 52021 and CV 3988 (**37**), with IC_{50} values of 48, 118, 318, 340 and 2320 nM, respectively (51).



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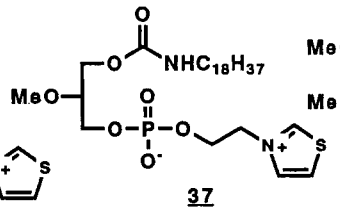

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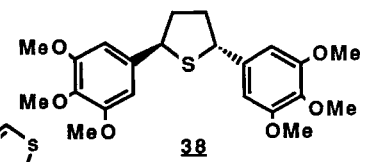
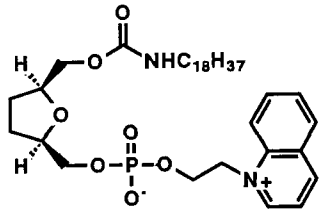


38

The role of PAF in cellular immune responses is complex (2). The IL_2-stimulated proliferation of T-lymphoblasts (52) is inhibited by L 652731 and CV 3988 which, together with L 653150 (**38**), also inhibit T-cell proliferation in mitogen-stimulated human peripheral blood mononuclear cell cultures (52, 53), possibly via induction of suppressor cell activity (54), as shown with BN 52021, WEB 2086 and CV 3988.

Pulmonary - The release of TxA_2 by lungs from normal or sensitized guinea pigs challenged with PAF or antigen is inhibited by BN 52021 (55) and WEB 2086, the latter antagonising bronchospasm only to PAF (56). Increases in basal perfusion pressure and potentiation of vasoconstriction to angiotensin II and hypoxia in perfused rat lungs produced by SRI 63-441 (**39**) and L 659989 indicate a role of PAF in the control of pulmonary vascular tone (57).



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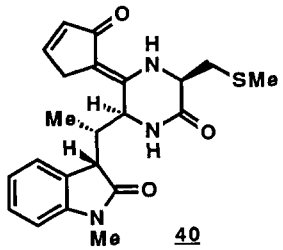

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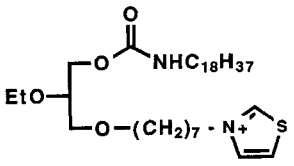


41

Bronchoconstriction to infused PAF in guinea pigs was inhibited by L 659989 both intravenously and orally, and by oral SDZ 64-412 (25). The pyridoquinazoline carboxamide (**27**) inhibited both the acute bronchospasm and hypotension induced by intravenous PAF (36). These responses to PAF and to antigen in passively sensitized guinea pigs were inhibited by oral (58) and intravenous (56, 58) WEB 2086. The latter either had inhibitory (58) or no effect (57) on antigen-induced bronchospasm in actively sensitized animals. Similarly, CV 3988 and L 652731, at doses which effectively inhibited PAF bronchospasm, failed to influence responses to antigen in either actively or passively sensitized animals (59). WEB 2086 and BN 52021 reduced both PAF and antigen-mediated accumulation of eosinophils into the lung (60), while SRI 63-441 and BN 52021 prevented the bronchial hyperreactivity to histamine induced by intravenous injection (61) or slow infusion (62) of PAF. Bronchospasm, hypotension and the intrathoracic accumulation of platelets induced in guinea pigs by intravenous PAF were inhibited by STY 2108 and WEB 2170 at low oral doses (63), with biological half-lives of 4 and 11 hours, respectively (32). The relative potencies of the isomers of WEB 2170 against PAF-induced bronchospasm reflected their antiaggregatory potencies, (-) WEB 2170 being about 20-fold more potent than the (+)-isomer (33). Oral WEB 2170 (1 $mg.kg^{-1}$) prevented antigen-induced lethality, bronchospasm and hypotension in actively sensitized guinea pigs (64). In inhalation challenge studies, intravenous and inhaled RO 19-3704, but not intravenous CV 3988, inhibited bronchospasm in guinea pigs induced by PAF or antigen (65). BN 52021 inhibited not only bronchospasm (55), but also bronchial hyperreactivity and eosinophil accumulation in the lung (66). The early bronchoconstriction to inhaled ascaris antigen is not inhibited by oral RP 48740 in dogs (67), nor by inhaled SRI 63-441 in Rhesus monkeys at a dose which prevented PAF bronchospasm (68). However, WEB 2086 inhibited the late bronchospasm (69) and bronchial hyperreactivity (70) in ascaris-sensitized sheep as did BN 52021 and L 659989 in ragweed-sensitized rabbits (71, 72).

Shock/Oedema - There is much evidence supporting a role of PAF in models of several shock syndromes. Thus, RP 48740, RP 59227, WEB 2086 and L 652731 protected mice against the lethal effects of intravenous PAF and, in sensitized animals, antigen (73). CV 3988, administered before or after PAF or antigen challenge, also reduced the histopathological changes (74). CV 6209 was a potent antagonist of PAF-induced mortality (15) in mice (ID_{50} = 9 $\mu g.kg^{-1}$, i.v.) and rats (ID_{50} = 14 $\mu g.kg^{-1}$) and also improved the survival of rats following haemorrhage (75) and trauma (76). SRI 63-675 prevented burn-induced hypotension (77) and SDZ 64-412

and SM 10661 protected mice against the lethal effects of endotoxin (35) and antigen (29), respectively.

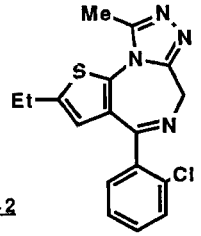
PAF-induced increases in haematocrit, reflecting a general enhancement of vascular permeability were inhibited by oral L 659989 in female rats (ED_{50} = 0.2 $mg.kg^{-1}$) only (24), while SDZ 64-412 was orally active in dog (ED_{50} = 5.1 $mg.kg^{-1}$), guinea pig (ED_{50} = 5 $mg.kg^{-1}$) and Cebus monkey (ED_{50} = 12.8 $mg.kg^{-1}$) (35). BN 52021 prevented PAF-induced vascular permeability increases in rat pancreas, kidney and heart (78) and in the bronchial microvascular bed of guinea pigs (79), as did SRI 63-675, which had no effect on permeability induced by histamine or LTB_4 (17). RP 48740 inhibited the increased pulmonary vascular permeability, neutrophil infiltration and epithelial cell damage produced by inhaled endotoxin in guinea pigs and hamsters (80). The increased cutaneous vascular permeability in rats produced by intradermal PAF is mediated by cyclooxygenase products, since indomethacin as well as CV 3988 inhibited the response (81).

Cardiovascular - The coronary vasoconstriction, decreased contractile force and release of LTC_4 and TxA_2 induced by PAF in perfused rat hearts are inhibited by BN 52021 and CV 3988 (82), while WEB 2086 inhibits the similar responses produced by antigen in guinea pig hearts (83). In contrast to heart and lung, PAF-induced increases in vascular permeability of isolated rat kidney (84), inhibited by L 652731, and contraction of rat colon, inhibited by FR 900452 (**40**) and CV 3988 with pA_2 values of 6.37 and 6.34, respectively (85), do not involve the release of other mediators.

The potent hypotensive action of intravenous PAF in rats was blocked selectively by RP 59227, both intravenously and for at least 5 hours after an oral dose of 0.5 $mg.kg^{-1}$ (86). SRI 63-675, L 652731, BN 52021 and WEB 2086 blunted PAF-induced hypotension in pithed rats (87). CV 6209 was 20, 74, 185 and over 2100 times more potent than ONO 6240 (**41**), CV 3988, BN 52021 and etizolam (**42**), respectively, in selectively preventing and reversing PAF-induced hypotension in rats (15). In addition, WEB 2170 and STY 2108 also prevented and reversed endotoxin-induced hypotension (32), as did SRI 63-441, which also inhibited the systemic and pulmonary vascular changes induced by endotoxin in sheep (88). SDZ 64-412 was orally active against PAF hypotension in the rat (ED_{50} = 14 $mg.kg^{-1}$) and dog (ED_{50} = 5.1 $mg.kg^{-1}$) (35).

Ischaemia/Reperfusion Injury - The contribution of endogenous PAF to the damage caused by ischaemia and reperfusion in several vascular beds has been investigated (10). CV 3988 protected rats against impaired cardiac function and reduced cellular integrity produced after coronary artery ligation (89). BN 52021 and SRI 63-441 reduced the incidence of ventricular ectopic beats, ventricular fibrillation and the fall in platelet count across the myocardium of greyhounds resulting from occlusion and reperfusion of the left anterior descending coronary artery (90). The efficacy of BN 52020 (**30**), BN 52021, BN 52022 (**32**) and BN 52024 (**33**) in ameliorating behavioral changes induced in gerbils by bilateral carotid artery ligation reflected their PAF antagonist potencies (91). BN 52021 also improved the recovery of renal function in rats following renal artery ligation (92). SRI 63-441 partially prevented the decrease in bile production and release of liver enzymes resulting from complete hepatic ischaemia (93).

Organ Damage/Transplantion - PAF antagonists protect several specific organ systems from a variety of insults. For example, BN 52063 (**34**) and BN 52021 markedly reduce the gastrointestinal ulceration in rats produced by PAF, endotoxin and ethanol, but not that due to pylorus ligation, aspirin or stress (94). Similarly, L 652731 reduced the mucosal damage and impaired gastric motility induced by both PAF and endotoxin (95). SRI 63-119 (**43**) totally prevented bowel lesions in rats produced by endotoxin (96), tumour necrosis factor (TNF) or TNF plus endotoxin (97), and chronic oral treatment with BN 52021 reduced the extent of colonic inflammation and ulceration induced by trinitrobenzene sulphonic acid (98). Renal function in rats is impaired by endotoxin, which was inhibited (99) by L 653150 and L 652731, the latter (99) and BN 52021 also reducing the similar changes induced by PAF (100). BN 52021 also prevented glomerular damage and proteinuria produced by adriamycin (101), as well as inhibiting increased pancreatic vascular permeability induced by intraperitoneal PAF or immune complex (102).



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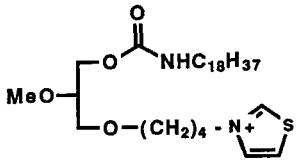

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BN 52063 delayed the rejection of livers transplanted from guinea pig to rat (103). SRI 63-441 improved the survival of liver (cat to rabbit) and heterotropic cardiac transplants in rats (104) and, whilst inactive alone, synergised with PGI_2 or PGE_1 in delaying rejection and maintaining function of pig to dog kidney transplants (105).

CLINICAL STUDIES

RP 48740, CV 3988, BN 52063 and WEB 2086 have been evaluated in normal subjects, and the last two also in patients. RP 48740 (250, 500, 1000 mg, p.o.) exhibited prolonged inhibition (plasma half-life 10.1 hr.) of PAF-induced platelet aggregation ex vivo (106), with some evidence of thromboxane synthetase inhibition at the highest dose (107). WEB 2086 produced marked and long-lasting inhibition of aggregation at oral doses as low as 5 mg (108) and also when administered intravenously or by inhalation (109). CV 3988 was infused at doses between 750 and 2000 $\mu g.kg^{-1}$, the latter dose increasing threshold aggregating concentrations of PAF by 356% (110). Oral BN 52063 increased PAF aggregation EC_{50} values by 21 and 47 fold at 80 and 120 mg, respectively (111), doses which also inhibit cutaneous wheal and flare responses to intradermal PAF, but not histamine (111, 112). Bronchoconstriction induced by inhaled PAF was inhibited 24% by BN 52063 at an oral dose of 120 mg (113), which also inhibited PAF cutaneous wheal and flare by 50% in atopic subjects (114). In the latter, however, flare responses to allergen were unaffected; the early wheal was inhibited 50% in half the subjects with a 50% reduction of the late wheal in all subjects (114). In 8 asthmatics, pretreatment with oral BN 52063 (40 mg t.i.d) for 3 days increased by a mean of 6-fold the dose of inhaled allergen required to increase airway resistance by 50% (115).

SUMMARY

The efficacy of PAF antagonists in a variety of animal models in which endogenous mediators are released, supports a major contributory role for PAF in several inflammatory and allergic pathophysiological conditions. Particular interest has focussed on delayed bronchospasm and bronchial hyperreactivity, shock syndromes, ischaemia and reperfusion injury and transplant rejection. With clinical trials for several antagonists already underway it should not be too long before it is known whether PAF antagonists represent a major new class of therapeutic agents.

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Chapter 10. Potassium Channel Openers: New Biological Probes

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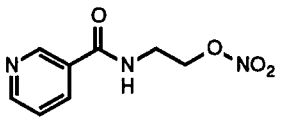
Introduction - Potassium (K) channel biology has undergone explosive growth in the last five years. Whereas it has long been known that K channels play a major role in neuronal excitability (1), it is now appreciated that K channels play a complex and critical role in the basic electrical and mechanical functions of a wide variety of tissues, including smooth muscle, cardiac muscle and glands (2). Two events have had a major influence on the rapid growth of this field: novel electrophysiological methods have been developed, including whole cell and patch clamp techniques, that permit K channel function to be carefully examined (3); and new classes of highly specific pharmacological substances have been developed which either open or block K channels (4). Fortunately, the rapidly expanding primary literature on K channels is supplemented with numerous, timely reviews (2-7). This chapter will summarize the recent literature of the chemistry and pharmacology of K channel openers (PCO's), a new class of compounds that specifically enhance K channel permeability of cell membranes.

Modulation of Potassium Channels - Electrophysiologic techniques have shown that K channels are gated (modulated) via three general avenues: by ligands (e.g., serotonin, GABA, calcium, or ATP), voltage, and G proteins (8). Ligands such as serotonin (5HT) have been shown to regulate predominantly neuronal K channels, while ATP affects glandular, cardiac and probably smooth muscle K channels. Calcium (Ca)-activated K channels are especially important in smooth muscle. Voltage-gated channels have been studied most extensively using a variety of cardiac and smooth muscle preparations, and K ions appear to carry a major portion of the current in these channels. Examples of three major types of voltage-gated K channels include the transient outward, the inward, and the delayed rectifier currents (9). Finally, a variety of chemical signals (e.g. acetylcholine, adenosine, somatostatin, GABA and 5HT) activate specific receptors, which in turn modulate K channels via G protein-coupled mechanisms (10).

Numerous endogenous compounds, such as catecholamines in smooth and cardiac muscle and acetylcholine in cardiac tissues, have long been known to alter the flux of K ions (11,12). Recently, several highly specific pharmacological probes for studying the K channel have been described. For example, apamin, a polypeptide toxin isolated from bee venom, selectively blocks the small-conductance Ca-activated K channel in a variety of smooth muscle cell types (13,14). Charybdotoxin, isolated from scorpion venom, blocks high-conductance, Ca-activated K channels in skeletal muscle, smooth muscle, and anterior pituitary cells (15). Dendrodotoxin, a snake venom, blocks transient outward K channels involved in the rapid phase of repolarization of

neurons in the CNS (16). A wide variety of synthetic compounds are also capable of nonspecifically blocking K channels, and recently compounds have become available that appear more selective for specific subtypes of K channels. For instance, compounds are available that can block the delayed rectifier channel in cardiac muscle (e.g. clofilium and sotalol) and which now serve as prototypes for a new class of antiarrhythmic drugs, the class III agents (17,18). Antidiabetic sulfonylureas block specifically the ATP-modulated K channel in the pancreas, an effect that mediates stimulation of insulin secretion (19).

Potassium Channel Openers - In vascular smooth muscle, the resting membrane potential of cells is more positive than the K equilibrium potential. Thus, a drug that increases membrane K permeability would be expected to increase the membrane potential (hyperpolarization) and render the cell resistant to neurotransmitter- or hormone-induced depolarization; in vascular smooth muscle, this would result in vasodilation. Several drugs which had been previously described as "nonspecific vasodilators" have been found to exert their effects by increasing K conductance. The earliest such compound was nicorandil (**1**), a nitrate-containing nicotinic acid derivative that was developed as a coronary vasodilator; its effects were thought to result from its ability to increase intracellular concentrations of cGMP via stimulation of guanylate cyclase, a mechanism of action common to the family of nitrate vasodilators such as nitroglycerin and nitroprusside (20,21). Further studies demonstrated the compound also produced a specific increase in K permeability leading to membrane hyperpolarization (22,23). The vasorelaxant effects of nicorandil may thus be mediated by at least two distinct mechanisms, which complicates attempts to use this agent to study K channel function.



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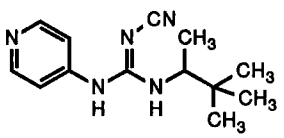

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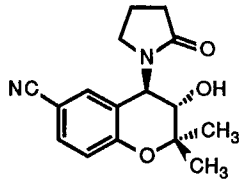

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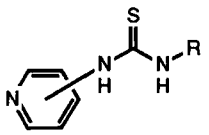
Pinacidil (**2**) and cromakalim (**3**) (BRL-34915) are vasodilators that appear more specific in their action than nicorandil. These compounds were discovered and developed as potent vasodilators with an undefined mechanism of action (24,25). Neither compound alters cGMP or cAMP levels, and the vasorelaxant activities of pinacidil and cromakalim do not involve interactions with Ca channels, adrenergic, serotonergic, cholinergic or histaminergic receptors (26-29). The compounds do not appear to release endogenous vasodilators such as EDRF, adenosine, or the prostanoids, and their effects are not modified by pertussis toxin (27,30). Since nicorandil was known to produce membrane hyperpolarization by increasing K permeability, the possibility that these compounds might act through a similar mechanism was investigated. Indeed, Weston and colleagues demonstrated that cromakalim increased ^{86}Rb (a surrogate ion for K) efflux from rat portal veins (preloaded with this radioactive tracer), and produced membrane hyperpolarization (31,32). Later, increases in Rb permeablity and membrane hyperpolarization were described for pinacidil, and the term K channel opener was applied to this general class of agents (33-35).

PCO's are derived from a variety of structural classes. Pinacidil resulted from a series of alkylpyridylthioureas (4) which were direct-acting vasodilators (36). The cyanoguanidine unit was explored as a bioisosteric replacement for the potentially toxic thiourea moiety,with a resultant increase in vasodilator potency. A variety of alkyl groups resulted in compounds with antihypertensive activity, and the best compounds possessed four to six carbons and branching α to the nitrogen. One of the most potent analogues was the *t*-pentyl congener. Both 4- and 3-pyridyl isomers were potent vasodilators, although the latter tended to be more potent; the 2-pyridyl isomers were essentially inactive (36).



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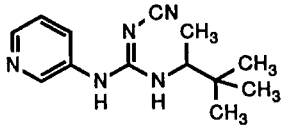

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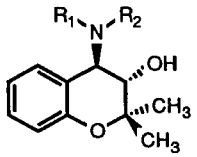
The two stereoisomers of pinacidil and its 3-pyridyl analogue (5) have also been prepared, and dramatic stereoselectivity was observed (37,38). Both (-)- and (+)-pinacidil relaxed phenylephrine-contracted canine cephalic veins, and EC_{50} values were 0.44 and 9.55 μM respectively, which represents a eudismic ratio of 22 (38). Similar eudismic ratios for pinacidil were obtained in rat vascular tissues (39). The EC_{50} values of the (-) and (+) enantiomers of (5) were 0.09 and 6.41μM, respectively; this represents a eudismic ratio of 71 (37). The disposition of pinacidil in man has been studied, and both stereoisomers appear to have comparable pharmacokinetics and metabolic fates (40). Pinacidil is metabolized extensively to the N-oxide, and this metabolite is 1/10th to 1/100th as potent as pinacidil as a vasorelaxant (41).

The synthesis and SAR of a series of *trans*-4-amino-3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-3-ols (6) had been previously reported (42,43). These compounds were studied as antihypertensives and served as progenitors of cromakalim. Transformation of the 4-amino substituent into a lactam moiety resulted in 10-fold increased antihypertensive potency (44). The *trans* relationship of the amide moiety and the hydroxy group was pivotal, and the 3,4-*cis* analogue was considerably less active. The size of the lactam ring could be increased to 6 members which increased potency, but further homologation was deleterious.



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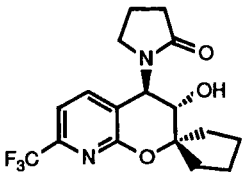

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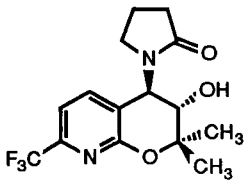

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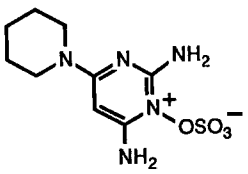
A powerful electron withdrawing substituent such as cyano at position 6, and the geminal methyl substituents at position 2 were important contributors to potency. Resolution of cromakalim, and testing of the optical isomers as antihypertensive agents in spontaneously hypertensive rats (SHR), revealed that the (-) enantiomer was at least 30 times more potent than the (+) enantiomer (44). In isolated portal vein preparations, the (-) enantiomer was considerably more potent in producing membrane hyperpolarization,

stimulating ^{86}Rb efflux, and producing venorelaxation. Such *in vitro* studies suggested that the (-) enantiomer was at least 100-200 times more potent than the (+) enantiomer (45). Pyridine isosteres of cromakalim have been prepared (46,47). The spirocyclopentane derivative 7 was less potent as a stimulator of ^{86}Rb-efflux than 8, which in turn was slightly less potent than cromakalim (46).

Several vasodilators, whose mechanism of action was previously unknown, may act at least in part by augmenting K flux (48,49). These would include minoxidil sulfate (9), the active metabolite of minoxidil (48), and possibly diazoxide (50,51) (10). Compound 49356-RP (11) is another vasodilator reported to have K channel opening activities (52).



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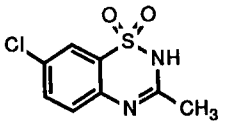

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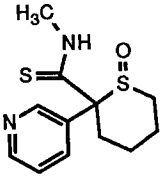

11

Effects on Vascular Smooth Muscle - Smooth muscles in general contain a variety of K channel subtypes, with high conductance (250-300 pS) Ca-activated K channels predominating (3). There is some evidence that the intermediate (180 pS) and low conductance (90 pS) K channels may be partly responsible for maintenance of resting membrane potential, while the high conductance K channel may be involved in repolarization (53,54). (Membrane conductance, as measured using patch clamp methodology, is usually defined in picoSiemens (pS). For a precise definition of this unit, see reference 55.)

Pinacidil and cromakalim are potent vasodilators and relax peripheral arteries, veins, and the coronary vasculature. In isolated tissues, they block contractions induced by a variety of spasmogens such as α-agonists, 5HT, and angiotensin (27). PCO's also antagonize contractions produced by low concentrations of K (below 20 mM). However, at higher K concentrations (ca. 80 mM) the difference between the K equilibrium potential and the membrane potential is minimized, such that little hyperpolarization results from an increase in K permeability. Moreover, at the depolarized potentials induced by high K concentrations, the voltage-sensitive Ca channels are fully activated and are sensitive only to Ca antagonists. This differential ability to inhibit low vs. high K contractions has been used to differentiate PCO's from Ca entry blockers (33, 56, 57). It has been emphasized, however, that nitrate-containing vasodilators also relax low K, but not high K-induced contractions (58). Both PCO's and Ca channel antagonists eventually reduce intracellular Ca concentrations, but definite pharmacological differences exist between these classes of drugs. Whereas pinacidil and nifedipine inhibit norepinephrine-induced contractions of human crural veins equally, PCO's appeared somewhat more effective in blocking angiotensin II-induced vasoconstriction of rabbit aorta (45,59). The sustained phase of norepinephrine-induced contraction, which may utilize intracellular Ca stores, is blocked by PCO's but not by Ca channel antagonists (60). PCO's may also decrease intracellular Ca concentrations by interfering with Na/Ca exchange mechanisms or the refilling of Ca by sarcoplasmic reticulum stores (61,62).

In whole animal studies the regional vasodilator effects of PCO's and Ca

channel blockers also differ. For example, in the anesthetized cat cromakalim increased renal blood flow, but nifedipine increased femoral blood flow (63). In a subsequent study in anesthetized rabbits, the (-) enantiomer of cromakalim was found to dilate preferentially the gastrointestinal, but not skeletal blood vessels (45). Calcium entry blockers strongly dilate skeletal muscle, but not gastrointestinal vascular beds (64,65). However, cromakalim increased nutritive blood flow to hypoxic skeletal muscle, whereas Ca channel antagonists did not (66). In anesthetized, ganglion-blocked SHR there appear to be major qualitative differences between the anti-vasoconstrictor activities of the two drugs. Cromakalim dose-dependently blocked the pressor responses to norepinephrine or phenylephrine, but did not block pressor responses induced by methoxamine, angiotensin II or vasopressin; conversely, nifedipine blocked all of these spasmogens, and was most effective against norepinephrine and angiotensin II. These data suggest the antihypertensive activities of the PCO's relate to a direct smooth muscle relaxant effect rather than a generalized antivasoconstrictor action (34,67).

Comparisons of PCO's among themselves and with other vasodilators have also been reported. In conscious dogs with prior coronary artery occlusion and reperfusion, both pinacidil and nitroprusside increased coronary flow in the nonischemic zone; however, ischemic blood flow increased and infarct size decreased only in the nitroprusside group, probably due to the reflexively mediated increases in cardiac work induced by pinacidil (68). Reflexively mediated increases in heart rate and cardiac output have also been demonstrated for pinacidil in conscious SHR (69). While the general pharmacological effects of known PCO's are qualitatively similar, some differences have been noted. For example, cromakalim is generally more potent as an antihypertensive and as a positive chronotropic agent, and may have a more marked effect on renal vascular resistance than pinacidil (34,70). Differences among PCO's regarding their susceptibility to nonspecific K channel blocking drugs have also been reported (71).

The specific K channel subtype(s) mediating the vasodilator effects of PCO's is under active investigation. A charybdotoxin-containing scorpion venom blocked cromakalim-induced increases in Rb flux, suggesting Ca-activated K channels may be involved (72,73). There is general agreement that the effects of nicorandil and cromakalim are not blocked by apamin, a specific blocker of the small conductance Ca-activated K channel (74). Although a wide variety of K channel blockers (e.g., tetraethylammonium, 4-aminopyridine, and procaine) can antagonize effects of PCO's, these blockers are relatively nonspecific, and do not discriminate among K channel subtypes (71,75,76). However, the vasorelaxant effects of pinacidil and cromokalim can be blocked specifically by antidiabetic sulfonylureas such as glypizide or glyburide (glibenclamide) (38,77). Since it is well-documented that these drugs interact with ATP-modulated K channels in pancreatic β-cells, it is possible that effects of cromakalim and pinacidil may involve a previously unrecognized ATP-modulated K channel in smooth muscle. Direct electrophysiologic evidence for the presence of this K channel in smooth muscle has not yet been reported, but its confirmation would have important implications regarding the control of resting membrane potential in smooth muscle.

Effects on Nonvascular Smooth Muscles - PCO's have important pharmacological effects on the uterus, bronchial smooth muscle, the urogenital tract (e.g., the bladder), and on gastrointestinal (GI) smooth muscle.

Cromakalim antagonized spontaneous and oxytocin-induced contractions in the rat uterus, but produced only modest hyperpolarization and had little effect on Rb efflux (78). The relaxant effects on uterus might be due to direct inhibition of pacemaker activity rather than alterations in membrane conductance *per se*. Small intestine peristalsis was inhibited by cromakalim in guinea pigs and mice, although small intestinal smooth muscle may be less sensitive to cromakalim than vascular smooth muscle (79). Both nicorandil and cromakalim have been shown to relax spontaneous tone in the guinea pig taenia caeci; the relaxant effects were associated with increases in Rb flux and were unaffected by apamin (32,80). As in vascular smooth muscle, pinacidil and nicorandil were more potent in relaxing spontaneous or low K (30 mM) contractions in GI muscle than in relaxing contractions caused by 100 mM K, whereas nifedipine was more potent in suppressing high K contractions (81). In guinea pig tracheal muscle, suppression of slow wave activity and membrane hyperpolarization produced by high concentrations of cromakalim (1-10 μM) were blocked by 4-aminopyridine and procaine, but not by apamin (82). Pinacidil relaxed spontaneous contractions of isolated guinea pig tracheal tissue, and was 9 times more potent in relaxing histamine-induced contractions than carbamylcholine-induced contractions; contractions induced by leukotrienes were also antagonized (83). In isolated human and rat bladder strips, pinacidil increased Rb efflux, inhibited spontaneous contractile activity, and antagonized the contractile responses to low but not high K. The inhibitory effects of pinacidil on contractile activity were greater in hypertrophied bladder caused by prior outlet obstruction (84).

Effects on Cardiac Muscle - K channels have been extensively studied in cardiac muscle. In addition to the aforementioned voltage-gated channels, one of the highest conductance K channels (80 pS) in cardiac muscle is an ATP-inhibited channel. It opens when internal ATP concentrations decrease to approximately 0.2 mM or below, and is also regulated by ADP (85). This K channel may open during periods of anoxia and could be important in tissue responses to acute ischemia.

The earliest studies involving PCO's in cardiac muscle were performed with nicorandil. This agent was shown to selectively decrease the action potential duration (APD) of dog atrial tissues and hyperpolarize the membrane (86). Experiments in canine Purkinje fibers demonstrated similar electrophysiological effects (87). Later studies in intact cells from dogs, as well as in isolated guinea-pig myocytes, also demonstrated marked decreases in APD without effects on the rapid upstroke phase of the action potential for both pinacidil and cromakalim (88-90). As in smooth muscle, the effects of PCO's are highly stereoselective, as the (-) enantiomer of either cromakalim or pinacidil was more potent in decreasing APD than the (+) enantiomer (38,91). Within a series of pinacidil analogs, a good correlation was achieved between the reduction of cardiac APD and the relaxation of isolated vascular smooth muscles, or antihypertensive effects. These data suggested that a common mechanism (K channel opening) may underly these effects in both tissues (92,93). The identity of the K channel affected by PCO's in cardiac muscle is being actively investigated. In guinea-pig myocytes, cromakalim activated a novel time and voltage-independent outward current, yielding a nearly ohmic relationship between current and voltage across a wide range of membrane potentials (89). Similar electrophysiological effects were noted for nicorandil and for pinacidil in the absence of effects on the Ca channel (94-96). In guinea-pig myocytes, the outward current activated by cromakalim was blocked by

glyburide, and patch clamp methods identified the ATP-inhibited channel as a likely target for the drug (96). As in smooth muscle, the stimulatory effects of cromakalim on Rb flux in cardiac tissue was unaffected by pertussis toxin (30).

Perhaps as a consequence of the dramatic shortening of APD by PCO's, a negative inotropic effect is apparent in isolated cardiac tissues obtained from both dogs and cats (90,92,97). In the blood-perfused canine heart preparation, negative inotropic effects were noted at 10-fold higher concentrations than those needed to dilate the coronary vasculature (98). Similar differences in potency between the effects on cardiac and smooth muscle *in vitro* were noted for pinacidil (92). In conscious dogs, no negative inotropic effects have been reported, probably due to the accompanying reductions in vascular resistance and reflex activity (68). Since decreases in K permeability may contribute to membrane abnormalities and cardiac arrhythmias, PCO's might possess antiarrhythmic activity (99). Indeed nicorandil, pinacidil, and cromakalim have all been demonstrated to decrease automaticity in isolated cardiac tissues exposed to various arrhythmogenic stimuli (87,90,92). In conscious dogs with arrhythmias caused by prior coronary artery ligation, pinacidil and hydralazine were given at doses that caused comparable decreases in blood pressure, but only pinacidil inhibited abnormal beats (100). Additional studies in intact animals using various models for arrhythmia induction are required to assess fully the significance of these observations. However, it should be noted that the marked decrease in APD (and refractoriness) may in some instances result in increased arrhythmias, especially those of the reentrant type (92,98).

Effects on Other Tissues - An ATP-inhibited K channel (20-50 pS) is prominent in pancreatic ß-cells. This channel is normally open and is responsible for the relatively high resting membrane potential of these cells. The intracellular ATP/ADP ratio is usually low and is increased in the presence of enhanced glucose or amino acid flux, resulting in depolarization, calcium influx, and ultimately the release of insulin (19). In rat pancreatic islets perifused with glucose, pinacidil increased Rb efflux, inhibited Ca influx and insulin release. Pinacidil-induced increases in K permeability were also noted in the presence of sulfonylureas (101). Diazoxide also opens ATP-inhibited K channels in mouse isolated islets when channel activity is reduced by tolbutamide (51). A variety of both ligand- and G protein-gated K channels exist in autonomic and CNS neurons (8,102,103). 5HT1a receptor agonists may hyperpolarize certain mammalian neurons via opening of K channels (103,104). Given i.c.v., cromakalim was found to be a potent antagonist of muscarinic-induced mouth movements in mice, an effect possibly mediated by Ca-dependent K channels (105). In guinea pig hippocampal slices, cromakalim depressed epileptiform neuronal activity (106).

Overview of Therapeutic Potential - Because of the prevalence and extreme diversity of K channels, PCO's could have a variety of potentially useful clinical effects. Due to their ability to relax vascular smooth muscle, hypertension was an obvious initial target, and pinacidil is currently marketed in several countries for this indication (107). Cromakalim has also been studied extensively in hypertensive patients and appears to be effective, but development of the racemate was discontinued after discovery of heart lesions in monkeys treated with high doses (108,109). The *l*-enantiomer (BRL-38227) is now under development for long-term management of hypertension (109). Whether vasodilation via opening of K channels confers any advantages relative to other antihypertensive mechanisms will not be clear until greater clinical experience

is obtained with PCO's. As indicated above, PCO's relax bronchial smooth muscle in animals, and studies with cromakalim in humans suggest these agents may be useful in the treatment of asthma. An oral 2 mg dose of cromakalim inhibited histamine-induced bronchoconstriction in healthy volunteers (110). Since PCO's relax the bladder, treatment of bladder hyperreflexia, a common cause of urinary incontinence, represents another logical target. The relaxation demonstrated in some gastrointestinal tissues also suggests possible clinical uses in hyperspastic bowel conditions such as ischemic bowel syndrome (79). Finally, the use of minoxidil in treatment of alopecia areata, and reports that pinacidil and diazoxide produce hypertrichosis, suggests that K channel opening may be involved in stimulating hair growth; whether this effect is related to K channel opening or is an epiphenomenon remains an unresolved question.

Conclusion - Full clinical exploitation of these potentially important agents, with their novel mechanism of action, will await a clearer understanding of the tissue selectivity that may be present with existing PCO's, and development of PCO's with enhanced selectivity. At the very least this burgeoning interest in PCO's will provide new chemical probes which will enhance our understanding of the physiology and pathophysiology of K channels.

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Section III - Chemotherapeutic Agents

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Chapter 11. Antibacterial Agents

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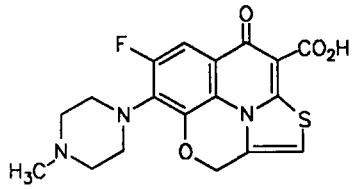
Introduction - Highlights in the antibacterial literature in 1988 include continued advances in the quinolone antibiotic area, new β-lactams exploiting the *tonB* transport system, and the breakthrough macrolides azithromycin and dirithromycin.

Quinolones - Ciprofloxacin (1,2,3) and ofloxacin (4) were reviewed in detail recently. A number of review articles have appeared covering structure-activity relationships (5,6,7,8,9), mechanism of action (10,11,12,13,14) and development of resistance (15,16,17,18). The role of DNA gyrase inhibition in the mechanism of action of these agents continues to be controversial. While it was previously reported that DNA gyrase isolated from *S. aureus* was not inhibited by the quinolones (19), a more recent paper described the inhibition of partially purified gyrase from *S. aureus* by norfloxacin, ciprofloxacin and ofloxacin at levels below 1 μg/ml (20). The DNA gyrase from *Citrobacter freundii* was purified and the inhibitory effects of the quinolones on supercoiling were found to correlate with antibacterial activity (21). Toxicological aspects of the quinolones were reviewed (22). Evidence continues to accumulate that inhibition of GABA binding to receptors is responsible for the epileptogenicity associated with some members of this class (23). Antiprotozoal (24) and antiviral (25,26) activity was exhibited by some of the newer quinolones.

The principal differentiating property of the new quinolones described this year is improved activity against Gram-positive organisms. A new oflaxacin analog, KB5246 (1), with an interesting ring fusion between the N-1 side chain and 2 position, was shown to be slightly superior to ofloxacin *in vivo* (27,28). A number of new 5-amino quinolones were described (29), and one, PD 124,816 (2), was selected for more extensive evaluation. This compound exhibited substantially improved activity *in vitro* against Gram-positive organisms and anerobes relative to ciprofloxacin (30). The substitution of an amino group for hydrogen at C-5 in the quinolones resulted in improved *in vitro* activity against Gram-positive organisms but generally resulted in reduced *in vivo* efficacy (31). Closely related in structure and antibacterial activity to the former compound is AT-4140 (3), which exhibited a $t_{1/2}$ of 16 hr in man (32,33). A new 7-morpholino analog (34), Y-25024 (4), was shown to be well distributed into all tissues excepting the brain, where levels one-tenth of those observed with ofloxacin were found (35). This partitioning, combined with lower affinity for GABA receptors, suggest a lower potential for CNS side-effects for this compound. A 1-*t*-butyl quinolone, BMY 40062 (5), was found to be more

efficacious than ciprofloxacin against methicillin-resistant *S. aureus* and equivalent against most Gram-negative organisms (36,37).



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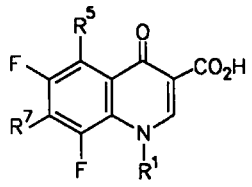

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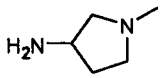
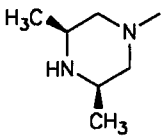
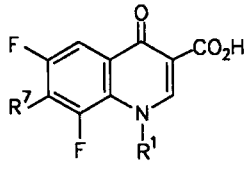
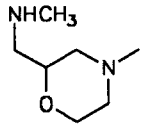
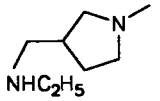
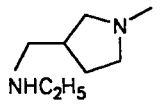
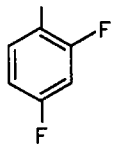
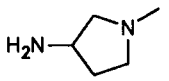
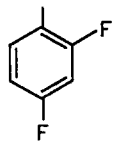
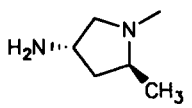
2		NH_2	
3		NH_2	

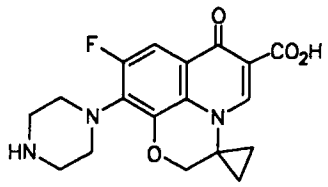


	R^1	R^7
4		
5	t-Bu	
6	Et	
7		
8		
9		

Details of the structure-activity relationships in the 8-substituted quinolone series related to CI-934 were disclosed. A study of variants at N-1 confirmed the superiority of cyclopropyl analogs observed in other series (38), and variation at C-7 and C-8 revealed the improved Gram-positive activity of the 8-fluoro-7-(3-ethylaminomethylpyrrolidinyl) derivatives CI-934 (**6**) and PD-117558 (**7**) (39).

Ten papers at the 28th ICAAC described work with A-61827 **8** (A-60669, T-3262), currently in Phase II in Japan (40). The influence of side-chain stereochemistry was probed and the *S*- enantiomer was found to be 2-4X more potent both *in vitro* and *in vivo* (41). To further optimize the physical properties in this series, an extensive investigation of substituted 3-aminopyrrolidines was undertaken, resulting in the synthesis of A-65485 (**9**), which is significantly more water soluble than the desmethyl analog (42). Two research groups independently reported the synthesis of ofloxacin-ciprofloxacin hybrid analogs with the general structure (**10**), which were found to be less active than either

parent structure (43,44). Similarly disappointing were the conformationally restricted aryl analogs (11) and (12), highlighting the subtle steric factors influencing activity in this series (45,46).



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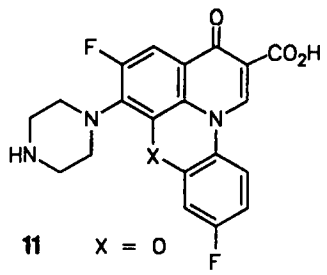
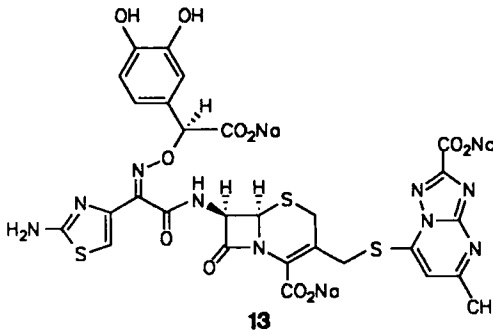


11 X = O

12 X = NH

β-lactams - Reviews covering SAR (47,48), penicillin binding proteins (49), and clinical applications (50,51) of this class of antibacterial agents were published. A number of reviews detailed the mechanisms of permeation of β-lactam antibiotics into bacteria and their role in bacterial resistance (52,53,54,55). Mechanistic and structural information on the penicillin-interactive serine proteases and trans-peptidases was reviewed (56). The synthesis and antibacterial activity of the penem class of antibiotics was the subject of a review (57). Force field parameters for the conformational analysis of penicillins were described (58), and applied to the modeling of the active site of a penicillin binding protein (59).

Exploitation of the *tonB*-dependent iron transport pathway continued to be a fertile source of agents with enhanced activity against members of the family *Enterobacteriaceae* and *Pseudomonas* (60,61). Among the new compounds of this type, M14659 (13) stands out as a significant advance (62). It exhibits improved potency against both *S. aureus* and *Pseudomonas* strains, and an extended serum half-life relative to ceftazidime (63). Another newly disclosed agent which likely exploits this pathway is the monocarbam U-78,608 (14) (64,65), a close structural analog of pirazmonam. The isocephems RU 45978 (15) and RU 46069 (16) were reported to exhibit improved potency against *S. aureus* and cephalosporinase-producing *Enterobacteriaceae* strains when compared to third generation cephalosporins, but they are only poorly active against *Pseudomonas* (66,67). A similar bioactivity profile was reported for the carbacephems LY 211256 (17) and LY 258360 (18), which have a quaternary nitrogen heterocycle directly attached to the nucleus (68). A detailed *in vitro* profile of E1040 (19) appeared. This compound is a potent anti-pseudomonal cephalosporin with excellent activity against strains derepressed for chromosomal type I β-lactamase (69).



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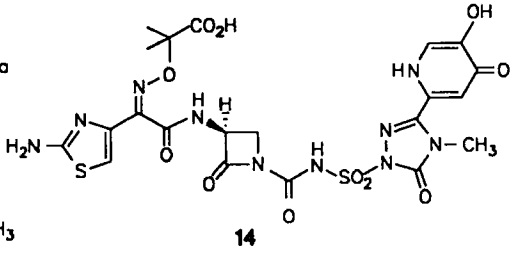

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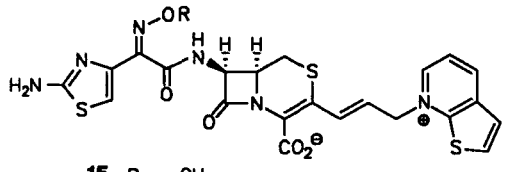
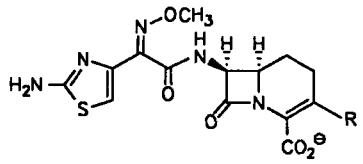
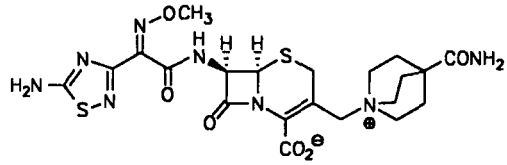


15 R = CH_3
16 R = CHF_2





19

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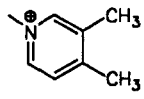

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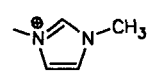
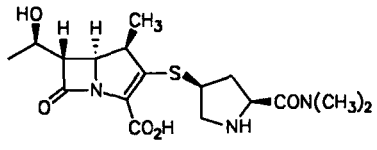


18

The 1-β-methylcarbapenem meropenem (**20**) (SM-7338, ICI-194660) was the subject of a number of papers at the 28th ICAAC meeting (70). Pharmacokinetic studies in human volunteers confirmed the improved metabolic stability of this drug, which exhibited urinary recoveries greater than imipenem alone and comparable to imipenem coadministered with the renal dehydropeptidase inhibitor cilastatin. The $t_{1/2}$ in man was found to be approximately one hour (71). Synthetic (72), preclinical (73) and Phase I studies on a new penem, CP-65,207 (**21**) were also disclosed at this meeting (74). This compound exhibited potent antibacterial activity against a broad range of Gram-positive and Gram-negative organisms, excluding *Pseudomonas*, and has a $t_{1/2}$ of one hour and a urinary recovery of 45% in man.

Several previously disclosed orally-active β-lactam antibiotics were described in more detail at the 28th ICAAC meeting. The cephalosporin prodrug ester BMY-28271 (**22**) was shown to be 2-24-fold more active than cefixime and cefteram against Gram-positive organisms, and was equivalent in potency to these agents against Gram-negative organisms (75). Human pharmacokinetic studies of lorcarbacef (KT-3777, LY 163892) (**23**) revealed both a longer $t_{1/2}$ and better oral bioavailablity than cefaclor (76). FK-482 (**24**) was shown to be considerably more active than cefixime and cefaclor against Gram-positive bacteria, and better than amoxicillin and cefaclor against susceptible Gram-negative organisms (77).



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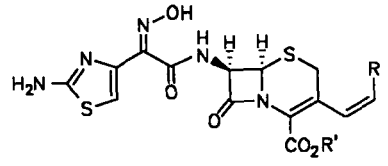
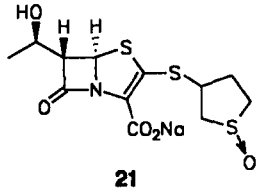


22 R = CH_3, R' = $CH(CH_3)OAc$

24 R = R' = H



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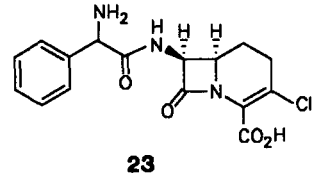
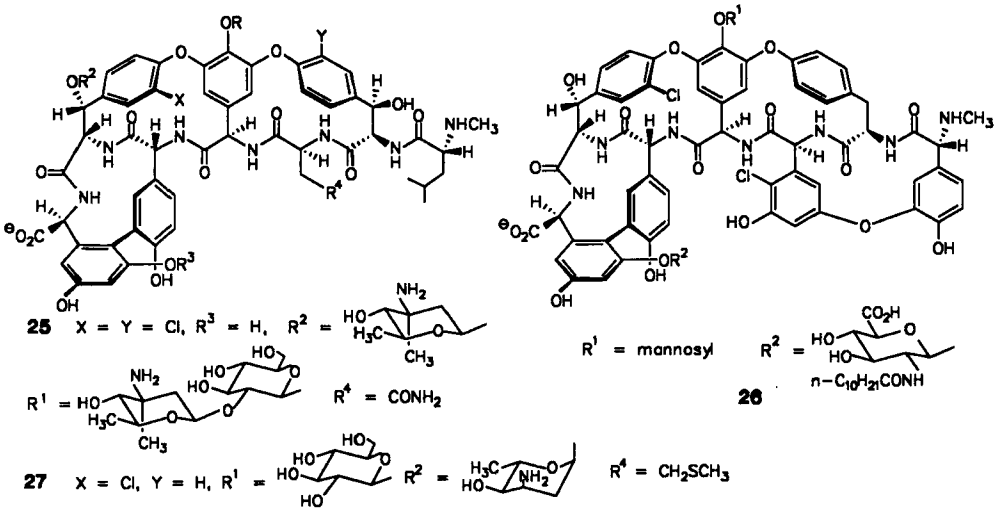


23

Glycopeptides - An excellent review of SAR in this area appeared this year (78), as well as an overview of current research on the mechanism of action (79). A review of the clinical experience with vancomycin was published (80). A detailed summary of microbiological results obtained with the newer glycopeptide, teicoplanin, appeared (81).

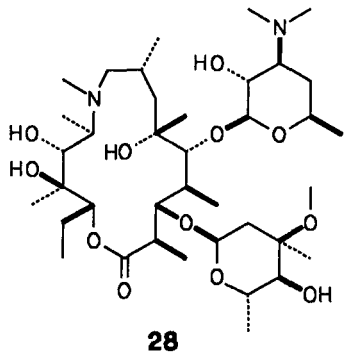
In spite of the fact that vancomycin has been in clinical use for almost thirty years, resistance development among the Gram-positive organisms has not been much of a problem. Recently, reports were published describing the appearance of inducible, transferable resistance to the glycopeptides in *Enterococcus faecium* and *Enterococcus faecalis* (82,83). In the latter case, induction of resistance was shown to be associated with the appearance of a new 39 kDa cytoplasmic membrane protein. The mechanism by which this protein confers resistance to glycopeptides has not been established, but it was shown not to function by destroying the antibiotic. Characterization of a mutant *E. coli* strain which was susceptible to glycopeptides suggested that the lipopolysaccharide layer in Gram-negative bacteria is reponsible for the broad resistance that these bacteria exhibit to agents such as vancomycin (84).



New glycopeptides (25) possessing a 4-epivancosamine sugar moiety were reported independently as the A82846 complex (85,86), and the orienticins and chloroorienticins (87,88). The chlorine-containing analogs were more potent than vancomycin against a number of Gram-positive organisms *in vitro* but exhibited no advantages *in vivo* (89). A new glycopeptide of the ristocetin structural type was independently discovered as parvodicin and A40926 (26) (90,91). This agent is distinguished by its exceptional activity against *Neisseria gonorrhoeae* and a long elimination half-life (92). Selective removal of the two sugar moeities revealed that the desmannosyl pseudoaglycone was more active against many Gram-positive organisms, particularly coagluase-negative strains, while the glycolipid group was essential for activity against *N. gonorrhoeae* (93). A semi-synthetic glycopeptide of the avoparcin type, SKF 104662 27 (94), was reported to have a longer $t_{1/2}$ and improved toxicological profile relative to vancomycin (95). Extensive investigations of N-acyl- and N-alkyl-vancomycin derivatives were reported (96,97). While the N-acyl derivatives had no advantages over the parent drug, lipophilic alkyl derivatives substituted on the N-methyl leucine nitrogen exhibited up to five times the potency of vancomycin and improved elimination half-lives. A new glycopeptide, M43F, was described which differs from vancomycin only in the substitution of an aspartate for an

asparagine residue (98). The ten-fold lower antibacterial activity of the new analog highlights the deleterious effect of a negative charge near the D-ala-D-ala binding pocket. Similarly, chemical removal of the terminal amino group in teicoplanin reduces the affinity for Ac-D-ala-D-ala tenfold and the *in vitro* activity against Gram-positive bacteria from three to five fold (99).

Macrolides - Reviews on spiramycin (100,101) and roxithromycin (102) were published. The comparative pharmacokinetics (103), clinical applications (104), mechanism of action (105) and gastrointestial side effects (106) of the macrolides were the subject of reviews. Azithromycin (CP-62,993) (**28**) was the subject of a number of papers (107,108,109). This 15-membered azamacrolide exhibited significantly improved activity against gram-negative bacteria (110), enhanced tissue penetration and an extended elimination half-life (111). These properties were exhibited to a somewhat lesser extent by dirithromycin (ASE 136, LY 237216) (**29**) (112), where high and persistant tissue concentration allows once daily dosing of 500mg to be bioequivalent to 500 mg of erythromycin *tid* (113,114). The extended spectrum and improved pharmacokinetics of these agents is dependent upon the presence of a basic nitrogen moiety near C9, as the acylated derivatives do not share these properties (115). An interesting intramolecular Michael addition was employed to introduce a nitrogen at C-11 in 6-O-methyl erythromycin, and several of the resulting analogs were more active than erythromycin against resistant strains (116,117). In the 16-membered macrolide series, a large number of tylosin analogs modified at the C-20 aldehyde function were reported (118). While many of these analogs exhibited improved bioavailability, probably due to the removal of the readily metabolized aldehyde group, they were less efficacious *in vivo*, particularly against *S. pneumoniae*.



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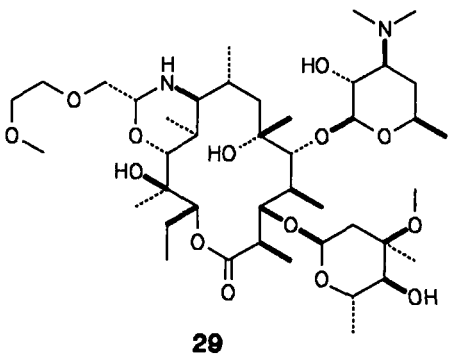
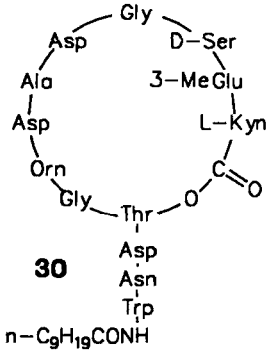


29

Miscellaneous - With the increasing incidence of nosocomial infections involving resistant Gram-positive organisms, interest has grown in agents with activity optimized against these pathogens. Phase III studies on the semisynthetic lipopeptide daptomycin (**30**) were reported at the 28th ICAAC meeting (119), and details of the synthesis and SAR in this series were recently disclosed (120). A new family of lipopeptides, exemplified by A54145A (**31**), was also disclosed at ICAAC (121). Like daptomycin, this agent was particularly effective againt Gram-positive organisms, including those resistant to methicillin and vancomycin (122). Lysobactin (**32**) and katanosin B (**33**) are two closely related dibasic peptides which exhibit a similar spectrum of activity against aerobic staphylococci and streptococci, and are also effective against a number of anaerobic species (123,124,125). The synthetic oxazolidinone DuP 105 (**34**) has a comparable antibacterial spectrum, although it's activity against anaerobes is

limited to some *Bacteriodes* species (126). This agent and its analog DuP 721 (**35**) are bacteriostatic, and appear to function by interfering with an early event during initiation of protein synthesis (127,128) The phenelfamycins (**36**) (phenelfamycin A) are structurally related to kirromycin and efrotomycin, but they lack the hydroxypyridone chromophore present in most of the other members of this class (129,130). The phenelfamycins exhibited potent activity against Gram-positive anaerobes, particularly *Clostridium difficile*. Phenelfamycin A also was active against *N. gonorrhoeae* and some streptococci, and was found to be effective in prolonging the survival of hamsters in an animal model of *C. difficile* enterocolitis (131).



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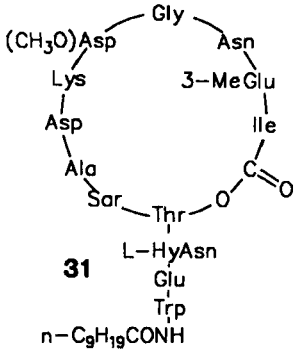

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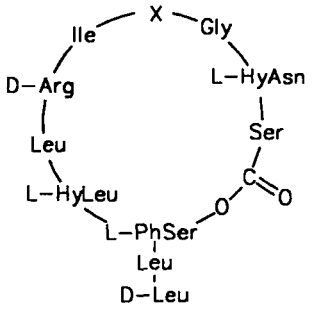
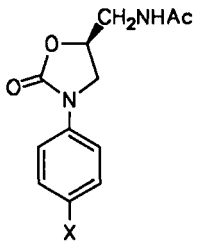
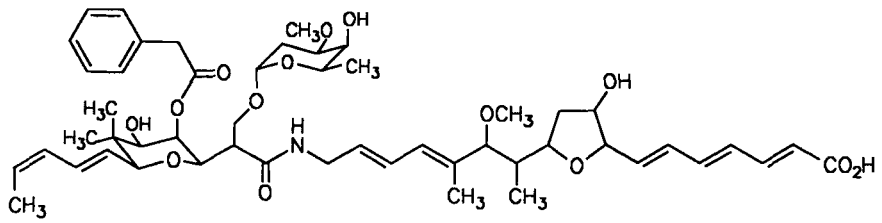


32 X = L-aThr
33 X = D-aThr



34 X = CH_3SO
35 X = CH_3CO



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Chapter 12. Recent Advances in Antifungal Agents

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Introduction- Modern research on antifungal agents has been devoted mainly to the oral therapy of primary and opportunistic pathogenic mycoses. Since the last review in this series (1), considerable progress has been made towards this endeavor. Due to the increased demands for treating opportunistic fungal infections, the need for safer, efficacious drugs, has grown rapidly. A comprehensive review on the recent clinical advances and current problems in the treatment of systemic infections has been published (2). In addition to well known opportunistic fungal pathogens such as *Candida*, *Aspergillus*, *Cryptococcus*, and *Mucor*, an increasing number of other molds and yeasts are now recognized as causes of infection in immunocompromised patients (3,4). The significant finding that *Pneumocystis carinii*, the most common lung infection in AIDS patients, may be a fungus should lead to a more systematic search for new agents to combat this serious condition (5).

Pulmonary aspergillosis is a major life-threatening infection among transplant recipients and patients on cancer chemotherapy (6). *Candida albicans* continues to be a major cause of systemic fungal infections in immunocompromised patients, and topical infections in healthy individuals (7). An overview of *Candida* infections (8), and a commentary on AIDS and *Histoplasmosis* (9) have appeared. The biochemical basis for activity of various anti-candida drugs has been reviewed (10). A comprehensive book on pharmaceutical (and agrochemical) aspects of sterol biosynthesis inhibitors has also appeared (11).

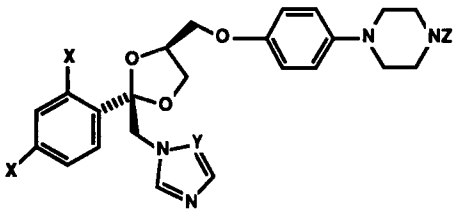
Until very recently, intravenous amphotericin B (AMB) has generally been the singular choice in the treatment of serious systemic fungal infections (12). 5-Fluoro-cytosine (5-FC) is often used as an adjunct for cryptococcal and candidal infections, and in some cases of aspergillosis (13). This situation may well change due to the addition of newer broad-spectrum agents with greatly improved systemic activity and safety.

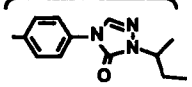
Polyenes- In a new model of bronchopulmonary aspergillosis, aerosolized AMB significantly delayed mortality compared with animals in a control group. Safety and additional pharmacokinetic studies are in progress for possible human trials (6). This route of administration for pulmonary infections may overcome at least some of the systemic toxicity of AMB. Reviews on pharmacokinetics, mechanism of action, activity, and clinical application of liposomal encapsulated AMB (L-AMB) have appeared (14,15). In suppport of previous studies, a recent trial in cancer patients showed that L-AMB was better tolerated than AMB alone (16). A report on treatment of fungal infections in heart transplant recipients using L-AMB has also appeared (17). An emulsion formulation of AMB improved its therapeutic index in murine candidiasis (18). Synergy between AMB and rifampin was demonstrated in vitro and possibly in vivo (19). When given at very high oral doses to mice (100 mpk), AMB had sustained blood levels of 0.2 μg/ml. In an experimental systemic *C. albicans* infection, mice treated orally with 12.5-100 mpk/day were completely protected. Favorable serum pharmacokinetics of oral AMB in humans may be of potential value in prophylaxis and therapy of systemic candidiasis and other deep-seated mycoses (20).

When nystatin (NYS) was reformulated as a pastille, it provided higher salivary concentrations than the original oral suspension. In immunocompromised patients with candidal stomatitis, the pastille version was shown to be equivalent in activity to clotrimazole (21). Liposomal NYS was as active as free NYS in vitro against a wide variety of yeasts and fungi and protected erythrocytes from the toxicity of free NYS (22). In mice iv administration of liposomal-NYS increased survival and reduced toxicity, making it an active systemic antifungal (23).

Azoles- The success of ketoconazole (1), the first broad-spectrum orally efficacious azole, stimulated the search for better agents in this class of antifungals. A minireview of azoles with emphasis on the newer triazole types has appeared (24), as has an editorial on classical and new azoles (25). A study of the effects of 14 azoles on various enzymes affecting steroid biosynthesis

(26), and articles on azole cytochrome P-450 interactions (27) have been published. The complexes of beta-cyclodextrin and some antimycotic azoles were more readily released from topical preparations than from the same vehicles without beta-cyclodextrin (28).

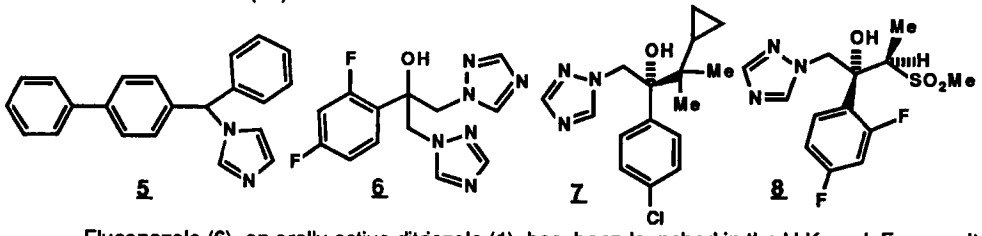


	1	2	3	4
X	Cl	Cl	Cl	F
y	CH	N	N	N
Z	Ac	iPr		

Terconazole (2) is a broad-spectrum topical antifungal, marketed in the U.S.. Several reports on influenza-like syndrome after topical treatment of vulvo-vaginal candidiasis with 2 have been documented (29). Bifonazole (5), another topical agent, had cure rates of 55-90% in onychomycosis clinical trials, which compared favorably with systemic treatment with other antifungals (30). In a histamine wheal test for antiinflammatory activity, 5 was found to be equivalent to hydrocortisone (31).

Itraconazole (3), an orally active broad-spectrum antifungal, was launched in Mexico and is approved in several other countries . A review of the clinical experience with 3 has appeared (32). In view of its relative lack of endocrine-related side effects, increased spectrum, and superior pharmacokinetics in normal volunteers, as well as in patients with renal dysfunction, 3 offers an attractive alternative to 1 (33,34). Itraconazole has shown clinical efficacy against progressive coccidioidomycosis (35), sporotrichosis, histoplasmosis, blastomycosis (36), aspergillosis (37) and vaginal candidiasis (32). The bioavailability of 3 was increased 2-3 fold when taken after a meal as compared with fasting (38). No cases of itraconazole-induced hepatitis have been reported in over 150,000 patients treated (39).

Saperconazole (4) is a close analog of 3. It has broad-spectrum in vitro and in vivo (p.o., topical and parenteral) activity against dermatophytes and yeasts. Against normal and immunocompromised albino guinea-pigs infected with *Aspergillus fumigatus*, 4 showed better efficacy than 1 and fluconazole (p.o., parenteral) with no drug-related side effects (40). Mice infected with *Aspergillus fumigatus* and treated with 4 (1.25 mpk/day) orally for 5 days had a mean survival time of 25.3 days, compared to 5.33 days for untreated controls and 7 days for those treated with itraconazole (41).



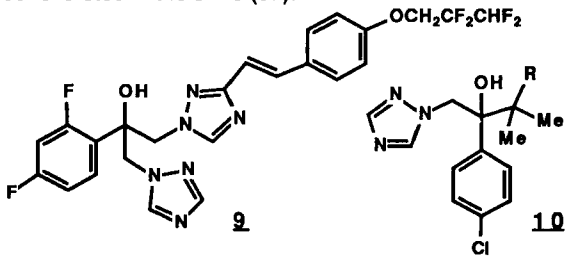
5 6 7 8

Fluconazole (6), an orally active ditriazole (1), has been launched in the U.K. and France. It is indicated for dermal and vaginal fungal infections, including patients with compromised immune functions (42). In a clinical study 6 was shown to have substantially greater selectivity than 1 as an inhibitor of fungal rather than mammalian steroid metabolism (43). Early clinical experience with 6 indicates substantial penetration into CSF, with good efficacy and minimal toxicity (44), and good activity in chronic mucocutaneous candidiasis (45). Patients taking 6 (50 mgs/14 days) for *candida*-associated denture stomatitis relapsed after therapy ended (46). A single oral dose of 6 (150 mgs) produced good results in vulvovaginal candidiasis (47). Unlike 3, the bioavailability of 6 was not influenced by food intake or gastric acidity (38). An iv formulation of fluconazole and additional indications such as cryptococcosis and invasive aspergillosis are being investigated (48)

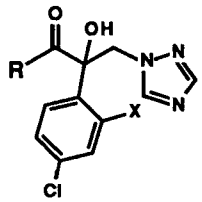
SDZ-89-485 (7), is a broad-spectrum orally active novel triazole undergoing clinical evaluation. It was more active in vivo than 1 and 3 in systemic models and less teratogenic in vivo

than both. The in vitro activity of 7 against *Aspergillus* and *Candida* was similar to 1 and 3. It had no effect on testicular and adrenal steroid synthesis. Oral 7 was effective in a disseminated coccidioidomycosis model in mice (49). SDZ-89-485 is the pure R(-) isomer; the corresponding S(+) isomer of 7 was much less active in vitro and in vivo (50).

Sch 39304 (8), a broad-spectrum novel triazole now undergoing Phase II clinical trials, is an orally, parenterally and topically effective antifungal more potent than 6, especially in immunocompromised animal models (51). In a rat pulmonary aspergillosis model, 8 (p.o.) was the most active azole compared to 1, 3 and 6 (52). Against a *C. neoformans* infection in mice, 8 consistently prolonged survival longer than 6 and lowered brain counts more than 6 following both intracerebral or intranasal challenge (53). In the treatment of immunocompetent and compromised mice infected with *Histoplasma capsulatum*, 8 was about 10-fold more active than 6 (54); against *C. neoformans* in rabbits it was at least as effective as 6 (55). In oral and topical treatment of vaginal *C. albicans* infections in hamsters, and *T. mentagrophytes* infections in guinea pigs, 8 was more active than 6 (56). In human volunteers the T_{max} for Sch 39304 given as a 50 , 100 , or 200 mg single dose was 2-3 hours. The elimination half-life was about 60 hours, with about 60% of the dose excreted in the urine (57).

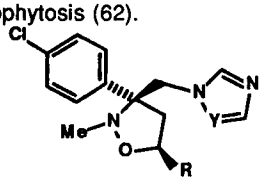


9 10

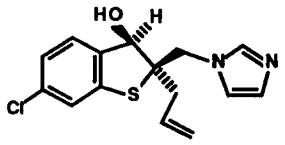


11 R=2,4-$Cl_2C_6H_3$, X=Cl
12 R=iso-propyl, X=H

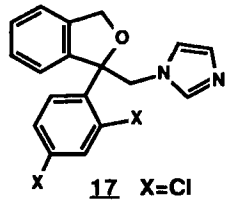
ICI-195-739 (9) is an orally active bis-triazole in preclinical evaluation (58). Against animal infections with *T. quinckeanum* and *C. albicans*, 9 (p.o. or topical) was efficacious at lower dosages than 1, 3 and 6. It was also very effective in protecting mice given lethal inocula of *C. neoformans* or *A. fumigatus* (59,60). Electrazole (BAY R3783, 10) is another broad-spectrum antifungal, active orally and topically in various infection models (61). The nature of group R in 10 has not been disclosed. The triazole, 11 was about twice as effective as 1 against a systemic candidiasis infection in mice, while the corresponding analog, 12 was more effective than 1 against guinea pig dermatophytosis (62).



13 R=p-Cl-C_6H_4, Y=CH
14 R=CH_2-O-C_6H_4-Cl-p, Y=CH
15 R=p-Cl-C_6H_4, Y=N



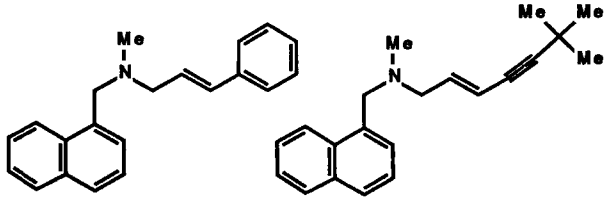
16



17 X=Cl
18 X=F

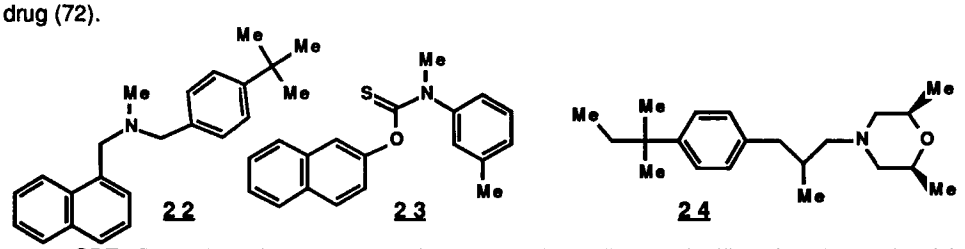
The isoxazolidines 13 and 14 showed excellent in vitro activity, but were less active than 1 in vivo (63). The triazole analog 15 , also demonstrated activity in an immunocompromised model of murine systemic candidiasis (64). The novel dihydrobenzothiophene 16 (and analogs) had excellent topical activity against a *C. albicans* vaginal infection in hamsters, but lacked oral activity in a mouse infection model (65). Likewise the isobenzofurans 17 and 18 had no significant oral activity, but had excellent topical activity against *Candida* and *Trichophyton* infection models. They were superior to miconazole in both infection models. Both 17 and 18 were similar in activity to clotrimazole in a topical *Trichophyton* infection (66).

Allylamines- The unique mechanism of action of the allylamines (squalene epoxidase inhibition) has attracted much attention to these potent antifungal agents (67). Since allylamines do not inhibit cytochrome P-450 linked reactions, side effects associated with P-450 inhibitors such as those seen with azoles may be avoided (68). Naftifine (19), was launched in the U.S. in a topical cream formulation. It has excellent clinical efficacy against dermatophytes (68).



19 20 21

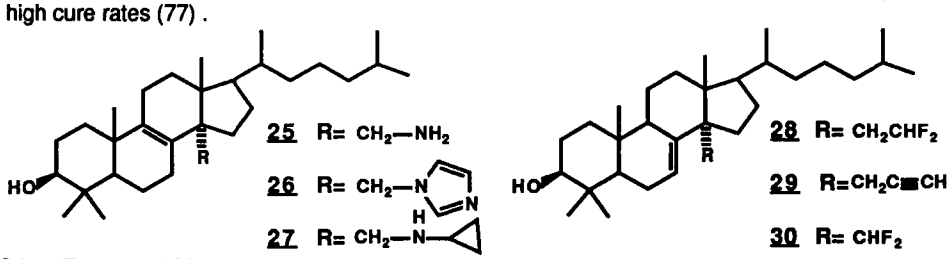
Terbinafine (20), is not only several times more active than 19, but also shows impressive oral activity. It is currently undergoing Phase III clinical trials in both systemic and topical formulations (69). When used topically (1%), terbinafine appeared more active than clotrimazole in terms of mycological cure rates, rapidity of clinical symptom regression and duration of therapy (70). Terbinafine was active against dermatophytes, *Aspergillus*, *Sporothrix* and *Candida* in vitro being either cidal or static (71). The clinical data indicates that 20 has no effect on cholesterol biosynthesis. Oral 20 has shown triphasic elimination with 85% of the drug recovered in feces and 15% in urine; 15 metabolites have been detected (68). The superior results seen in the oral treatment of dermatophytosis of nails with 20, have been attributed to the fungicidal activity of the drug (72).



22 23 24

SDZ-87-469 (21), is a new allylamine analog of 20. Due to significantly enhanced activity of 21 against *C. albicans*, further modifications are in progress (73). The allylamine functionality does not appear to be critical for squalene epoxidase inhibition as evidenced by KP-363 (22), tolnaftate (23) and tolciclate (74,75). The antifungal activity of 22 was compared with 19, 23 and clotrimazole. In general, 22 was more active than the other drugs except against *Candida sp.* where clotrimazole was superior (74).

Morpholines- The activity of this class of antifungals has been attributed to the accumulation of abnormal sterols, resulting mainly from inhibition of Δ^{14}-reductase and Δ^{8}-Δ^{7}-isomerase enzymes (76). Amorolfine (24) is currently undergoing Phase III clinical trials as a topical agent. It is highly efficacious against *Candida*, dermatophytes, dematiaceous fungi and dimorphic pathogens. In vaginal mycoses, 50 mg intravaginal tablets of 24 were well tolerated and produced high cure rates (77).



25 R= CH_2—NH_2

26 R= CH_2—N(imidazole)

27 R= CH_2—N(cyclopropyl)

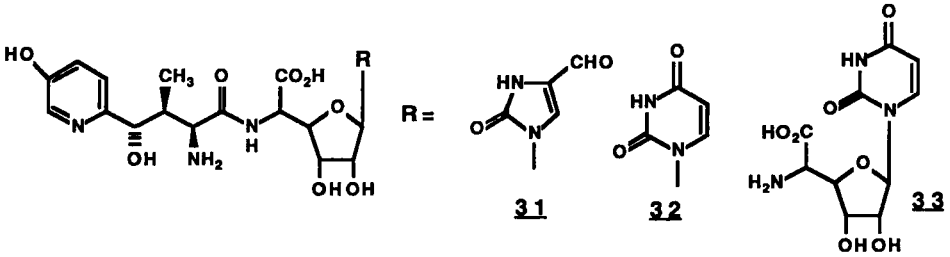
28 R= CH_2CHF_2

29 R= $CH_2C{\equiv}CH$

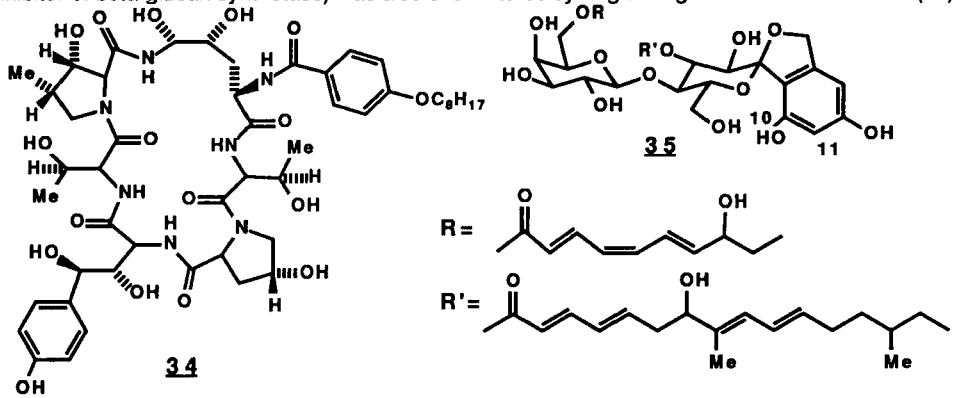
30 R= CHF_2

Other Ergosterol Biosynthesis Inhibitors- A logical approach to ergosterol biosynthesis inhibition would be a heme binding component at the 14α-methyl position of lanosterol. Based on this concept, two groups have independently reported on analogs 25 - 30 (78,79). In terms of MICs, the compounds 25 and 26 were more active against dermatophytes than *Candida* strains. Compound 26 was as effective an inhibitor of ergosterol synthesis as 1. However, there was no correlation between IC_{50}s in rat liver cytochrome P_{450} and MICs (78). Compounds 28 to 30 were powerful inhibitors of lanosterol 14α-methyl demethylase (79).

Fungal Cell-Wall Inhibitors- Two excellent reviews on the cell envelope and nutrient transport in *C. albicans* have appeared (80,81). Chitin synthetase inhibition continues to be a viable approach for the design of antifungal agents (82). Recently a new enzyme termed chitin synthetase 2 (Chs 2), was isolated from a *Saccharomyces cerevisiae* mutant. Gene disruption experiments led to the surprising conclusion that it was Chs 2 (and not the previously isolated major enzyme Chs 1) which was responsible for the septum formation and regulation of chitin synthesis (83). These results have yet to be verified in *C. albicans.*



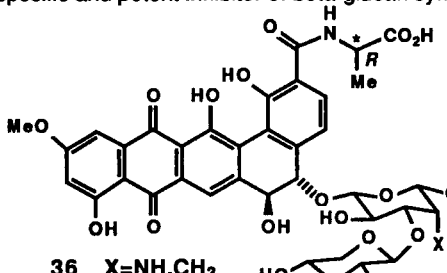
Nikkomycins X and Z (31, 32) showed efficacy in a disseminated candidiasis model in mice, but on discontinuation of treatment the animals relapsed (84). Nikkomycin Z was active in mice against a systemic *Coccidiodomycosis immitis* infection; had only moderate activity against *Blastomyces dermatitidis*, and had no activity against *Histoplasma capsulatum* (85). These are the first examples of a chitin synthetase inhibitor showing activity in systemic animal infections. A corn protein was reported to be synergistic with 31/32 and reduced the MICs of nikkomycins 100-fold (86). Semisynthetic analogs derived from uracil polyoxin C (33), have been reported (87). Several new nikkomycins were isolated from culture filtrates of *Streptomyces tandae,* and shown to vary in their amino acid side chains (88). Two new analogs of nikkomycins Z and J, having a C-glycosidic linkage between uracil and 5-amino-deoxy-D-allo-furanuronic acid, have been isolated from *Streptomyces tandae.* These CC-nucleoside nikkomycins exhibit inferior antifungal activity than the CN-nucleoside nikkomycins (89). A combination of 31/32 and papulacandin B, (35) (an inhibitor of beta-glucan synthetase) was also shown to be synergistic against intact *C. albicans* (90).



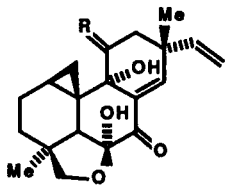
Another class of antifungal agents under investigation are beta-glucan synthesis inhibitors. Cilofungin (34), a semi-synthetic echinocandin B derivative, is a narrow spectrum parenteral antifungal with specific activity against *C. albicans* and *C. tropicalis* (91). It is undergoing extensive preclinical studies (92). In a comparison with several clinically useful antimycotics, 34 was shown to be more active (MICs) against 75 strains of *Candida sp.* (93) A combination of 34 and anticapsin was synergistic and more active against *Candida* strains than 34 alone (94). In disseminated candidiasis in rabbits, 34 was equivalent to AMB in renal and occular tissue, but more effective in cardiac tissue (95). Cilofungin was shown to be a non competitive inhibitor of *N. crassa* (1-3)-beta-D-glucan synthase activity (96).

The difference between the good in vitro and poor in vivo results with papulacandin B (35) was explained in terms of a possible relationship between inhibition of the enzyme and enzyme conformation in whole and broken cells (97). Some of the 10-alkyl ether and 11-acylamino

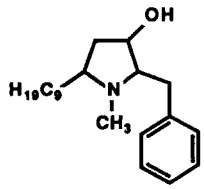
derivatives of **35** exhibited much improved in vivo activity (98). The activity of aculeacin A as a specific and potent inhibitor of beta-glucan synthase in *C. albicans* was confirmed (99).



36 X=NH.CH$_3$
37 X=OH
38 X=NH$_2$

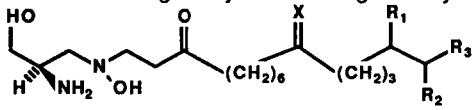


39 R=O
40 R=H, H

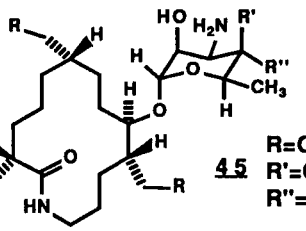


41

Natural Products- Pradimycin A (**36**) , isolated from a new strain of *Actinomadura hibisca* , exhibited broad-spectrum fungicidal activity including activity against many strains that were resistant to other antifungal agents. The in vivo efficacy of **36** against a variety of *Candida sp* was compared with 5-FC, a number of azoles and AMB (100). In a cryptococcal meningitis model, **36** (PD_{50} 22 mpk, i.p.) was more active than the azoles and 5-FC, but less active than AMB (PD_{50} 2.2 mpk, i.p.). Although not as active as AMB, **36** shows significantly less acute toxicity in mice (LD_{50} > 144 mpk vs. 2.6 mpk, i.v.). Benanomicins A (**37**) and B (**38**) isolated from a culture of *Actinomycete sp*, are closely related to **36** . They were markedly effective against *C. albicans* and *A. fumigatus* infections in mice with low acute toxicity (101). The novel diterpenes myrocin B (**39**) and C (**40**) produced by *Murothecium verrucaria* showed in vitro activity against *C. albicans* and *A. niger*, with **39** being more active (102). L-657,398 **41** is a novel broad-spectrum antifungal (in vitro) isolated from *Aspergillus ochraceus* fermentations (103). The antifungal spectrum of **41** against both filamentous fungi and yeasts was significantly broader than anisomycin (104).



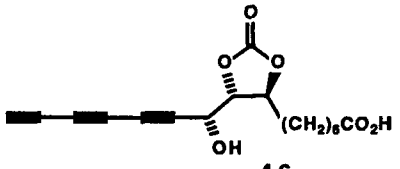
42 R_1=R_2=CH_3; R_3=H; X=O
43 R_1=H; R_2=R_3=CH_3; X=O
44 R_1=R_3=H; R_2=CH_3; X=



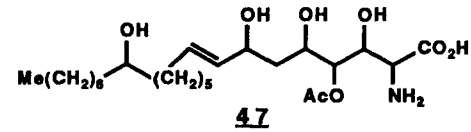
45 R=CH3, H
R'=OH, H
R"=H, OH

The three neoenactin congeners **42** (B_1), **43** (B_2) and **44** (M_2) were active in vitro against yeasts and fungi. They also potentiated the activities of polyene antifungals (105). A series of novel macrolactams (**45**) produced by *Actinomadura vulgaris* and *fulva* subspecies were reported (106). They had marked activity against *Candida sp.* but were much less active (in vitro) against dermatophytes. Comparative in vitro antifungal activity of these compounds **45** against *Candida sp.* was also presented. A novel triynecarbonate **46** (Sch 31828/L-660,631) produced by an unusual *Microbispora sp.* (107) and *Actinomycete* cultures (108), exhibited good in vitro activity against *C. albicans* and dermatophytic fungi. Inhibition of the sterol biosynthetic pathway prior to mevalonate synthesis was regarded as the primary site of action of **46** against *C. albicans* (108).

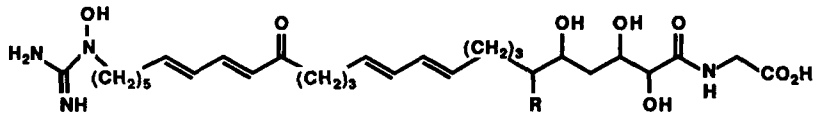
Fumifungin (**47**), isolated from a *A. fumigatus sp.*, showed in vitro activity against yeasts and filamentous fungi (109). Two new antibiotics octacosamicins A (**48**) and B (**49**) were isolated from a strain of *Amycolatopsis*. Both **48** and **49** had broad-spectrum in vitro antifungal activity against *Candida sp.*, *C. neoformans* and *A. niger* (110). Mulundocandin is a new lipopeptide antifungal closely related to the echinocandins (111). Another new lipopeptide antifungal agent, L-671,329 (**50**), related to echinocandin B was isolated from a filamentous fungus *Zalerion arboricola* (112,113). L-671,329 possesses potent anti-*Candida* activity including *C. parapsilosis*



46

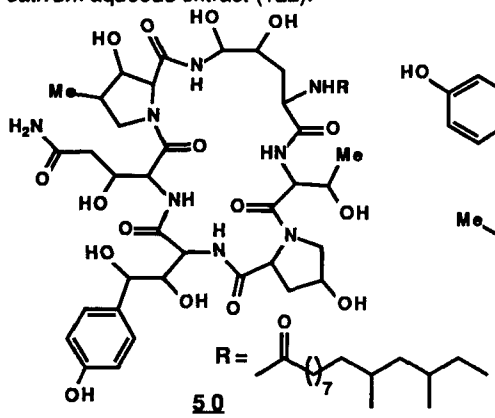


47

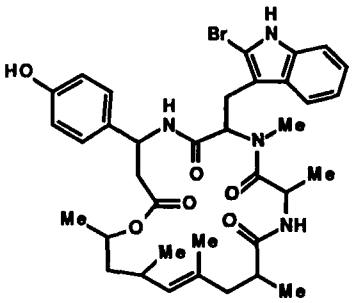


48 R=H 49 R=CH_3

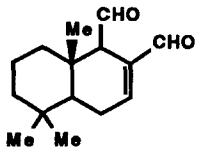
(114). It significantly prolonged survival of mice infected with *Candida albicans* (ED_{50} 3.38 mpk, ip) and was more active than aculeacin (ED_{50} 6.44 mpk, ip). Jasplakinolide (51) , a cyclopeptide isolated from *Jaspis sp.* was similar in topical efficacy to miconazole against a murine vaginal *C. albicans* infection. The toxicity and narrow spectrum activity of this marine natural product may limit its development (115). A review on antifungal substances from marine invertebrates has appeared (116). Xylocandin, a complex of novel peptides produced by a *Pseudomonas cepacia* sp. displayed potent in vitro activity against *Candida* and dermatophytes. The extensive inactivation in the presence of serum and vaginal fluid may account for it's lack of in vivo activity (117). Polygodial (52), a sesquiterpene dialdehyde, was shown to facilitate fungal cell-membrane permeability (118) as did geraniol and iturin A (119,120). A chitin soluble extract was found to be effective in preventing persistent candidal vaginitis in hormone-treated mice. The nature of the active component that also blocks adhesion of *C. albicans* (in vitro and in vivo) is not known (121). Blockage of lipid synthesis is probably an important component of the anticandidal activity of *Allium sativum* aqueous extract (122).



50



51



52

Screening Methodology - Some basic aspects of pure and applied studies with in vivo models for testing antifungal agents have been presented (123). A new model for the rapid in vivo evaluation of antifungal agents was described (124). This four-infection murine model includes vaginal and systemic *C. albicans* infections, a dermal infection with *T. quickeanum* and a lung infection with *C. neoformans* . Studies with eight antifungal agents showed no interference between the four infections in the determination of drug activity. A considerable degree of information with a minimal amount of compound and effort may encourage other laboratories to adopt this new procedure. A minireview highlighting some of the current issues relating to the reproducibility and relevence of antifungal susceptibility testing has appeared (125). A reproducible method for the determination of the MICs of azole antifungals with sharp endpoints using either yeasts or molds was described (126). A ready-to-use micromethod using the same synthetic medium for a diverse class of antifungals has also been described . Ready-made microdilution plates containing 5-FC, AMB and azoles allowed MIC determinations of seven antifungal agents. The reproducibility average for over 4200 antifungal agent-yeast results in this new assay procedure was an impressive 96.3% (127).

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Chapter 13. New Approaches to Antitumor Therapy

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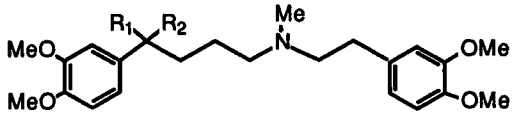
Introduction - Tumor biology at the experimental and clinical level has provided new opportunities for the treatment of cancer. Particularly important has been the recent identification of previously unrecognized biomolecular targets for therapy. This chapter will concentrate on three representative new approaches to anti-tumor therapy: the circumvention of multidrug resistance; the antagonism of mitogenic growth factors, particularly gastrin releasing peptide; and finally the inhibition of oncogenic tyrosine kinases. Each area holds promise for the evolution of novel therapeutic agents, and specific molecular approaches to each are emerging.

Multidrug Resistance - The utility of current antitumor chemotherapies generally relies on narrow gradients in sensitivity between the target tumor cells and non-target tissues. Small changes in the sensitivity of tumors to cytotoxic therapy can ablate that differential sensitivity. Numerous factors have been identified which may contribute to the resistance of tumor cells to antitumor agents. Genetic aspects of several forms of resistance to antitumor agents have been recently reviewed (1). Changes in cellular enzymes linked to antitumor drug resistance were summarized in Volume 23 of Annual Reports (2). Insufficient vascularization of the tumor mass and resulting hypoxia may also alter drug efficacy by altering drug delivery, cellular pH, and reducing active growth fractions (and hence susceptibility to cell cycle active agents). It may also shift the internal and external cellular environments to a more reducing state, leading to enhanced capacity for sulfhydryl based detoxification and repair mechanisms (3). In addition to increased glutathione detoxification pathways which may impair activity of electrophilic or oxidatively activated agents (4,5), elevated metallothionein may also contribute to tumor cell resistance (6). Conversely, metabolic activation of redox-potentiated agents such as mitomycin C may be reduced in resistant tumor cells (7). Active intracellular transport of some agents, such as nitrogen mustard, melphalan, and antimetabolites such as cytarabine and fluorodeoxyuridine, can be altered, reducing effective intracellular exposure at the biochemical target (8).

Multidrug resistance (MDR) is characterized by cross-resistance to a group of structurally and mechanistically distinct antitumor agents, including the anthracyclines, doxorubicin and daunorubicin; the vinca alkaloids, vincristine, and vinblastine; actinomycin D, colchicine and the epipodophyllotoxins. This resistance does not extend to all agents; for example, antimetabolites such as methotrexate, cytarabine, thioguanine, steroids such as dexamethasone, alkylating agents such as BCNU and cyclophosphamide are not affected. Reviews of the biological, biochemical, and clinical features of MDR have appeared (9-11). This section will focus on recent characterization of the gene product responsible for the MDR phenotype, its clinical expression, and emerging pharmacology associated with inhibition or reversal of the phenotype as a possible novel approach to antitumor therapy.

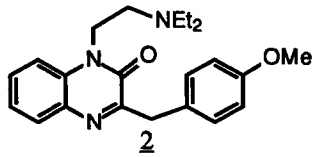
MDR can be induced by the expression of human *mdr1* cDNA, and also by gene transfection (12-14). The gene encodes a membrane associated phosphoglycoprotein (Pgp) of approximately 180kD mass, which shows extensive homology with the bacterial transport proteins, including apparent ATP binding sites (15,16). Mutations in the *mdr1*-gene can alter the profile of cross resistance to antitumor agents (17). The protein is labelled by an ATP photoaffinity reagent (18). Immunopurified protein has been shown to possess ATPase activity (19). Reduced drug accumulation in *mdr1*-expressing cells correlates with the degree of resistance, and conversely drug efflux from the cell increases with resistance. Vinblastine efflux from vesicles prepared from *mdr1* expressing cells shows an ATP dependency as demonstrated by inhibition with the non-hydrolyzable ATP analog 5'-(beta-gamma-imido)ATP and the ATPase inhibitor vanadate (20). Immunohistochemical localization of the Pgp in MDR cells shows highest occurrence on the surface of the plasma membrane and on the luminal side of the Golgi stack membranes (21). These features are consistent with the characterization of Pgp as a membrane-associated, energy-dependent transporter of antitumor agents.

Expression of the *mdr1* gene occurs in normal human tissues such as colon, lung, liver and kidney, tissues which are frequently exposed to xenobiotics (22-25). The intrinsic resistance to antitumor chemotherapy of tumors originating from these sites may be related to *mdr1* expression in the tissue of origin, as well as the acquisition of the MDR phenotype by selection for *mdr1*-expressing tumor cells by antitumor drug treatment (26-28). Selective expression of Pgp in the capillary endothelia of the brain and testes may contribute to the difficulty in treatment of metastases in these tissues (29). Thus inhibition or reversal of the MDR phenotype appears as a promising clinical target for improvement of current antitumor therapy.



1 R_1 = iPr , R_2 = CN

6 R_1, R_2 = -S(O$_2$)CH$_2$CH$_2$CH$_2$S(O$_2$)-



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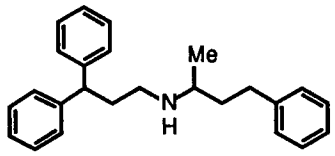

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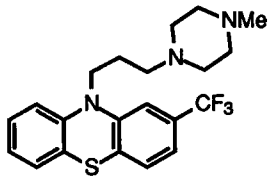

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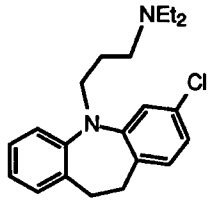

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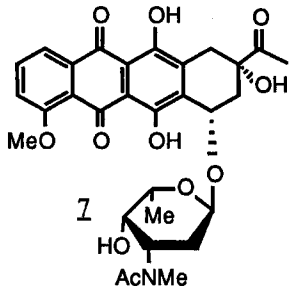
Early studies of the pharmacology of MDR showed that a number of calcium antagonists, including verapamil (1), caroverine (2), prenylamine (3), trifluoperazine (4) and clomipramine (5), are effective in restoring sensitivity to cytotoxic agents (30). This led to the proposal that calcium metabolism may be directly linked to MDR. However, the reversal of MDR by these agents is not correlated with effects on calcium transport or action, indicating independent pharmacologies exist (31,32). While verapamil has been used extensively as a prototypical agent for the study of MDR, and binds to and apparently directly inhibits Pgp function (33), it also restores reduced plasma membrane potentials in MDR cells, which may also affect cellular resistance (34). Its efficacy in reversing drug resistance in MDR cells may thus be due to several pharmacologic properties, including selective potentiation of topoisomerase/DNA acting agents (35). Verapamil and trifluoperazine increase the phosphorylation of Pgp, and this may have a role in regulation of its function (36). While neither vincristine nor doxorubicin affect the ATPase activity of immunopurified Pgp, both verapamil and trifluoperazine increase the enzymatic activity (37). Verapamil and a number other inhibitors of MDR induce a rapid ATP consumption in MDR tumor cells, while the verapamil analog tiapamil (6), which retains calcium antagonist activity but has little effect on MDR, shows no appreciable effect on ATP consumption. Neither vincristine nor daunorubicin significantly affect ATP levels at cytotoxic concentrations, and the authors suggest that these results may reflect a competition by the antitumor drugs and MDR reversal agents for a common energy-dependent extrusion mechanism (38).



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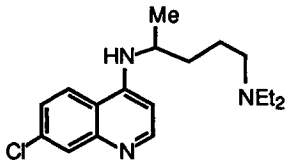

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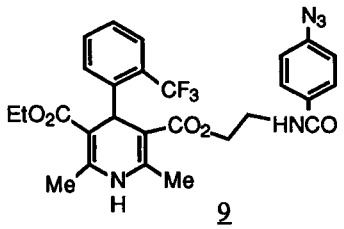
In addition to calcium antagonists, a diverse range of structurally and pharmacologically distinct drugs have been shown to reverse MDR. N-acetyl daunorubicin (7) and other anthracycline analogs are also effective, with a good correlation between hydrophobicity and effect on daunorubicin accumulation (39,40). The antimalarial agents quinidine and chloroquine (8) have

been shown to inhibit MDR (41,42). Interestingly, malaria resistance to chloroquine shows parallels with tumor MDR, including reduced uptake of drug, as well as reversal by verapamil, and other calcium antagonists (43,44) A study of physicochemical properties of compounds that modulate multidrug resistance in human leukemic cells led to a proposal that lipid solubility, cationic charge, molar refractivity and some structural similarities, including a broad requirement for an aromatic domain and a nitrogen atom, may be important properties for activity (45).

Cyclosporin A and non-immunosuppressive homologs modulate doxorubicin resistance in MDR cell lines but this effect may be associated with restoration of plasma membrane potentials rather than direct effects on Pgp (46,47). Cyclosporin A also has been shown to enhance vinblastine accumulation in MDR cells, and cyclosporin accumulation itself is reduced in *mdr1*-expressing kidney cells, but can be restored by verapamil (48). The anti-estrogen tamoxifen has been shown to restore sensitivity to doxorubicin and vinblastine in MDR cells, but without an effect on doxorubicin accumulation (49). Another multidrug resistance phenotype, described as "atypical" multidrug resistance has been described. It differs from *mdr1* -linked MDR in that affected cells show a lack of cross-resistance to the vinca alkaloids, and no apparent defect in drug accumulation (50,51).



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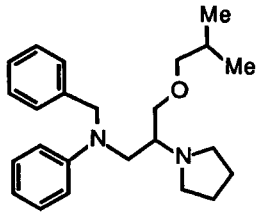

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Binding of (^{3}H)vinblastine to Pgp expressing MDR cells is increased relative to sensitive or revertant cells, and is inhibited by vinblastine, vincristine and daunomycin, but not actinomycin D. Binding is also inhibited by verapamil, which reverses the MDR phenotype (52). The Pgp is modified by the vinca alkaloid photoaffinity label, N-(4-azido-3-^{125}I)salicyl-N'-(2-aminoethyl)vindesine, and this is blocked by vinblastine and vincristine, but not colchicine (53). The dihydropyridine calcium channel blocker, ^{3}H-azidopine (9) also photolabels the Pgp, and this can be inhibited by agents that reverse MDR, including nimodipine, nitrendipine, nifedipine, diltiazem, and verapamil, but is stimulated by two other MDR inhibitors, prenylamine and bepridil (10) (54). Binding is inhibited by doxorubicin, actinomycin D, vinblastine and colchicine. A murine monoclonal antibody, MRK-16, which reacts with the Pgp, increases the uptake of vincristine in MDR cells, and potentiates the activity of verapamil in restoring uptake of vincristine and daunorubicin (55). These data suggest that the Pgp not only plays a role in MDR, including binding of certain antitumor agents, but also has direct interaction with a number of agents that reverse the phenotype.

Experimental clinical studies of inhibitors of MDR in conjunction with established antitumor agents have been described. Trifluoperazine and doxorubicin have been evaluated in a Phase I/II study in patients with intrinsic (no initial response to prior therapy) and acquired drug resistance (initial response followed by treatment failure), and the authors conclude further evaluation is warranted based on the response rate seen (56). Studies with verapamil and doxorubicin in patients multiple myeloma have been initiated (57). Verapamil is also being studied in conjunction with vinblastine and etoposide in pediatric relapsed patients, with dosage of verapamil limited by cardiotoxicity (58).

Mitogenic Growth Factors- Increasing evidence points to the role of mitogenic growth factors in sustaining the growth of a number of important tumor cell types, including colon and gastric cancers (gastrin(59,60)), breast cancer (epidermal growth factor (EGF) (61,62)), and small cell lung cancer (gastrin releasing peptide or GRP, and vasoactive intestinal peptide, or VIP(63)). Tumor dependence may reflect the responsiveness of the tissue of origin to the growth factor or an inappropriate expression of either the growth factor or the growth factor receptor, giving rise to an exocrine, paracrine or autocrine pathway signal for inappropriate growth (64). Growth factor involvement in maintenance of the transformed phenotype is, like most aspects of the phenotype, likely to be heterogeneous at the tumor level. While this may restrict the therapeutic utility of growth factor antagonists to a responsive subpopulation of a target tumor, the possible tumor or tissue selectivity

of such agents may still provide a therapeutic advantage. A discussion of inhibition of growth factor action as an approach to cancer chemotherapy has recently appeared (65). Gastrin releasing peptide (GRP) (11) or the related amphibian peptide bombesin (BN) (12), have been studied in particular detail as therapeutic targets.

GRP or related peptides have been implicated in maintaining the growth of small cell lung carcinoma (SCLC) which comprises approximately 25% of lung cancer cases (66). GRP and BN represent a class of peptidic neuro-endocrine hormones with mitogenic activity, characterized by a highly conserved C-terminal region. A brief review of the family of peptides related to GRP and their antagonists has been published (67). A more comprehensive review on BN, BN antagonists and SCLC has appeared (68).

11 V-P-L-P-A-G-G-G-T-V-L-T-K-M-T-P-A-G-N-H-W-A-V-G-H-L-M-NH_2

12 pGlu-Q-R-L-G-N-G-W-A-V-G-H-L-M-NH_2

It has been suggested that in SCLC, these proteins support neoplastic growth in an autocrine manner. Both the proteins and their receptors are expressed by cells in these tumors that are also responsive to their mitogenic effects. Growth of SCLCcell lines *in vitro* and *in vivo* can be inhibited by a monoclonal antibody to BN, as well as by peptides that antagonize GRP and BN binding (69-72). However, the autocrine dependence of SCLC lines on GRP has been debated, and may be a function of culture conditions (73). GRP involvement in primary human disease may not be proven in advance of clinical evaluation of specific antagonists.

A number of peptidic antagonists of GRP and BN, and also monoclonal antibodies to BN, inhibit growth of SCLC lines, as well as BN-stimulated growth of murine Swiss 3T3 cells. Modified derivatives of substance P ((13), and (14)) inhibit the cellular effects of GRP and vasopressin in Swiss 3T3 cells, and also inhibit growth of SCLC cells in serum free medium (74). While the growth inhibition of SCLC lines by these peptides is reversed when the inhibitor is removed, it is not reversed by addition of GRP in the presence of the antagonists. Concentrations of the antagonists required to block cell growth are roughly ten-fold greater than those required to block GRP binding in Swiss 3T3 cells.

Another BN/GRP antagonist, spantide, (15), shows similar differences between growth and BN binding inhibition in SCLC lines, and growth inhibition is not reversed by exogenous BN (75). The antagonists were effective in inhibiting growth of A549 cells, a non-SCLC, non-BN producing cell line that lacks BN receptors. Thus 15 may inhibit SCLC growth by an independent mechanism, leading the authors to question the role of GRP in autocrine growth of SCLC cells. Several of the antagonists derived from substance P also antagonize other neuro-endocrine peptides, such as substance P (76) and vasopressin (77,78), and interpretation of their effects on cellular events may be complicated by this promiscuity.

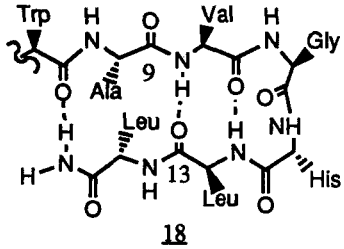
13 (D)R-(D)P-K-P-Q-Q-(D)W-F-(D)W-L-L-NH_2

14 (D)R-P-K-P-(D)F-Q-(D)W-F-(D)W-L-L-NH_2

15 (D)R-P-K-P-Q-Q-(D)W-F-(D)W-L-L-NH_2

16 p-Glu-Q-R-L-G-R-Q-W-A-V-G-H-L-CH_2-L-NH_2

17 Acetyl-H-W-A-V-G-H-L-M-NH_2



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A potent series of BN antagonists has been derived from (Leu^{14})-BN. Replacement of C-terminal amide carbonyl residues with methylene groups effectively disrupts agonist function (79). Structure-activity relationships of these modified bombesin derivatives have been interpreted in terms of a requirement for a C-terminal beta-turn for agonist activity in (Leu^{14})-BN, which is disrupted by replacement of carbonyls (of Ala^9 or Leu^{13}) with methylene residues (18). One of these compounds, (16), inhibits BN-stimulated growth of Swiss 3T3 cells (IC_{50} = 18nM), as well as bombesin binding to pancreatic acinar cells (Kd = 60nM). While 16 inhibits BN-stimulated amylase release from pancreatic acinar cells at 35nM, it shows no appreciable inhibition of the effect of other neuro-endocrine peptides at a concentration of 300uM. Unlike a number of the substance

P-based antagonists, 16 does not inhibit vasopressin-stimulated Swiss 3T3 cell mitogenesis at a concentration (1uM) that fully suppresses GRP-stimulated growth. Furthermore, the inhibition of GRP effects is completely overcome by increased GRP concentration (80). Inhibition by 16 of BN-stimulated increases in inositol triphosphates and Ca^{2+} levels in SCLC lines has also been demonstrated, providing additional evidence for a GRP-based mechanism of SCLC growth inhibition (81). Thus 16 appears to be a highly selective antagonist for study of GRP/bombesin in cellular systems. No reports of *in vivo* activity of these derivatives have been published.

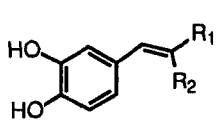
The importance of the C-terminal domain for GRP binding and mitogenicity has also been delineated by a systematic N-terminal deletion of GRP-20-27 derivatives; the octapeptide N-acetyl GRP-20-27 (17) shows full agonist activity. When the native C-terminal sequence of GRP is retained, the potencies for binding to the GRP receptor and GRP-agonism (mitogenic stimulation of Swiss 3T3 cells) show strong concordance. This indicates His^{20} and Trp^{21} (corresponding to Trp^{8} of BN) have a significant role in receptor binding (82). The clarification of the minimal ligand features required for binding and agonist effects may provide useful information for smaller peptidic or non-peptidic inhibitors.

In addition to peptidic GRP antagonists, a murine monoclonal antibody 2A11, raised against Lys^{3}-bombesin and cross reactive with a number of GRP and BN derivatives, but not other neuro-endocrine peptides, blocks clonogenic growth of human SCLC lines *in vitro*, in a manner which is reversed by addition of exogenous BN (83). This selective growth inhibition is also demonstrable *in vivo,* against a BN-responsive human SCLC xenograft, without any effect against the growth of a BN-nonresponsive melanoma. 2A11 also inhibits growth of a BN sensitive pancreatic cancer cell line, which may have implications for GRP as a therapeutic target in this cancer (84). The antibody appears to be well tolerated in dogs at doses (50mg/dog) well above those required for inhibition of the physiological response to exogenous GRP or BN (>2mg/25kg dog), and thus appears to have potential for study of GRP involvement in SCLC in man (85).

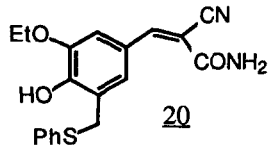
Protein Tyrosine Kinases - The protein tyrosine kinases, which appear to play important roles in the transduction of signals initiating cellular replication and transformation, represent one type of an ever-increasing family of regulatory protein kinases . Although protein phosphotyrosine constitutes a small percentage of total protein phosphorylation, the critical nature of the protein tyrosine kinases in the regulation of cellular growth makes them potential targets for drug development. Not only have many oncogene products been identified as protein tyrosine kinases, but the cellular receptors which normally transduce growth factor signals frequently phosphorylate protein tyrosines as well. Importantly, several oncogene products are actually altered growth factor receptor tyrosine kinases. Protein tyrosine kinases and their role as oncogenes and growth factor receptors have been reviewed (86-90). The phosphotyrosyl protein phosphatases, which may have important regulatory functions in protein phosphorylation cycles, were also recently surveyed (91).

Protein tyrosine kinases has now been associated with several human cancers. Examples include the activation of c-abl in chronic myelogenous leukemia (92), the expression of the erb-B-2 oncogene in human breast cancer (93-94) and the increased tyrosine kinase activity of c-src in colon carcinomas (95,96). As human tumor tissues are increasingly probed for activating mutations and over-expression of protein tyrosine kinase activity, the role of these and other oncogenes in human cancer will be clarified.

The plethora of protein kinases makes the development of an inhibitor specific for a particular target a formidable task. A variety of approaches, including reversible and irreversible protein and nucleotide substrate-based inhibitors are possible, and have been reviewed (97). Most of the inhibitors described to date do not show a high degree of specificity for tyrosine kinases and affect serine protein kinases and other nucleotide dependent enzymes. Several dicyanostyrenes and cinnamic acid derivatives do show selective inhibition of the EGF receptor kinase (98). Compound 19 preferentially inhibits the EGF receptor kinase (IC_{50} = 0.85uM) over insulin receptor tyrosine kinase (IC_{50} = 640uM), as well as EGF-dependent, but not EGF-independent cell growth. These compounds are related to another series of 4-hydroxycinnamide derived tyrosine kinase inhibitors, one of which, ST638 (20), inhibits the EGF receptor (IC_{50} = 0.37 uM) without affecting the activity of the six other protein kinases tested (99). Thus, these results suggest that selective inhibition of target protein kinases is possible, and can result in selective growth inhibition. Several other polyhydroxylated aromatic derivatives are inhibitors of tyrosine kinase activity, including erbstatin (100), genistein (101) and a number of flavonoids(102,103). Erbstatin (21) competitively inhibits the EGF receptor tyrosine kinase with respect to a peptidic substrate , whereas genistein (22) and the flavonoids show competition with ATP (104).



19 $R_1 = CSNH_2$, $R_2 = CN$
21 $R_1 = NHCHO$, $R_2 = H$



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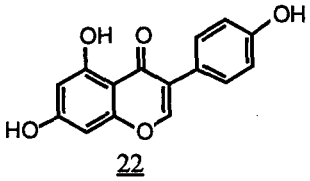

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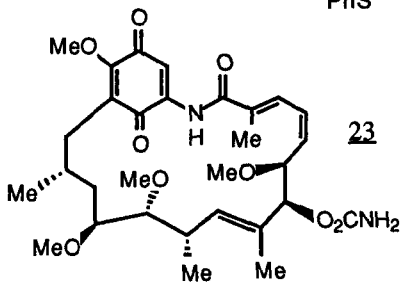

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Multisubstrate inhibitors, mimicking adenosine triphosphate and tyrosine inhibit v-abl tyrosine kinase (105,106), but these compounds appear to function as adenosine analogs and are not sensitive to structural changes in the tyrosine substrate residue. Low affinity peptidic substrate analogs have also been prepared as tyrosine kinase inhibitors (107). Proteins functioning as physiological inhibitors of tyrosine phosphorylation exist and play important regulatory roles. Mullerian inhibiting substance, (MIS), induces regression of the Mullerian duct in the embryo. This protein has been shown to inhibit EGF receptor phosphorylation and cell growth in A-431 cells at picomolar concentrations (108). The benzoquinoid ansamycins, including geldanamycin (23), inactivate the tyrosine phosphorylation of v-src kinase in intact cells and revert the oncogene transformed cells to a normal morphology (109). Although these antibiotics do not directly inhibit the v-src kinase, they do support the rationale for developing specific inhibitors of key protein tyrosine kinases.

Conclusion - The approaches described above are founded on the advances in our understanding of unique aspects of tumor biology that have recently emerged. As specific pharmacologic agents emerge from these areas, their value in antitumor therapies can only be judged in the most relevant model for cancer therapy, human clinical trials. As these approaches fall outside the conventional paradigms of classic cytotoxic therapies, the definitive testing of the hypotheses they represent will require creative and skillful solutions to the challenging clinical problems their evaluation will present.

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Chapter 14. Antiviral Agents

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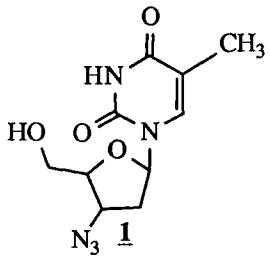
Introduction - Progress in the chemotherapy of RNA and DNA viral infections has recently been reviewed (1). Several targets have been defined as potential approaches for the treatment of AIDS (2). Knowledge concerning the structure of the virus has, over the past few years, reached remarkable proportions. What remains to be done is to utilize this information to design more potent drugs with the goal of curing rather than retarding the disease.Work continues in the picornavirus area with some clinical studies being reported; however, no drug as yet has achieved therapeutic clinical efficacy. Additional compounds have been reported to be effective against herpes 3virus and hepatitis B infections, but none of these have been marketed. This review will cover advances in the chemistry of HIV, picornavirus and herpes virus infections.

RNA VIRUSES

HIV Infections - Two clinical studies have recently been reported on the effect of AZT (1), in children (3,4), the results of which suggests that an optimal i.v. dose of between 0.9 and 1.4 mg/kg/hr results in an improvement in disease related neurological abnormalities. Maternal transfer of AZT was shown to be sufficient to protect neonatal mice from challenge with a retrovirus and prevent development of disease (5). AZT has been found to act synergistically with foscarnet (2), in inhibiting the replication of HIV in human peripheral blood mononuclear cells (6). A combination of 100 mg of AZT and 800 mg of acyclovir administered orally every 4 hours was found effective in AIDS patients and was well tolerated (7).



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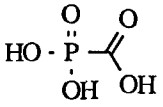


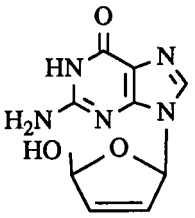
2

Dextran sulfate was found to be a potent agent against HIV-1 *in vitro* (8,9). Recently, it was shown to block the binding of the virions to target T lymphocytes and thus inhibit virion binding to CD_4^+ cells. It also suppressed the replication of HIV-2 *in vitro* (10).

The use of antisense oligodeoxynucleotides in the inhibition of HIV replication has been previously discussed (11). Three classes of phosphate modified antisense oligodeoxynucleotides exhibited 95% inhibition of HTLV-III in tissue culture at ~20 μM, and preliminary toxicity studies in mice indicated that a dose of 40 mg/Kg of body weight showed no overt toxicity.

Carbovir (3), was reported to exhibit significant activity against HIV in both ATH8 and MT-2 cells with an MIC_{50} in the range of 0.25 to 0.50 μg/mL (12). A series of

phosphonomethoxyethyl purines have been evaluated against HIV and found to be effective (13). PMEDAP (4), was effective at 1μMol while PMEA (5), exhibited an ED_{50}



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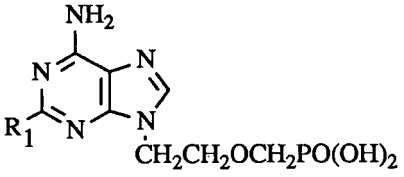


4 $R_1 = NH_2$

5 $R_1 = H$

of 2 μMol in either MT-4 or H9 cells. Thus far the mechanism of action has not been determined. The HIV protease has been shown to be an aspartic-type protease (14,15). Pepstatin A was shown to inhibit part of the intracellular HIV-specific gag processing. This suggests yet another interesting approach to the inhibition of HIV replication. A clinical study with cyclosporin (7.5 mg/kg/day) was designed to examine the possibility that this drug could inhibit replication and prevent the effect of HIV (16). A sustained increase of T_4 cells with a decrease in T_8 cells occurred and lymphadenopathy disappeared in 14/16 patients. After withdrawal, all of the parameters returned to pretreatment status.

Considerable efforts have been directed toward preventing adsorption of HIV by either blockade of or use of soluble recombinant CD_4 receptors, which have been shown to be essential for virus entry into the host cell (17). Recombinant soluble CD_4 purified from a Chinese hamster ovary cell line, as well as from a baculovirus expression system, was shown to be a potent inhibitor of both virus replication and virus-induced cell fusion *in vitro* (18,19). Soluble CD_4 was also expressed in several cellular environments and was found to bind to the envelope glycoprotein (gp 120) of HIV and inhibit the binding of virus to CD_4+ lymphocytes (20,21). A purified CD_4 [83-94] protein dibenzylated at cysteine 86 and glutamate 87 has exhibited antisyncytial activity at 125 μM. It was suggested that dibenzylation at the peptide may mimic the conformational constraints of the holoreceptor peptide fragments imposed by the Cys^{86} to Cys^{18} sulfur bond in the native molecule (22). Soluble recombinant CD_4 (rcd_4), conjugated to the plant toxin ricin was found to kill HIV infected H9 cells with an IC_{50} of $1.5 \pm 0.53 \times 10^{-10}$M, and was non-toxic to non-infected cells. The free rCD_4 was only 1/1000 as effective (23). A toxin produced by E. coli has been shown to contain the HIV-binding portion of the human CD_4 molecule linked to active regions of pseudomonas exotoxin (24). HIV infected cells have been shown to be sensitive to this toxin which displays selective toxicity towards cells expressing HIV envelope glycoprotein. Considerable efforts have been made to characterize the CD_4 receptor in order to identify the critical elements for binding to gp 120 (25,26). Several truncated forms of CD_4, such as CD_4 - CD_8 chimaera which contains only the amino terminal 177 residues of CD_4, act as viral receptors suggesting that these elements are all that is needed for HIV entry into the cell (25).

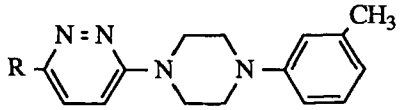
A decapeptide composed of the cleavage site between the alpha and p32 domains of the avian sarcoma leukosis virus (ASLV) reverse transcriptase- integration polyprotein (pol), with changes in the amino acids that flank the cleavage site of the peptide, acts as an inhibitor of the retrovirus encoded protease (27).

Picornaviruses - A recent review of RNA viruses other than HIV in this publication focused on influenza viruses (1). We shall, therefore, concentrate on recent developments in chemotherapeutic agents for the common cold in this review.

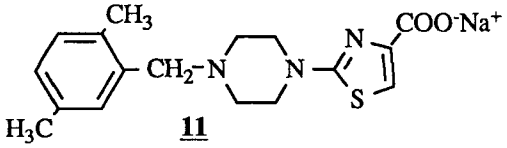
The human rhinovirus (HRV) group of the picornavirus family represents one of the most serologically diverse group of human pathogens. Over 100 serotypes of rhinoviruses have been catalogued thus far (28). Approximately 40% of the common cold syndromes in man can be attributed to infection with the HRVs (29). Because infection

with one HRV serotype affords little or no protection upon subsequent challenge with a different serotype, conventional vaccine approaches to curing of the common cold have essentially been abandoned. Anti-peptide vaccines, although potentially broad-spectrum in nature, induce only weak neutralizing antibody responses (30). While showing efficacy in a prophylactic regimen (31), therapeutic intranasal administration of recombinant alfa-2b interferon to patients with naturally occurring colds was both ineffective and associated with toxicity (32). Attempts to block replication of HRV with a monoclonal antibody which binds to the cellular receptor for 90% of the HRV serotypes were unsuccessful at ameliorating clinical symptoms (33). Consequently, treatment with anti-HRV compounds, although largely unproven in the clinic, offers the greatest hope for the eventual treatment of the common cold in man.

A direct *in vitro* comparison of a "new" generation of more potent antirhinovirus compounds has been reported (34). Dichloroflavan (BW683C) and enviroxime, were included as reference compounds. Also included were the pyradazines (R61837, **6**) and (R60164, **7**) the chalcone (Ro 09-0410); benzamides (Ro 09-0881, **8**), (Ro 09-0535, **9**) and (Ro 09-0696, **10**) and the thiazol-4-carboxyllic acid (89.365, **11**). The minimal inhibitory concentrations (MIC) of the compounds against HRV-9 ranged from 0.0037 ug/ml for **7** to > 1.0 ug/ml for compound **11**. In general, the pyridazines (**6** and **7**) were approximately 10-fold more active *in vitro* against HRV-9 than were the chalcone compounds and enviroxime. 4′, 6 Dichloroflavan had an MIC of 0.25 ug/ml against this serotype. In HRV-2, **7** was approximately equi-potent with the chalcones (0.001-0.006 ug/ml), but **6**, compound **11**, 4′, 6 Dichloroflavan, and enviroxime were all at least 10-fold less active



6 R= CH_3O
7 R = Br



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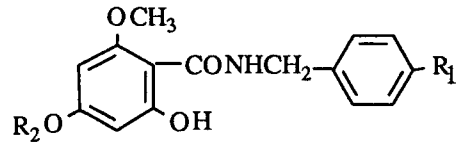


8 $R_1 = NHCH_3$, $R_2 = C_2H_5$

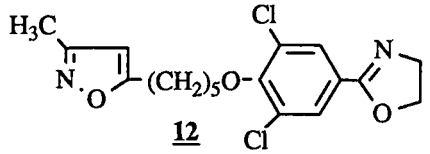
9 $R_1 = OCH_3$, $R_2 = C_2H_5$

10 $R_1 = OCH_3$, $R_2 = (CH_3)_2C{=}CHCH_2$

R 61837 (**6**), exhibits broad spectrum activity against 40 typed and 3 untyped HRVs (35). The concentrations of compound which inhibited 50% and 80% of the serotypes tested were 2.0 and 6.0 ug/ml, respectively. Prophylactic, intranasal administration of R 61837 to human volunteers resulted in a 5-fold reduction in mean total daily clinical score and a three-fold reduction in mean total secretion weight after challenge with HRV-9 (36). Both indices rose upon discontinuation of drug administration. The data suggest that R 61837 could be considered as a new candidate for the prophylactic, and perhaps even therapeutic treatment of the common cold in man.

The antirhinoviral activity of a number of natural and synthetic 3-methoxyflavones were recently reported (37,38). The 3-methoxy function was found to be critical for the antiviral activity of this series, while hydroxy functions at the 5 and 4′ positions significantly reduced both acute and chronic cytotoxicity *in vitro* (38). Introduction of methoxy functions at the 5, 6, 7, and 8 positions of the A-ring of 3-methoxy-5, 4′-dihydroxyflavone increased the therapeutic index of the compound by further lowering the cytotoxicity in the absence of an effect on antiviral activity. Substitution of the A-ring with electron-withdrawing or electron-donating functions resulted in a lower therapeutic index than the naturally occurring 3-methoxyflavones (3-methylquercetin and 3,3′,7-trimethylquercetin) (38).

Win 54,954,(12), an analogue of disoxaril, has been shown to possess potent, broad-spectrum activity *in vitro* against the rhinoviruses and enteroviruses. The compound exhibited an MIC_{80} against 52 HRV serotypes of 0.2 ug/ml (39) and was also effective against several of the enteroviruses (40). A concentration of 0.2 ug/ml inhibited replication of 80% of the most commonly isolated enterovirus serotypes (41). In addition, Compound (12) showed *in vivo* efficacy prophylactically against Coxsackievirus A-9 and Echovirus-9 in suckling mice (40).



12

X-ray crystallographic analysis of a number of compounds related to disoxaril complexed with HRV-14 has shown that the drugs bind into a hydrophobic pocket in the VP1 beta-barrel beneath the canyon floor (42). Upon binding, the compounds induce dramatic conformational changes in the floor of the canyon of up to 4.5 angstroms in the C-alpha chain over the native conformation (43). Because the rhinovirus canyon is thought to be involved in receptor binding for the HRVs (44,45) the effect of these compounds on the ability of HRV-14 to bind to HeLa cell membrane fragments was examined (46). All eight of the compounds examined blocked adsorption of HRV-14 at approximately the same concentration required to block replication of the virus in intact HeLa cells (46). A naturally-occurring, drug resistant mutant of HRV-14 which had a single amino acid substitution in the drug-binding pocket (a leucine at VP1 position 188 in place of a valine) (43), was also resistant to the adsorption blockade (45,46). Molecular modeling of three of the compounds into the mutated pocket indicated that steric interactions could inhibit drug binding (43). This is the first report of a series of compounds capable of blocking attachment of HRV serotype to its receptor. Furthermore, these data indicate that these compounds may exhibit a dual mechanism of action, since disoxaril has previously been reported to block uncoating but not the adsorption of HRV-2 or poliovirus type 2 (47,48).

DNA VIRUSES

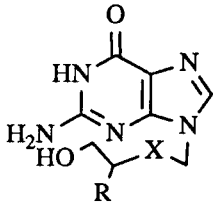
Acyclovir (ACV, 13) remains the drug of choice for control of HSV infections. A multi-center, double-bind, placebo-controlled trial confirmed the safety and efficacy of intravenous ACV for therapeutic treatment of first episode genital herpes (49). However, like oral therapy, iv therapy had little effect on the frequency of recurrences. A new oral dosage regimen (twice rather than five times daily) was found to be better tolerated for episodic treatment of recurrent genital herpes (50). Several double-blind, placebo-controlled trials demonstrated that long-term oral suppressive therapy (up to 4 years) was well tolerated and more effective than episodic therapy for treatment of genital herpes (51-59). Long-term oral therapy (up to 6 months) had no effect on sperm production in men with frequently recurrent genital herpes (60). ACV was found to be effective for therapy of first-episode herpes proctitis (61), eczema herpeticum (62), neonatal encephalitis (63), and herpetic tracheobronchitis (64). In addition, low dose oral ACV for 90 days effectively prevented HSV infections in patients undergoing bone marrow transplantation (65). Resistance to ACV has not yet been clinically significant, but may become an increasing problem in AIDS patients and require alternate forms of therapy (66). New ACV derivatives were synthesized in which one or more nitrogen centers were blocked by methylation or incorporation into a ring but none was found to be better than ACV (67). Pyrollopyrimidine (14) analogues of ACV were synthesized, one of which was ten fold more active *in vitro* than ACV against human cytomegalovirus (HCMV) (68-69).

Ganciclovir (DHPG,15) is still being evaluated for treatment of CMV disease. A randomized prospective comparative study demonstrated that DHPG delayed the progression of CMV retinitis in AIDS patients (70). Prophylactic DHPG was found to be effective against CMV labyrinthitis and hearing loss in a guinea pig model (71). A

toxicity trial in dogs showed that DHPG at 3 mg/kg/day administered during the immediate post bone marrow transplant period was well tolerated (72). The diisopropyl ether of DHPG, HOE-602, was shown to have comparable antiviral activity to DHPG *in vivo* and a much greater oral bioavailability (73). The methyl cytosine analogue of DHPG was found to have equivalent *in vitro* activity against HCMV (74). The carbon analogue of DHPG, BRL 39123, (**16**), was shown to be more potent than ACV against HSV-1 and HSV-2 in several animal models (75). BRL 39123 has a longer duration of action than ACV, possibly because the triphosphate is accumulated in virus-infected cells and has a long half-life (~ 10 hours) (76). The oral adsorption of **16** was less than 20%. However, the oral adsorption for the prodrug, BRL 42810 **17**, was over 80% with a 50% conversion to BRL 39123 (77).

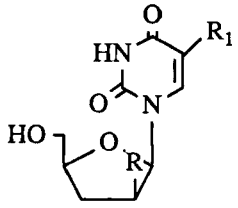
The antiviral effect of BCV (**18**) against HSV was shown to be due to its effect on the viral DNA polymerase (78). BCV showed no toxicity in several animal species (mice, rats, dogs, and rabbits) (79). 2HM-HBG (**19**) was effective against simian varicella zoster virus (SVZV) in monkeys and may have potential for treating varicella zoster virus (VZV) infections in humans (80).

Two bromovinyl nucleoside analogues, BVDU (**20**) and BVara U (**21**) were compared to ACV against Epstein-Barr virus (EBV) *in vitro* (81). BVDU was five-fold more potent than BVaraU or ACV and had a greater therapeutic index. BVaraU and Vara U, (**22**) were not as active as BVDU against HSV-1 keratitis in rabbits (82). The 4-triazolyl derivative of BVDU was synthesized and found to have similar *in vitro* activity as BVDU against HSV-1 and VZV (83). Various 4-O-difluoromethyl analogues of 5-substituted uridine, 2′-deoxyuredine (DU), and ara U nucleosides were synthesized with only 5-methyl-ara U (**23**) showing comparable anti-HSV-1 and 2 activity *in vitro* to ACV (84).

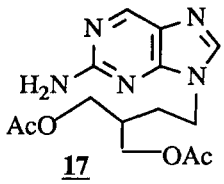


13 R = H, X = O
15 R = CH_2OH, X = O
16 R = CH_2OH, X = CH_2
18 R = OH, X = CH_2
19 R = CH_2CH_2OH, X = CH_2

14



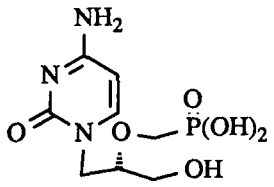
20 R_1 = CH=CHBr, R = H
21 R_1 = CH=CHBr, R = OH
22 R_1 = CH=CH_2, R=OH
23 R_1 = CH_3, R = OH



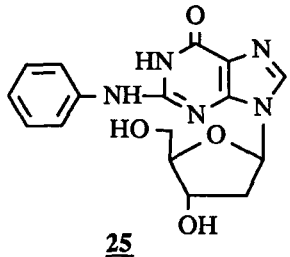
17

The use of vidarabine (araA) and ara-AMP to treat HSV and hepatitis B virus (HBV) infections has been limited because of several shortcomings, including toxic side effects. The lactosaminated human serum albumin conjugate of ara-AMP (L-HSA-ara-AMP) was found to inhibit HBV replication in chronically infected patients at one-third to one-sixth the dose of free ara-AMP with no adverse effects (85). The anti-HIV agent, (**1**) effectively inhibits EBV DNA replication *in vitro*, but has no effect on CMV, VZV, or HSV DNA replication (86).

Of the potent anti-herpes phosphonates, (S) - HPMPC (**24**) (87) appears to be the best candidate. It showed no toxicity in mice at 200 mg/kg/day, high *in vivo* potency (better than DHPG) in a mouse CMV model, and high *in vivo* potency (better than ACV) in HSV 1 and 2 guinea pig models.



24



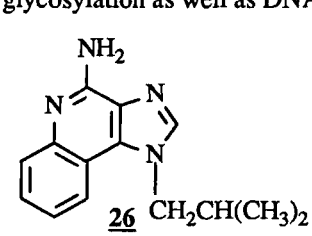
25

Foscarnet (phosphonoformate, PFA) (2) cream (3%) showed no significant efficacy in a multi-center, randomized, double-blind study for treatment of oralabial herpes (88). Because limited efficacy of PFA and phosphonoacetate (PA) is thought to be due to inability to cross membranes, the approach of encapsulating the drugs in liposomes was studied and shown to result in superior efficacy against HSV-2 in tissue culture (89).

Several studies were done to assess the value of combinations of antivirals on treatment of HSV and HBV. Combinations of nucleoside analogues and interferon (α-IFN) were effective against HSV-2 in a weanling mouse model for disseminated neonatal disease (90). Treatment with recombinant IFN (α) following steroid withdrawal was effective in treating chronic hepatitis B in a randomized control trial (91). The HSV ribonucleotide reductase inhibitor, acetylpyridine thiosemicarbazone, (A723U), showed synergy with ACV in cell culture (92).

Several natural products have been tested for activity against HSV, CMV, or HBV. HBV carriers treated with a preparation of the plant *Phyllanthus amarus* lost HBV surface antigen for up to 9 months (93). Methyl gallate, purified from the leaves of *Sapium sebiferums* (94-96), as well as an extract of *Chamecyparus lawsoniana* were active against HSV-2 in cell culture at 0.2 and 0.5 uglml, respectively. Oxetanocin G, a derivative of the novel nucleoside oxetanocin A from *Bacillus megaterium*, was active against both HSV-2 and HCMV in cell culture (1 and 3.5 mg/ml, respectively) (97).

Additional agents which interfere with herpes virus DNA synthesis as well as other stages in the virus life cycle were reported. Kanamycin derivatives inhibited HSV-2 immediate early transcription and translation (98). Prostaglandin synthesis inhibitors decreased HCMV replication *in vitro* and may affect early transcription or translation (99). N-phenyldeoxyguanosine (25) was shown to be a selective inhibitor of the HSV-1 thymidine kinase (100). 7-aminoquinolines inhibited HSV-1 DNA synthesis *in vitro* (101) Megalomycin C showed *in vitro* activity against HSV-1 and inhibited glycosylation (102). UDP-glucose inhibited HSV-1 glycosylation as well as DNA synthesis (103).



26

Recombinant α-2a IFN modified the severity of VZV in immunocompromised patients with cancer in a multi-center, placebo-controlled, double-blind study, but was associated with frequent side effects (104). It had little long-term effect on suppression of HBV replication in Chinese patients with chronic HBV infection (105). Recombinant mouse IFN-β protected mice against acute lethal infection of HSV-1 and prevented reactivation, but was not able to prevent the establishment of latency or eliminate a previously established latent infection (106). The immunomodulator, S26308 (26), induced IFN production and reduced CMV infection in immunocompetent and immunocompromised guinea pigs (107), and prevented acute and recurrent HSV-2 disease in guinea pigs and reduced latency (108).

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Chapter 15. Transport of Antibiotics into Bacteria

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Introduction - Gram-positive bacteria are surrounded by a single membrane, the cytoplasmic membrane, whereas in Gram-negative bacteria, there is a further membrane bilayer in the form of the outer membrane. Since many clinically useful antibiotics have target sites that are either located within the bacterial cell (e.g. inhibitors of DNA and protein synthesis), or within the cytoplasmic membrane (e.g. beta-lactam antibiotics),the antibiotic molecules may have to cross either one or two membranes, depending upon whether the organism is Gram-positive or Gram-negative. It is therefore not surprising that the ability of antibiotics to cross bacterial membranes is a major determinant of their efficacy (1).

The topic of antibiotic transport has been extensively discussed in recent years. Therefore this article will only focus upon very recent developments. Major groups of antibioticsare considered separately in alphabetic order. Certain important antibiotics (e.g. chloramphenicol, tetracyclines) have been omitted from this review simply because there have been no recent advances in our understanding of their transport into bacteria. For a comprehensive appraisal of antibiotic transport mechanisms, the present article should be read in conjunction with earlier reviews (1-14).

AMINOGLYCOSIDE-AMINOCYCLITOL (AGAC) ANTIBIOTICS

The AGAC group of antibiotics comprise a large number of clinically useful drugs which are able to enter both Gram-positive and Gram-negative bacteria to interfere with protein synthesis. Uptake of the AGAC group across bacterial outer membranes has been covered in previous reviews (3,4-6,11-13). In E. coli some evidence points towards porin-mediated uptake (whereby drugs diffuse passively through porin channels which span the outer membrane) but, in Pseudomonas aeruginosa, these antibiotics seem able to promote their own transfer across the outer membrane by causing localized distortion of the bilayer (4-6,13).

Passage of the AGAC group across the bacterial cytoplasmic membrane is essential for anti-bacterial action and the possible (energy-dependent) transport mechanisms that may be involved have been extensively reviewed in recent years (3-6,11-13). One recent and somewhat controversial aspect of AGAC transport across the bacterial cytoplasmic membrane is worthy of comment here.

It has recently been proposed that uptake of streptomycin depends upon accumulation of mis-translated proteins in the cytoplasmic membrane forming transmembrane aqueous channels that allow the positively charged antibiotic molecules to diffuse from the exogenous compartment into the cytoplasm (15-17). Misreading of proteins during translation is a characteristic feature of

streptomycin action at the ribosome and the following sequence of events has been envisaged : a) A small amount of streptomycin enters the cell and its interaction with chain-elongating ribosomes causes misreading of mRNA and production of defective proteins. b) Some of the defective protein is incorporated into the cytoplasmic membrane creating channels that permit influx of antibiotic. Further accumulation of drug molecules leads to an autocatalytic process of increased misreading, channel formation and drug uptake. However, various cogent arguments against the above hypothesis have recently been made (18). The principal difficulty relates to the irreversible nature of streptomycin transport which implies a more specific transport process than one involving channels. Essentially, it is not expected that a channel-mediated uptake system can account for the irreversible kinetics of accumulation. Nevertheless, although the hypothesis that mis-translated membrane proteins form pores for streptomycin uptake has been rejected, the mechanism of streptomycin uptake (and indeed all AGAC drugs) is still not completely understood (18).

BETA-LACTAMS

The beta-lactams comprise a large group of antibiotics that inhibit bacterial growth by binding to penicillin-sensitive enzymes (PSEs) (also known as penicillin binding proteins, PBPs) that are located on the surface of bacterial cytoplasmic membranes (19). Therefore, in Gram-negative organisms these antibiotics must cross the bacterial outer membrane to exert their inhibitory effects. Much attention has recently been focused upon the molecular basis of beta-lactam uptake across Gram-negative outer membranes and the information gained has been the subject of extensive reviews (5,7,8,10,13). Therefore, only a brief consideration of the topic is presented here, with the emphasis on very recent findings.

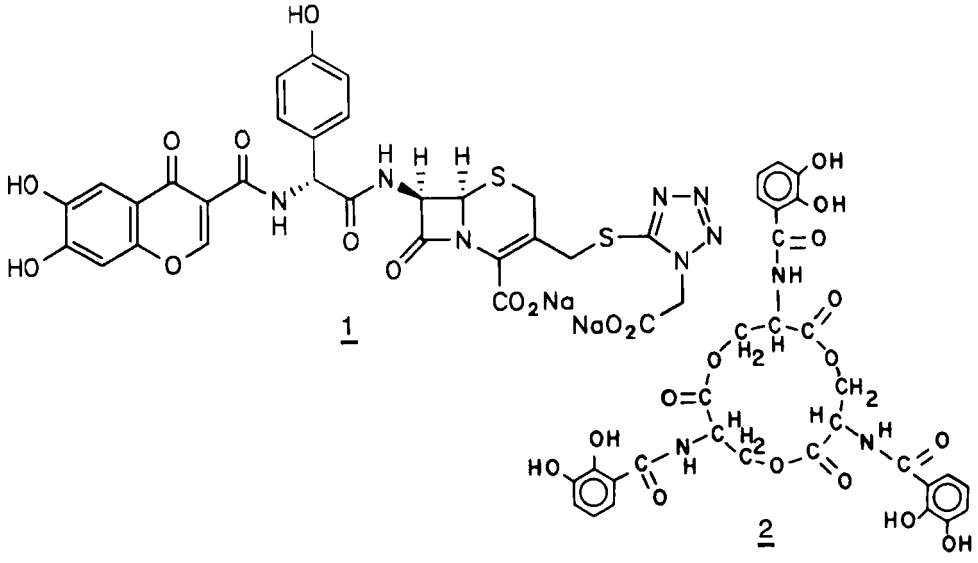
Uptake across the E. coli outer membrane - The majority of beta-lactams cross the outer membrane by passive diffusion through porin channels, primarily the OmpF and OmpC porins (5,7,8,10,13). The rate of influx of beta-lactam molecules through the porins is influenced by a number of factors that include drug hydrophobicity, size and net charge (7,8). In general, increases in hydrophobicity, size or net negative charge tend to decrease the rate of permeation of beta-lactams through porin channels. However, these parameters frequently interact with one another so that it is the gross physico-chemical properties that influence the rate of permeation of these antibiotics through the porin channels (7,8).

Uptake across the outer membrane of other Gram-negatives -Studies on the passage of beta-lactams across the outer membranes of organisms other than E. coli have recently begun (20-24). Pseudomonas aeruginosa is intrinsically resistant to most beta-lactams, a situation resulting from poor uptake of drug molecules across the outer membrane (20,21). However, the basis of this impermeability is somewhat obscure. Ps. aeruginosa contains an abundant outer membrane protein, designated OmpF, which is apparently able to form porin channels in liposome reconstitution experiments (25). Surprisingly, the pores formed in vitro by the OmpF protein appear to have a large exclusion limit, allowing diffusion of molecules with molecular weights of 2000-3000 daltons (25). In contrast, the exclusion limit for the E. coli OmpF and OmpC pores is about 800 daltons (25). However, using intact cells of Ps. aeruginosa, the exclusion limit of the outer membrane has been shown to be much smaller than that predicted from the in vitro studies (22). Therefore, the quantity of OmpF protein and the size of the pores it apparently forms, are at variance with other data on the permeability of intact cells to beta-lactams and other solutes. A possible explanation for this

confusing situation is now available. The role of OmpF as a porin can be questioned on the basis that mutational loss of the protein does not affect outer membrane permeability to beta-lactams; the previous liposome experiments may have been flawed; and the amino acid sequence of OmpF shows greater homology with the E. coli outer membrane structural protein OmpA, rather than E. coli porins (21,22). Therefore in some respects, the behavior of Ps. aeruginosa resembles that of porin-deficient mutants of enteric bacteria such as E. coli. Possibly, uptake across the outer membrane of essential solutes required for growth of Ps. aeruginosa is mediated primarily by specific porins and this organism is naturally deficient in general diffusion porins through which beta-lactams might pass (25).

Outer membrane permeability to beta-lactam antibiotics has also recently been investigated in Yersinia enterocoalitica and Bacteroides fragilis (23,24). In Y. enterocoalitica two outer membrane proteins, designated YOMP-C and YOMP-F, with molecular weights of 37,000 daltons were identified as porins through which beta-lactams (and other solutes) pass across the outer membrane (23). Although porins have not been specifically identified in B. fragilis, several features of beta-lactam permeation in this organism are consistent with the use of porins by certain beta-lactams (24). However, in this organism hydrophobic beta-lactams may cross the outer membrane by the so-called "hydrophobic pathway" (i.e. across the lipid rich regions of the outer membrane bilayer) because increased hydrophobicity of drug led to increased penetration into the periplasm (24). This contrasts with the situation in E. coli which does not have a "hydrophobic pathway" for uptake (5,7,8,13).

TonB dependent uptake of catecholic beta-lactams - While there is little doubt that porins play an important role in the passage of many beta-lactams across the outer membrane, other means of entry are also possible (13). For instance the cephalosporin E-0702 (1) and other catechol bearing structures (3, 5, 6, 8) are transported across the bacterial outer membrane by the tonB-dependent iron transport system (26,27). Apparently, the catechol moieties in these compounds permit their accumulation by the pathway normally operating for the naturally occurring catecholic iron siderophore, enterobactin (2) (also known as enterochelin).



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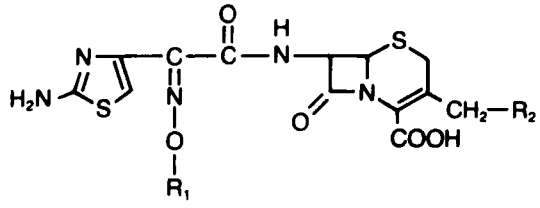
This approach to the delivery of antibiotics into the cell will be especially appealing if (a) the uptake pathway illicitly transports a range of molecular structures and (b) resistance due to mutation in the transport system does not arise. Structure-activity studies with compounds **3** - **8** showed that the tonB-dependent transport system is relatively tolerant of chemical variability in the substrate (27). However, analogs which lack adjacent free hydroxyl groups (e.g. **4** and **7**) and are therefore incapable of forming bidentate complexes with iron are not accumulated by the tonB pathway.

Mutation to catechol-cephalosporin resistance involving the tonB locus is observed in the laboratory (27). However, the inability of tonB mutants to acquire iron in the iron-depleted in vivo environment probably precludes their survival in the host (27).



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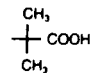
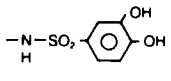
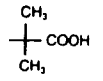
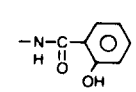
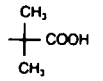
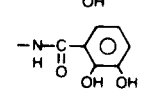
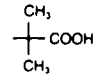
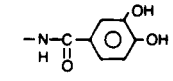
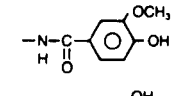
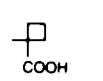
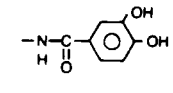
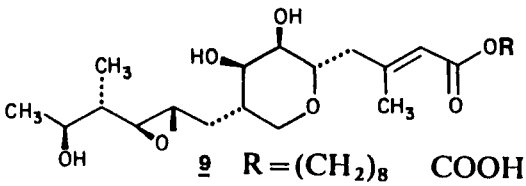
3		
4		
5		

Compound	R_1	R_2
6		
7		
8		

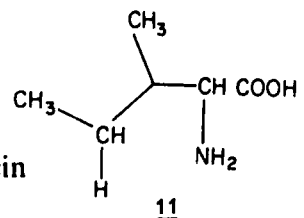
MUPIROCIN

The antibiotic mupirocin (**9**) consists of a short fatty acid side chain linked to a larger molecule, monic acid, the tail end of which mimics the amino acid isoleucine (**11**).



9 R = $(CH_2)_8$ COOH: mupirocin

10 R = H: monic acid



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Mupirocin competetively inhibits iso-leucyl transfer RNA synthetase and thus, by preventing the incorporation of iso-leucine into growing polypeptide chains, arrests protein synthesis (28,29). The hydrophobic nature of mupirocin suggests that it will not easily penetrate the outer membrane of most Gram-negative bacteria. This is consistent with the observation that mupirocin has poor activity against E. coli, but is about 500-fold more active against S. aureus (29). Thus, although the E. coli outer membrane probably "shields" the inner membrane from the antibiotic, it is able to cross the cytoplasmic membrane of Gram-positive bacteria. Transport of mupirocin into B. subtilis and S. aureus was recently investigated (29). Accumulation was energy independent, but temperature dependent. These features are consistent with non-carrier mediated passive diffusion across the cytoplasmic membrane.

QUINOLONES

The 4-quinolone antibacterial agents comprise a group of synthetic drugs that inhibit the enzyme DNA gyrase (DNA topoisomerase II) which is responsible for introducing negative supertwists into the bacterial chromosome (30,31). When DNA gyrase is prevented from supercoiling chromosomal DNA by the action of 4-quinolones, both chromosome replication and gene transcription cease leading to rapid bacterial death (30,31). Since DNA gyrase is located intracellularly, the 4-quinolones must be able to cross both outer and cytoplasmic bacterial membranes. However, compared with knowledge on other antibiotic transport mechanisms, relatively little is known about the uptake of 4-quinolones into bacteria.

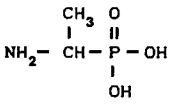
Uptake across the outer membrane - Most quinolones are low molecular weight (i.e < 400 daltons) hydrophilic molecules (32). These properties imply that the drugs could well cross the outer membrane through porin channels (13). Quinolones are indeed known to penetrate the outer membrane of E. coli through OmpF and OmpC porins. This has been demonstrated both by the use of defined porin-deficient strains and by the isolation of quinolone-resistant mutants that lack porins (33-36). However, other evidence has suggested that the passage of quinolones through the outer membrane is not limited to porins (32,37). The recent finding that changes in lipopolysaccharide structure confer resistance to quinolones is also consistent with a second non-porin mediated uptake pathway for these drugs across the outer membrane (38). The question of whether there is a second uptake pathway for quinolones across the E. coli outer membrane has now been more directly addressed (35). Evidence was obtained for self-promoted uptake of drugs across the outer membrane whereby divalent cations are displaced, leading to membrane destabilization and further insertion of antibiotic molecules into the outer membrane bilayer. Therefore both porin and non-porin pathways can contribute to total uptake of quinolones (35).

Uptake across the cytoplasmic membrane - The mechanism of uptake of quinolones across the bacterial cytoplasmic membrane is at present uncertain. Some evidence points towards an energy-independent passive diffusion mechanism, whereas other data suggest that an energy dependent mechanism is responsible (35,39). However, it is difficult to explain how passage of hydrophilic molecules like the quinolones can occur across the hydrophobic phospholipid bilayer of the cytoplasmic membrane without the involvement of an energy coupled transport process (39). Perhaps the recent advent of new and relatively simple assays for quinolone uptake will encourage further experiments to resolve the apparent discrepancy concerning transport of quinolones across the cytoplasmic membrane (40).

EXPLOITATION OF TRANSPORT SYSTEMS FOR THE DELIVERY OF INHIBITORS TO THE BACTERIAL CYTOPLASM

In recent years it has become apparent that several inhibitors of bacterial metabolism can be more effectively delivered to their intracellular targets by chemical linkage to naturally occurring transported molecules. This method depends upon the ability of the transport system to accumulate the synthetic adduct in addition to the normal molecule.

The most familiar example of this approach concerns the incorporation of L-aminoethylphosphonic acid (**12**) into a dipeptide to produce L-alanyl-L-aminoethylphosphonic acid (**13**), also known as alaphosphin. L-aminoethylphosphonic acid inhibits the bacterial enzyme alanine racemase *in vitro*, but does not inhibit bacterial growth because it is not accumulated by the cell (13,41). Alaphosphin is actively transported into the cell by peptide permeases, followed by intracellular peptidase cleavage to yield L-aminoethylphosphonic acid, the active inhibitory molecule or "warhead".



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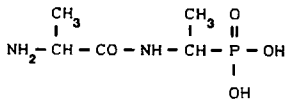

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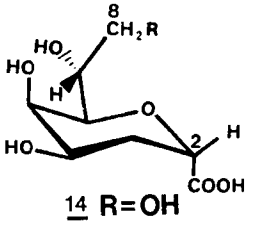
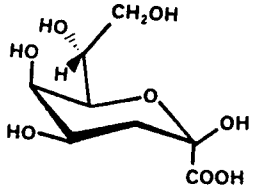
The principle of linking impermeant "warheads" to peptides for delivery into the bacterial cell has also been applied in two separate studies to inhibitors of lipopolysaccharide (LPS) synthesis in Gram-negative bacteria (13,42,43). One of these studies will serve as an example here (42). A synthetic analog, 2,6-anhydro-3-deoxy-D-glycero-D-talo-octonic acid (**14**), of the essential LPS component 3-deoxy-D-manno-2 octulosonate (KDO) (**16**) was prepared and found to be a potent *in vitro* inhibitor of the enzyme 3-deoxy-manno-octulosonate cytidyltransferase (CMP-KDO synthetase) that converts KDO to cytidine-5' monophosphate-KDO (CMP-KDO). CMP-KDO is the substrate for a series of CMP-KDO-transferases that incorporate KDO into LPS (Fig. 1). Although the KDO analog (**14**) is a potent inhibitor of CMP-KDO synthetase *in vitro*, it displays poor antibacterial activity because it is unable to cross the cytoplasmic membrane to produce inhibitory concentrations at the site of the target enzyme. However, coupling an L-L-alanine dipeptide to the 8-amino-8-deoxy derivative of **14**, i.e. producing 8-amino-2,6-anhydro-3,8-dideoxy-D-glycero-D-talo-octonic acid (**15**), produced an antibiotic (abbreviated to Ala-Ala-I) with good activity against a range of Gram-negative bacteria. The dipeptide analogue (Ala-Ala-I) is actively accumulated by the oligo-and tri-peptide transport systems of *E. coli* and hydrolysed in the cell to produce a high proportion of the free inhibitor I (Fig.1). Subsequent inhibition of CMP-KDO synthetase leads to accumulation of LPS precursors and bacterial death.



14 R=OH
15 R=NH_2



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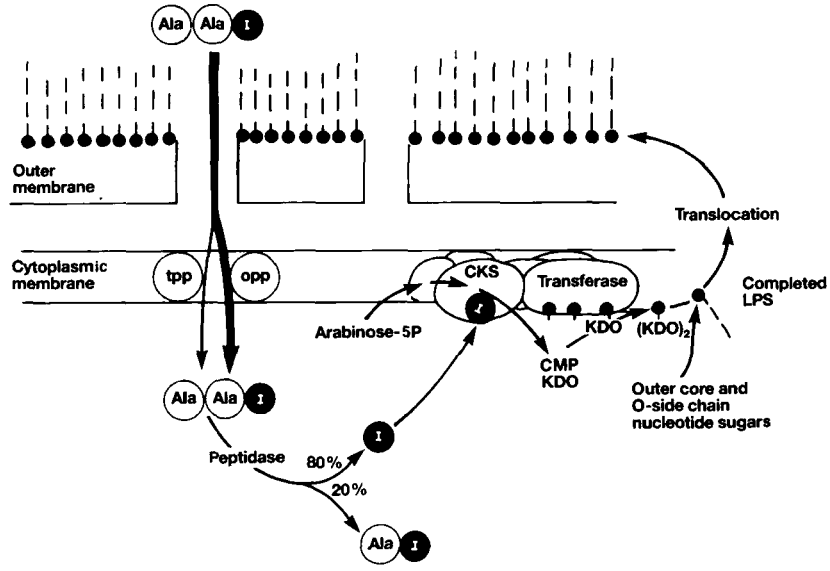

Fig. 1. Transport and release of the lipopolysaccharide inhibitor 8-amino--2,6-anhydro-3,8,dideoxy-D-glycero-D-talo-octonic acid (I) in E. coli. Linkage of I to the carboxyl group of dialanine permits transport of Ala-Ala-I via the oligo-and tripeptide permeases (opp, tpp respectively). I is an inhibitor of CMP-KDO synthetase (CKS). Reprinted by permission from Nature Vol. 327 pp 732 Copyright (c) 1987 Macmillan Magazines Ltd.

Conclusions - In some cases the mechanisms of antibiotic uptake into bacteria are quite well defined in molecular terms. However, in other cases either the precise basis of transport is unknown, or the mode of uptake differs from that predicted by simple considerations of antibiotic properties. Therefore there are still considerable gaps in our understanding of the mechanisms by which antibiotics enter bacteria. The design of novel antibacterial agents that can utilize existing bacterial transport systems for entry into bacteria is an attractive prospect for the therapy of infectious diseases. This can be applied both to the delivery of antibiotics across the outer membrane (e.g. the catecholic beta-lactams) and the inner membrane (e.g. alaphosphin and LPS inhibitors). Further studies on antibiotic transport will probably suggest other ways of improving drug delivery to target sites.

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Section IV - Endocrinology, Immunology and Metabolic Disorders

Editor: William F. Johns, Sterling Research Group
Rensselaer, New York 12144

Chapter 16. Treatment of Hypercholesterolemia

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Introduction - Hypercholesterolemia (HC) encompasses a heterogeneous group of disorders in lipid metabolism characterized by substantially elevated levels of plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C). The origins of these defects are varied, including a deficiency of hepatic LDL-receptors (familial HC), overproduction of apolipoprotein B (familial combined hyperlipoproteinemia) and diet (1). HC, with or without accompanying hypertriglyceridemia, is the one independent risk factor definitively linked to increased mortality due to myocardial infarction (2). Guidelines for treating HC have been established by the National Institutes of Health (3) and the European Atherosclerosis Society (4).

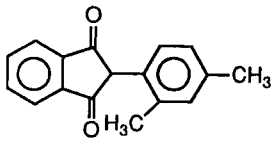
Since cholesterol (C) enters the body pool from only two sources, either by absorption from diet (300-500 mg/day in man) or endogeneous synthesis (700-900 mg/day) (5), the major focus of this review will be inhibitors of either process which have appeared since the last review of this subject in Annual Reports (6). The remainder of the review will deal with compounds which affect C metabolism by other mechanisms.

CHOLESTEROL BIOSYNTHESIS INHIBITORS

The inhibition of endogenous cholesterogenesis has become the most attractive method of lowering plasma TC and LDL-C in animals and man. The biosynthetic pathway to C involves more than 25 different enzymes. The major rate limiting step in this pathway is regulated by the enzyme HMG-CoA reductase (HMGR), which catalyses the conversion of HMG-CoA to mevalonic acid. The general subject of cholesterol biosynthesis inhibition has been recently reviewed (7).

Inhibition Pre-HMG-CoA Reductase - The indan-1,3-dione (1) causes a 50% inhibition of cytoplasmic S-acetyl coenzyme A synthetase, the enzyme responsible for acetate activation in the first biosynthetic step. 1 is also reported to inhibit ATP dependent citrate lyase and acyl CoA: cholesterol acyltransferase (ACAT). It reduced LDL-C and increased HDL-C in normal rats (8). A series of polymethylenemethane thiosulfonates (2) have been shown to inhibit cytoplasmic acetoacetyl-CoA thiolase, which catalyses the reversible condensation of two molecules of acetyl-CoA to yield acetoacetyl-CoA (9). The hypocholesterolemic effect of these compounds in chow fed rats, however, was variable. F244 (L-659,699, 3), a naturally occurring β-lactone, has

been found to inhibit HMG-CoA synthetase (10). The β-lactone moiety has been shown to be essential for activity (11).



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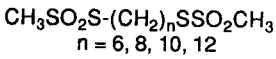

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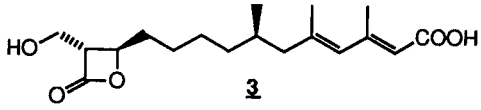

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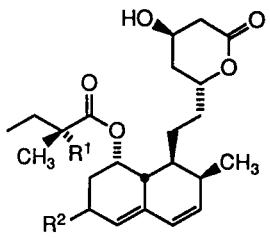
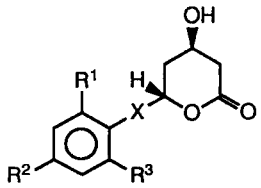
HMG-CoA Reductase Inhibitors - Compactin (4) and lovastatin (5), the first fungal metabolite inhibitors of HMGR, have been the subject of many reviews (12-14). Since its rapid approval in 1987, lovastatin has been shown to be the most effective treatment for lowering both TC and LDL-C (30-50%) in patients with primary hypercholesterolemia. Combination with either a bile acid sequestrant or nicotinic acid reduces LDL-C by 45-60% (15). Although effective and well tolerated clinically, the long-term safety of lovastatin has come under scrutiny. Preclinical studies demonstrated that high doses of lovastatin (60-180 mg/kg) caused cataracts, neurotoxicity and optic nerve degeneration in dogs after 1-2 years of treatment (16). The major documented clinical side effects have been increased serum liver transaminases and myositis (17-19). There is conflicting evidence concerning whether 5 causes lens opacities in man (17,18). Lovastatin toxicity, both in animals and man, appears to be related to increasing plasma levels of active drug (16,19,20).



4, R^1 = H, R^2 = H
5, R^1 = H, R^2 = α-CH_3
6, R^1 = CH_3, R^2 = α-CH_3
7, R^1 = H, R^2 = β-OH
(dihydroxyacid, sodium salt)



8, R^1 = OCH_2-(4-F-Ph), R^2 = R^3 = Cl, X = -CH_2CH_2-

9, R^1 = (2-F, 4-CH_3-Ph), R^2 = R^3 = Me, X = -CH=CH-

10, R^1 = R^2 = CH_3, R^3 = $(CH_2)_3O$-(4-F-Ph), X = -O-CH_2-

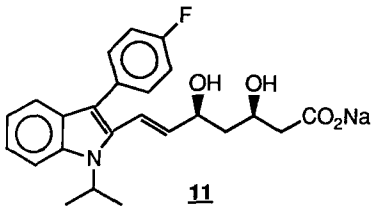
A systematic study of side chain esters of 5 has resulted in the identification of simvastatin (6), reported to be three times more potent in vitro than the parent (21) and also more potent at lowering LDL-C in man (17,22). 5 and 6 exhibit similar tolerability and safety profiles (23).

A third fungal metabolite under clinical development is pravastatin (7). Clinical efficacy for 7 comparable to 5 has been demonstrated (24). It has also been reported that 7 is more selective for inhibiting HMGR in the liver than 5, both in vitro and in vivo (25,26). Recently, it has been shown that 7 is 100-fold less potent at inhibiting cholesterogenesis in ex vivo rat lens compared to 5 or 6 (27). It was postulated that this

may translate into superior ophthalmologic safety. However, other authors have reported that 7 is more widely distributed in rats than 5 or 6 (28).

There has been intense activity in the design and synthesis of synthetic analogs of 4 and 5. A series of 5-keto analogs of 4 has been prepared and shown to be equipotent (29). It has also been shown that the decalin moiety of 4 plays a purely hydrophobic role in binding inhibitors to the enzyme (30). Work on a series of 5-substituted-3,5-dihydroxypentanoic acids established certain parameters that were essential for potent inhibition (31). Most potent inhibitors have the lactone moiety flanked by a bulky lipophilic group and an alkyl group, e.g., 8 and 9. 9 exhibited three times the intrinsic activity of 4 *in vitro* (31). Rendering the biphenyl rings in compounds such as 9 coplanar produced a series of fluoren-9-ylidenyl compounds which were less potent (32), as were a series of silicon containing biphenyl compounds (33). Replacement of the ethylene bridge by an alkoxymethylene bridge (10) also reduced activity (34,35).

A wide variety of different structural variants encompassing both carbocyclic and heterocyclic replacements for the decalin moiety of 4 have been reported. From a series of indole mevalonolactones, XU 62-320 (11) has been identified as an extremely potent inhibitor *in vitro* and *in vivo* (36). Recently, however, it has been shown that 11 induces bilateral posterior cataracts in beagle dogs at a low dose of 24 mg/kg and mortality at 36 mg/kg (37). A number of heterocycles replacing the indole in 11 have been shown to possess excellent *in vitro* potency, including imidazoles (12) (38), indolizines (39), pyrazoles (40), quinolines (41), pyrroles (42,43), furans (43), thiophenes (43), naphthalenes (44) and indenes (45). Efforts to simplify these compounds, while retaining the strict spacial arrangement of substituents, generated compounds such as 13, which exhibited *in vitro* potency comparable to 5 and lowered LDL-C 33% in rabbits (46). A structurally similar compound, BMY 21950 (14), retained excellent potency and displayed greater tissue selectivity than 5 or 6 in a variety of cell dispersions (47,48).



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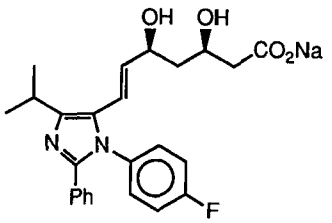

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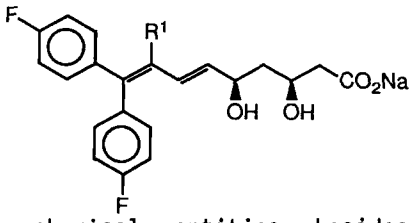
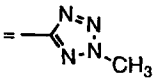
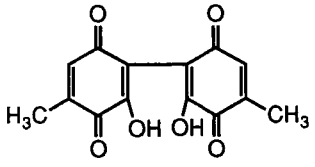


13, R^1 = i-Pr

14, R^1 = 

Other chemical entities besides mevalonolactones and their derivatives have been shown to inhibit HMGR. Phenicin (15), isolated from *Penicillium phoenicium*, has been shown to be an irreversible inhibitor of rat liver microsomal HMGR (49). Purification of the nonpolar fraction of high protein barley by HPLC gave 10 major

components, one of which, d-α-tocotrienol (16), was shown to inhibit HMGR and lower TC in chicks fed a C-free diet (50).



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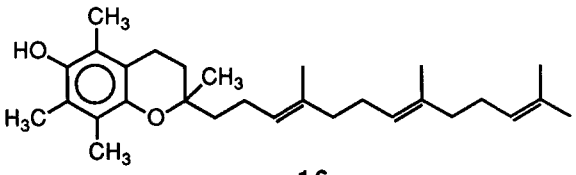

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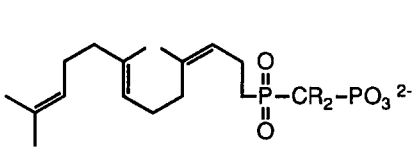
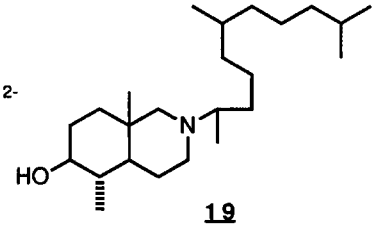
Some steroids bearing a second oxygen function are known to inhibit HMGR in vitro, possibly by a feedback mechanism (51). 22- and 25-Hydroxycholesterol derivatives and a 15-ketosterol (5α-cholest-8(14)-en-3β-ol-15-one) have been shown to be hypocholesterolemic in primates (52-54). 6-Nitrocholesterol has been shown to cause a 50% reduction in HMGR activity in vitro (55).

Inhibition Post-HMG-CoA Reductase - Fluorinated mevalonate analogs have been shown to inhibit mevalonate-5-pyrophosphate decarboxylase (56). Several stable analogs of farnesyl diphosphate (17 and 18), have been shown to be potent, competitive inhibitors of squalene synthetase, the first pathway specific enzyme in C biosynthesis (57). 2,3-Oxidosqualene-lanosterol cyclase has proven an attractive target for inhibition (58). 2-Aza-2,3-dihydrosqualene and 19 have been shown to be potent inhibitors of this enzyme in vitro (59). 7-Oxo-24,25-dihydrolanosterol has also been shown to be an inhibitor of C biosynthesis, possibly at lanosterol 14α-demethylase (60). Ketoconazole, another inhibitor of this enzyme, has recently been shown to be hypocholesterolemic in man (61). BM 15766 (20) inhibits 7-dehydrocholesterol-Δ^7-reductase and markedly reduces TC in rats (62). It has been shown that 20 induces a proliferation of peroxisomes in perivenous rat hepatocytes (63).



17, R = H; 18, R = F



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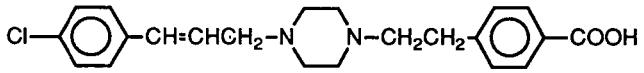

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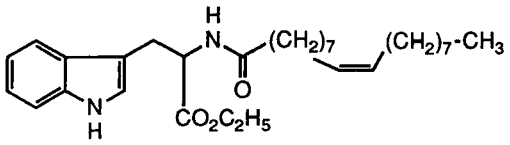
CHOLESTEROL ABSORPTION INHIBITORS

Plant Sterols - Plant sterols are commonly present in the human diet in edible vegetable oils and fats. The hypocholesterolemic effect of plant sterols, most commonly β-sitosterol, has been well documented in animals (64) and man (65). Various mechanistic proposals, such as a reduction in the micellular solubility of cholesterol and the formation of a nonabsorbable complex with cholesterol in the intestinal lumen, have been postulated (66). In mice, β-sitosterol, has been shown to decrease not only C absorption, but also liver C concentration and bile acid synthesis (67). Recently a low dose (1.5 g/day) of β-sitostanol, administered for

4 weeks to hypercholesterolemic patients, was shown to produce a 10-15% reduction in LDL-C (68).

ACAT Inhibitors - Several enzymes have been postulated to be responsible for cholesterol esterification. These are lecithin cholesterol acyl transferase (LCAT), cholesterol esterase (CEH), and ACAT (69). Upregulation of ACAT in animals fed a high-C diet is well documented and most authors now agree that the ACAT catalyzed esterification of C is the rate limiting step in intestinal C absorption (70,71). Recent studies suggest that there are subtypes of this enzyme which may be differentially induced by C feeding (72,73).

Melinamide, the α-methylbenzamide of linoleic acid, was found to be an absorption inhibitor in rats and to inhibit ACAT (74). SaH 57-118 (21) and SaH 58-035 (22) are ACAT inhibitors which significantly reduce TC in C-fed rabbits (75). Extensive pharmacokinetic and drug metabolism studies with 22 in rats and dogs have revealed that this agent is poorly absorbed and highly metabolized (76). Absorption in man is improved with a high-fat meal (77). Several anilides of oleic, linoleic and shorter chain fatty acids have also been reported to inhibit rabbit intestinal ACAT and lower plasma TC in C-fed rats and rabbits (78-80).



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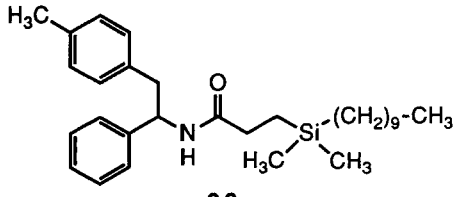

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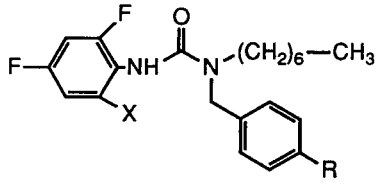
Several members of a series of tri-substituted ureas, e.g., CL 277082 (23) and CL 283546 (24), have been reported to potently inhibit intestinal ACAT and to have profound hypocholesterolemic activity in rats, rabbits, and monkeys (81-83). Recently, a series of disubstituted urea ACAT inhibitors was reported (84).

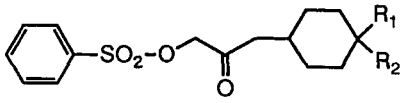


23, R = - CH_2 - $C(CH_3)_3$, X = H
24, R = - $(CH_2)_2$ - $CHCH_3)_2$, X = F

The antihypertensive agents propranolol, metoprolol, prazosin, and chlorthalidone and the tranquilizer diazepam have been reported to modestly reduce ACAT activity in normal rat and atheromatous rabbit aorta (85,86). Chlorpromazine, an antipsychotic drug, has also been shown to inhibit arterial ACAT when given orally for 12 weeks to rabbits on an atherogenic diet (87).

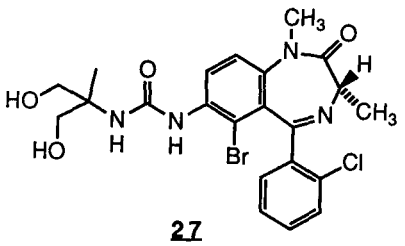
Other Absorption Inhibitors - Pancreatic cholesterol esterase has also been implicated in the esterification of exogenous C. A series of

arenesulfonate derivatives (25 and 26) has been reported to potently inhibit this enzyme and to reduce C absorption acutely in rats fed a high fat, high-C meal (88).



25 , $R_1 = -CH_3$, $R_2 = -(CH_2)_2-CH_3$
26 , $R_1 = R_2 = -(CH_2)_5-$

Ro 16-0521 (27), a benzodiazepine which does not bind to the brain benzodiazepine receptor, has been shown to reduce plasma and liver TC by 27% and 61% respectively when given to rats on a high-C diet (89). However, 27 did not reduce TC in patients with hyperbetalipoproteinemia (90). Recently, a new antihypertensive agent, SM-2470 (28), has been reported to decrease intestinal C absorption and plasma TC in rats (91).



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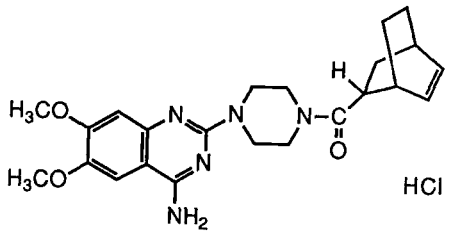
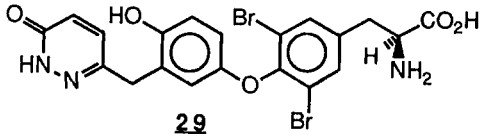


28

MISCELLANEOUS HYPOLIPIDEMICS

Bile Acid Sequestrants - Treatment of HC with bile acid sequestrants remains the first line and most cost effective method of drug therapy (92). Cholestyramine has been proven to reduce the incidence of myocardial infarction in a primary intervention trial (2). Resin therapy has also been shown to retard the progression and/or induce the regression of coronary atherosclerotic lesions (93,94). Bile acid sequestrants also produce effective combinations with HMGR inhibitors, fibric acid derivatives and probucol (95). Two new resins, MCI-196, claimed to be 4 times more potent than cholestyramine (96), and filicol, an apparently more palatable resin (97), have been reported.

Thyromimetics - There has been a renewed interest in thyroid hormone analogs due to mechanistic research which suggests that they exert their hypocholesterolemic effect by stimulating production of hepatic LDL receptors (98). Recently, a new thyromimetic, SKF L-94901 (29), has been identified which binds selectively to the hepatic thyroid hormone receptor, thereby causing a decrease in plasma TC in rats, without increasing cardiac activity (99).

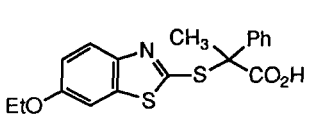


29

Fibric Acid Derivatives - The effect of derivatives of clofibrate on plasma lipids and lipoprotein metabolism has recently been reviewed

(100). In patients with both elevated serum TC and triglycerides (TG), fibric acid therapy is often employed (101). Despite the widespread use of these drugs, their mechanism of action is unclear. Some authors have speculated that there is a relationship between biological activity, hepatic enzyme induction (102) and peroxisome proliferation in rodents for this class of compounds (103). The most significant development in this area was the publication of the results of the Helsinki Heart Study, a 5-year primary prevention trial with gemfibrozil in middle-aged men with dyslipidemia. In this study, gemfibrozil caused a 11% increase in HDL-C, a 11% reduction in LDL-C and a 35% reduction in TG. This was correlated with a statistically significant 34% reduction in cardiac endpoints (104). The therapeutic use of gemfibrozil has recently been reviewed (105). Recent efforts have focused on dissociating the hypolipidemic effects from peroxisome proliferation (106). Tazasubrate (30) produces minimal induction of peroxisomes (107).

Miscellaneous - Screening in chow or C-fed rat models has uncovered a variety of compounds which reduce plasma TC by unknown mechanisms. Some of these include xenalipin (31), also reported to be effective in C-fed monkeys (108); gem-phosphonate-phosphate (32), reported to selectively elevate HDL-C in rats (109) and TA-1801 (33), which is almost identical to clofibrate in reducing TC and TG in rats and at inhibiting platelet aggregation in vitro (110,111). Pyrazole 34 has been reported to exert its hypocholesterolemic effect by multiple modes of action, including inhibition of C absorption (112). Itanoxone (35) also displays an activity profile similar to clofibrate in rats (113).



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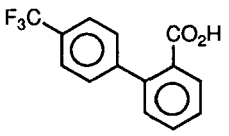

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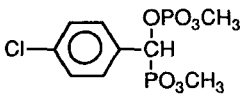

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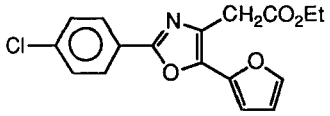

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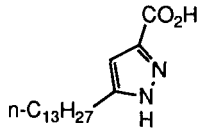

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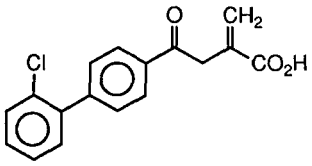

35

Conclusion - The importance of HC as a causative factor in coronary artery disease has been firmly established by long-term primary intervention trials. The efficacy of bile-acid sequestrants and HMGR inhibitors in treating HC in man is now well documented. Although recent research into C absorption inhibitors has uncovered a number of interesting drug types, such as ACAT inhibitors, their clinical efficacy still remains unproven. In the future, increased knowledge of the underlying causes of HC will allow drug therapy to be targeted for specific defects in lipid metabolism. It can also be expected that recent efforts to understand the molecular biology and regulation of the synthesis and secretion of the LDL receptor (114), the apolipoproteins (115,116) and the less studied lipoproteins, such as the atherogenic lipoprotein Lp(a) (117,118), will open up new avenues for drug intervention.

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Chapter 17. Recent Advances in the Design and Evaluation of Inhibitors of Phospholipase A_2

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Introduction - The last twenty years have produced a greater appreciation for the role of phospholipase A_2 (PLA_2), in the inflammatory process. PLA_2 action results in the liberation of fatty acid, predominantly in the form of arachidonic acid (AA), from phospholipid (PL). Once formed, the two products, free AA and lysophospholipid, are available for conversion to potent proinflammatory mediators , the eicosanoids (prostanoids, leukotrienes, lipoxins, hydroxy fatty acids) and platelet activating factor (PAF), respectively (1, 2). At basal levels, PLA_2 is responsible for deacylation-reacylation processes required for cell membrane repair and maintenance. Its activity is enhanced significantly during inflammation. High levels of PLA_2 activity have been found in joint fluid from patients with rheumatoid arthritis (RA) (3), from serum of patients with endotoxin shock (4) or pancreatitis (5), and in psoriatic lesions (6). In some cases PLA_2 activity is positively correlated with disease severity (3,4). The source of this "inflammatory" PLA_2 is not known, but various proinflammatory mediators are known to initiate the cellular release of PLA_2 such as thrombin-induced PLA_2 release from rat platelets (7) or interleukin-1 (IL-1) induced increase in cell-associated and secreted PLA_2 (8,9). The recent isolation and characterization of a PLA_2-activating protein (PLAP) has provided additional evidence for the inflammatory role of PLA_2 (10). PLAP potentiates the activity of PLA_2 enzymes ,is associated with enhanced eicosanoid generation and has been detected at high levels in the synovial fluid of RA patients (10,11). Recent reports indicate that articular injection of PLAP into the rabbit knee joint induces arthritis characterized by eicosanoid production and neutrophil migration (12). To further conceptualize the therapeutic value of a PLA_2 inhibitor it is appropriate to review the potent anti-inflammatory action of glucocorticoids and their congeners. They are thought to act in part by the induction of a PLA_2 inhibitory protein (lipocortin (13)). Evidence exists that lipocortin or various cloned moieties display *in vivo* anti-inflammatory activity (14,15). While the direct inhibitory action of lipocortin against the enzyme is currently being debated (16,17), greater problems related to its therapeutic usefulness are surfacing. Titers of anti-lipocortin antibodies are reported in patients suffering from RA, suggesting that anaphylactic side effects or tachyphylaxis of anti-inflammatory activity may result with direct lipocortin administration. Interestingly, high antibody titers correlate with patients that do not respond to steroid therapy (18). Taken together, these facts form a strong rationale for the development of an inhibitor of PLA_2 as a novel approach for the design of anti-inflammatory agents.

DETECTION AND ASSESSMENT OF PLA_2 INHIBITORS

A thorough review of PLA_2 mode of action was recently written (19). Two major objectives in designing an assay system to identify and assess inhibitors of PLA_2 are to choose the most appropriate enzyme for the therapeutic target and demonstrate the predictability of the assay for drug activity *in vivo*. The PLA_2 enzymes present special *in vitro* problems that include not conforming with classical Michaelis-Menten kinetics; therefore, careful consideration must also be taken in the choice of substrate type and the mode of analysis used. The decisions made can influence the selectivity and specificity of inhibitors identified as well as their potential *in vivo* anti-inflammatory efficacy.

Selection of Enzyme - PLA_2 refers to a large class of acylhydrolytic enzymes that specifically act at the sn-2 position of phospholipid substrate. They are found ubiquitously in nature in both cell-associated and extracellular forms. Even though aspects of PLA_2 catalysis, such as the role of enzyme dimerization (1, 20), activation *via* lipid:water interfacial recognition (21), the role of Ca^{++} (22) and the reaction of acylhydrolysis (21) are now better understood, questions and controversy still exist. It is not known if the catalytic mechanism is universal for all PLA_2 enzymes. The majority of the literature covering structural and functional aspects of the PLA_2 enzyme has been acquired using either the snake venom derived PLA_2 enzymes or the pancreatic PLA_2 enzymes due to their easy accessibility and quantity . The snake venom and pancreatic enzymes have a high degree of structural homology, particularly in the catalytic site and hydrophobic regions (23,24). It is yet unclear whether interspecies or for that matter intertissue/intercellular PLA_2 structural and/or catalytic differences exist. Amino acid sequence homology has been reported between PLA_2 from human synovial fluid, human placenta (25), rabbit ascites fluid (26), rat platelet (26), human platelet (27,28) and *Crotalus atrox* venom PLA_2 (28,29), but not human pancreatic PLA_2. Structural comparison studies of PLA_2 utilizing cross-reactivity against anti-rat liver mitochondria PLA_2 antibodies demonstrated no reactivity with rat or pig pancreas PLA_2 or *Crotalus atrox* snake venom PLA_2, but did with PLA_2 from rat platelet (30). This disparity could be due to the existence of enzyme recognition sites other than the catalytic site that are more variable and less likely to react with antibody. The contribution of non-homologous structural components is unclear and could possibly convey variations in three dimensional conformation generating isotypes. Evidence of this includes enzyme differences in overall surface protein charge (acidic vs basic) noted between enzyme types (25,31), the natural existence of certain enzymes as monomeric or polymeric forms (20) and exhibition of characteristics of either a Type I or Type II PLA_2 (enzymes categorized by the type and position of sulfur containing amino acids and the length of and extension of the carboxyl terminus into the catalytic site) (32). Additionally, aspects of enzyme catalysis from different PLA_2 sources are reported to vary. Examples include variable reactions to substrate charge, influences of buffer ionic strength (33), pH optima, selectivity in PL affinities (29,34,35), requirement for detergent micelles (36) and the dependence on Ca^{++} (27,37). While one must be cautious in interpreting these studies, the bulk of the information suggests that PLA_2 enzymes from various sources may possess enough active site variation to design selectivity into the PLA_2 inhibitors. This will offer an advantage to the drug such that potential side affects arising from non-specific inhibition of all PLA_2 enzymes may be obviated. Since the similarity between PLA_2 of different species is not clear it may be essential to design inhibitors against human enzymes. The isolation and purification of human PLA_2 from the synovial fluid of patients with RA (26,28,29), the placenta (25), neutrophils (36), platelets (27) and the serum of septic shock victims (38), is just now being reported. These enzymes could offer an enzyme target better than the snake venom or pancreatic PLA_2 for inhibitor development.

Nature of Substrate Used - Phospholipid substrate must be in an aggregated form for optimal presentation to the enzyme (39). The lipid aggregate forms used to assess PLA_2 activity include monolayer dispersions, pure or combined lipid mixtures (liposomes), and detergent-lipid emulsions or micelles. The lipid chosen will form aggregates in various molecular forms, such as bilayer or hexagonal array. Parameters such as pH, ionic strength, temperature and type of PL further influence the physical form of the aggregate. Introduction of detergents such as Triton-X or deoxycholate results in micelle formation. The overall characteristics such as packing density and lipid surface charge can have a marked effect on PLA_2 activation and hydrolysis (40). Substrate sources are derived from two broad categories: 1) synthetic PL or 2) natural "membrane" lipid forms. Membrane lipid composition is fairly complex and must be determined whereas synthetic PL aggregates are easily designed and controlled. However, synthetic PLs are by no means "natural lipid" presentation forms; they display artificial lipid-aqueous interfaces and surface charges. Moreover, subtle effects of other membrane components (such as cholesterol or constitutive proteins), lipid domains, or bilayer asymmetry are not represented in the synthetic substrate milieu (41). Finally, synthetic PL aggregates have a very low tolerance

to assay manipulations such as pH, ionic strength, substrate concentration or product formation (42). The weight of these considerations in choosing the appropriate substrate for *in vitro* assessment depends on the target enzyme used and its physiological role.

Mode of In Vitro Analysis - Several well-characterized methods exist for determination of acylhydrolysis (19, 40, 43). Mammalian PLA_2 enzymes are generally less abundant; thus, assay sensitivity becomes a major consideration. Both colorimetric (44,45) and fluorometric (46) assays have been reported utilizing specific PL probes or product complexes with compounds which have measurable functional groups. Tracers, specifically radionuclides, have been extensively used and provide a more sensitive assay (47,48).

In Vivo Anti-inflammatory Assessment - When a PLA_2 inhibitor has been identified, the next stage of development is assessment of its bioactivity. Depending on the clinical target, several cellular and whole animal models can be exploited to determine the efficacy of a PLA_2 inhibitor in modulating an inflammatory response. Initially, PLA_2 inhibitors can be assessed for their ability to alter cellular AA mobilization and metabolite formation e.g. free AA, eicosanoid and PAF generation. This can be conducted with a wide variety of immune cell populations such as mouse peritoneal macrophages (49), casein or glycogen elicited peritoneal cell population (predominantly made up of polymorphonuclear lymphocytes (50)), or pleural cavity exudate cells (51), just to list a few. Due to the lack of potent and selective inhibitors very little literature exists. The bulk of the work reports the utilization of weak and or non-specific compounds such as mepacrine or *p*-bromophenacyl bromide (BPB) and therefore is not easily interpreted. Recently, a potent PLA_2 inhibitor, manoalide, was demonstrated to reduce prostaglandin, leukotriene and AA release by cultured mouse macrophage at nanogram concentrations (52). A secondary functional response generally accompanies stimuli-induced activation of cellular PLA_2 and lipid mediator release that can be evaluated, e.g., measurement of IL-1, histamine, free radical generation, phagocytosis or degranulation. These studies are valuable in that PLA_2 inhibitors can be examined in a more controlled setting with the premise that fewer complicating factors exist to interfere with correlations made between functional activity and PLA_2 inhibition. Additionally, direct effects on cellular lipid changes can be monitored as well as drug toxicity.

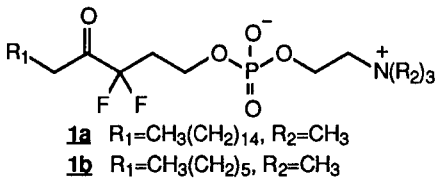
Evaluation of PLA_2 inhibitor *in vivo* anti-inflammatory activity can be achieved using routine inflammation animal models particularly since it is thought that most models owe some aspect of their etiology to activation of PLA_2 and eicosanoid production (various paw edema models, mouse zymosan peritonitis, reverse Arthus pleurisy or various skin inflammation assays) (53). While these models will reflect the drug's ability to alter an inflammatory response, they cannot address the specificity of drug action. Moreover, the models were in many cases set up to detect classical non-steroidal anti-inflammatory drugs (NSAID) and it may be important to separate the potential NSAID-like properties of PLA_2 inhibitors from their novel non-NSAID activities. New models are therefore being developed. The use of PLA_2 administration as the initiating inflammatory insult are the most documented and well characterized models in terms of evaluating the proinflammatory activity of PLA_2. Recently, a model has been extensively characterized using purified *Akristadon piscivorus piscivoris* (D-49) PLA_2 injections into mouse paws (54). Interestingly, whereas PLA_2 action is the initiating event, the model is sensitive to antihistaminic/antiserotonin agents as well as PAF antagonists, PLA_2 inhibitors and glucocorticoids, suggesting that edema formation is due to a concert of mediator events. Other PLA_2 have been used to induce paw edema; one of particular interest is HSF-PLA_2 - induced mouse paw edema which was reported to be inhibited by coinjection of aristolochic acid (55). Snake PLA_2 induced cutaneous vascular permeability in guinea pig (56) or rabbit (57), PLA_2 induced rat pleurisy (58), and guinea pig bronchoconstriction initiation by doses of aerosolized snake PLA_2 (59) have also been reported but are less characterized.

Because of the measurement of PLA_2 activity in human serum or inflammatory fluids, the classical inflammation animal models are being reevaluated for the presence of PLA_2 activity. Recent examples include the measurement of PLA_2 activity during glycogen induced rat peritonitis (60), zymosan-induced mouse peritonitis (61), zymosan-induced rat pleurisy (62) and reverse Arthus pleurisy in rats (58). In all cases, PLA_2 activity correlates with various inflammatory parameters during the course of the episode. Due to the early stage of model development little or no PLA_2 inhibitor drug data is available. The role of this enzyme activity is not clear since the activity is not always associated with eicosanoid measurements, cellular influx or protein extravasation. Certainly, much more work is required, particularly studies performed with selective PLA_2 inhibitors, to understand the role of this enzyme in the inflammatory response.

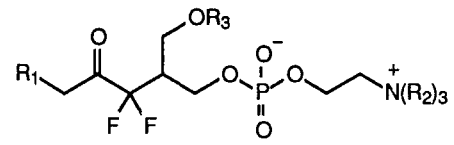
INHIBITORS OF PHOSPHOLIPASE A_2

Substrate Analogs - As an extension of previous work (63), a series of fluorinated phospholipid transition state analogs **1** and **2** and their corresponding alcohol reduction products were prepared and tested as inhibitors of PLA_2 from *Naja naja naja* venom (N-PLA_2) in a mixed micelle system (64). The best inhibitors (**1a**, **1b** and **1c**, IC_{50} values of 0.7 mM, 1.6 mM and 0.07 mM, respectively) were single chain fluoro ketones which were shown by ^{19}F NMR to be partially hydrated in the micelle. NMR studies with the two chain analogs **2** demonstrated that these compounds were present in the micelle only as the ketone (64). Reduction of **1a** to the corresponding fluoro alcohol resulted in a dramatic loss of inhibitory activity (<5% inhibition at 4 mM) indicating that fluoro alcohols do not serve as good transition state analogs in this system.

The transition state analog approach was then used to design phosphonate-containing phospholipid analogs **3a-c** (65). Phosphonate **3c** was significantly more potent than the fluorinated ketones **1** and **2**. The methyl phosphonate analog of **3c** was 250 times less potent than **3c** itself.



1a $R_1=CH_3(CH_2)_{14}$, $R_2=CH_3$
1b $R_1=CH_3(CH_2)_5$, $R_2=CH_3$
1c $R_1=CH_3(CH_2)_{14}$, $R_2=H$



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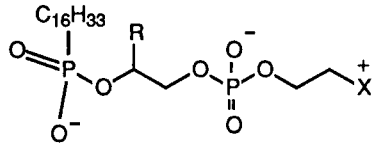
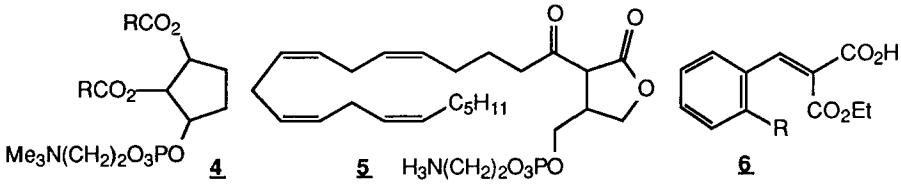


3a $X=NH3^+$, R=H: IC_{50} = 0.75 mM
3b $X=NMe_3^+$, R=H: IC_{50} = 2.3 mM
3c $X=NH_3^+$, $R=CH_2OC_{16}H_{33}$: IC_{50} = 5 μM

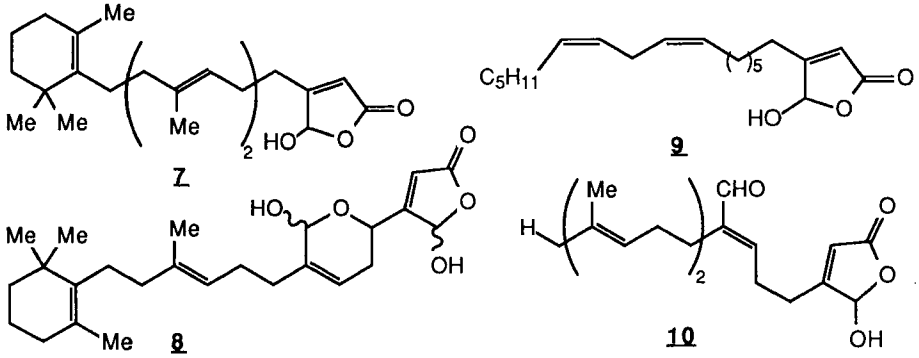
The activity of PLA_2 is greatly enhanced when the substrate is above its critical micelle concentration (CMC). Several models have been proposed to account for this. To see if this activation results from a change in the substrate conformation in the micelle (substrate theory of activation), two groups (66 - 69) have reported on the synthesis and susceptibility towards PLA_2 hydrolysis of constrained phospholipid analogs **4**. While the (+)-enantiomer of the all *cis* compound **4a** ($R=C_5H_{11}$, absolute configuration undetermined) was an inhibitor of L-dihexanoyl-PC hydrolysis (50% inh. at 3 mM, N-PLA_2), the (-)-enantiomer, **4a**, was a substrate for PLA_2 and its rate of hydrolysis did not increase above the CMC. This supported the substrate theory of activation since it was felt that the constrained nature of the ring would not allow a conformational change upon reaching the CMC. If the increased rate of hydrolysis above the CMC for unconstrained substrates was due to a change in the enzyme conformation as opposed to the substrate conformation, one would

have expected to see the effect with the constrained compound as well. What is unknown is whether the ring constrains the compounds in the activated or unactivated conformation.

In a molecular recognition study, various conformations (*cis/trans* isomers and keto/enol tautomers) of the constrained analog (±)-**5** were modelled and overlapped with the best enzyme docked conformation of di-lauryl-PC (70). The best fit was obtained with (3S,4R)-**5**. Racemic **5** was shown to inhibit rat neutrophil cell-free PLA_2 (IC_{50}, 64 μM) and rat macrophage PLA_2 (IC_{50} 44 μM).



5,8,11,14-Eicosatetraynoic acid (ETYA) was found to inhibit the PLA_2 of rabbit peritoneal neutrophil sonicates and acid extracts (IC_{50}, 12 and 22 μM, respectively) (71). Others also reported its inhibitory activity , as well as AA analogs **6** (R=alkenyl or alkynyl) (72). The most potent compound, **6a** (R = C≡C-$C_{11}H_{23}$, IC_{50} 0.7 μM, N-PLA_2), was evaluated *in vivo* as an inhibitor of SRS-A release in a rat passive peritoneal model of anaphylaxis. At 200 μM, **6a** produced 51% inhibition at a contact time of 2.5 min. while ETYA (IC_{50} 20 μM vs. N-PLA_2) produced weak and inconsistent inhibition. Compounds related to **6** have been reported in the patent literature by the same workers (73).



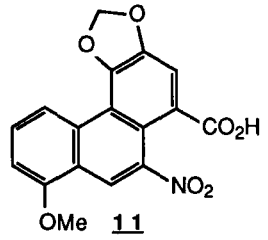
Natural Products and Analogs - The sesterterpene luffariellolide (**7**) was shown to inhibit hydrolysis of phosphatidyl choline by purified bee venom PLA_2 (IC_{50} 230 nM) (74). Compound **7** is closely related to another sesterterpene, manoalide (**8**) which irreversibly inhibits bee venom PLA_2 (75). Unlike **8**, the inhibition by **7** was partially reversible. At a dose of 50 μg/ear, **7** was also found to inhibit topical 12-O-tetradecanoate phorbol-13-acetate (TPA)-induced inflammation in the mouse ear.

In order to determine the basis for the irreversible inhibition by **8**, **9** (resembling **7**) and **10** were prepared (76 - 78). It was found by ^1H NMR that the closed and opened forms of **9** are in rapid equilibrium between pH 4 and 9, and are present in roughly equal amounts between pH 7.5 and 8.5. Although the opened form allows for the possibility of a conjugate addition or Schiff base formation with the enzyme, inhibition of N-PLA_2 by **9**, like **7**, is reversible. On the other hand, **10**, like **8**, acts irreversibly, indicating that both the opening of the ß-lactone ring, as well as the presence of an α,ß-unsaturated aldehyde (opened hemiacetal in **8**) are necessary for irreversible inhibition.

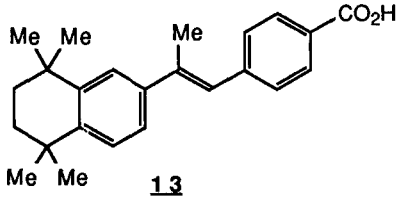
The importance of comparing inhibitor activity against the same enzyme can be illustrated with **8**. Its IC_{50} value for inhibition of PLA_2 hydrolysis has been measured against a number of enzymes and varies over almost three log units (0.05-0.12 μM, bee venom PLA_2; 0.7 μM, rattlesnake venom PLA_2; 2 μM, cobra venom PLA_2; 30 μM, porcine pancreatic PLA_2) (52). Additionally, it is probably not enough to ascertain whether the same enzyme was used; one should also look further at the assay to make sure that the substrate and any detergents are also the same.

The alkaloid aristolochic acid, **11**, was found to inhibit *Vipera russelli* venom PLA_2 hydrolysis of phosphatidyl choline with an IC_{50} value of 50 μM and a K_i of 9.9×10^{-4} M (79). As shown in a circular dichroism study, it binds to this enzyme in the absence of substrate (80). By following the intensity of the extrinsic CD band at 320 nm, an association constant of 5.0×10^3 M^{-1} was calculated (ΔG° = -5.1 kcal/mole). A kinetic analysis showed the inhibition to be non-competitive. However, against the basic PLA_2 from *Trimeresurus flavoviridis* venom, **11** had an IC_{50} value of 25 μM and a K_i of 3.9×10^{-7} M, but the inhibition was competitive. It was also shown to inhibit the edema inducing activity of both *Vipera russelli* and *Trimeresurus flavoviridis* venom PLA_2 injected into mice paws (81). Both i.m. and i.p. routes of administration of **11** were effective.

Recent studies reported the inhibition of N-PLA_2 and human synovial fluid (HSF) PLA_2 by retinol, retinal, all-*trans*-retinoic acid and 13-*cis*-retinoic acid (N-PLA_2 IC_{50} values of 24, 25, 37 and 45 μM, respectively; HSF PLA_2 IC_{50} values of 165, 6, 10 and 15 μM, respectively) (82, 83). Three analogs, **12a**, **12b**, and **13**, were also found to inhibit the N-PLA_2 enzyme (IC_{50} values of 83, 34, and 37 μM, respectively), and **13** inhibited HSF PLA_2 (IC_{50} 12 μM). In the case of **12b**, activity against cyclooxygenase and 5- and 12-lipoxygenase was also found.



11



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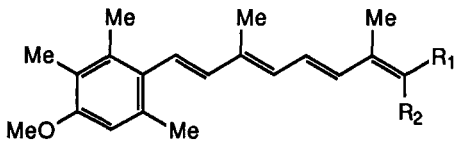
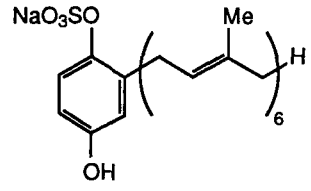


12a R_1=CO_2Et, R_2=H
12b R_1=H, R_2=CO_2H

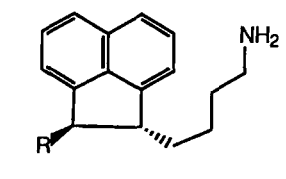


14

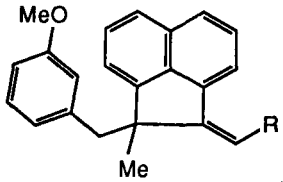
The inhibitory effects of these retinoids on the release and metabolism of AA from rat peritoneal macrophages challenged with A23187, zymosan and TPA were also studied. Generally, the compounds which inhibited ^{14}C-release upon A23187 stimulation (all-*trans*-retinoic acid, 13-*cis*-retinoic acid, **12b** and **13**) also inhibited its release with zymosan stimulation. However, TPA stimulation was inhibited only with all-*trans*-retinoic acid and **13** while 13-*cis*-retinoic acid and **12b** were inactive. This suggested the involvement of a different lipase upon TPA stimulation, or that these compounds are not acting as direct PLA_2 inhibitors.

Several other natural products have also been reported to inhibit PLA_2. Gossypol, a male non-steroidal antifertility agent, has been shown, at 100 μM, to completely inhibit

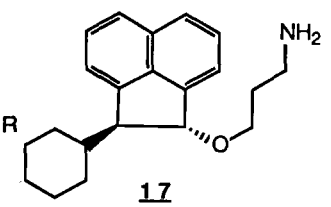
intact human spermatozoa PLA_2 hydrolysis of monolayers of phosphatidylglycerol (84). The bioflavonoid quercetin has been found to inhibit [^{3}H]-AA release from prelabeled neutrophils stimulated with zymosan (85). It was later shown to inhibit the PLA_2 of rabbit peritoneal neutrophil sonicates and acid extracts (IC_{50}, 57 and 100 μM, respectively) (22). In the same system, the antioxidant nordihydroguaiaretic acid was shown to inhibit hydrolysis by PLA_2 (IC_{50}≈10 μM for both sonicates and extracts). The membrane lipid antioxidant vitamin E has also been shown to inhibit PLA_2 activity, this time from rat platelets (86). At vitamin E concentrations of 116 μM, hydrolysis of varying amounts of phosphatidyl choline was inhibited by 20-50%. A common characteristic of gossypol, quercetin, nordihydroguaiaretic acid and vitamin E is their antioxidant potential; however, it is not obvious how this feature could affect PLA_2 hydrolysis of phospholipids. Finally, the hexaprenylhydroquinone sulfate, **14**, isolated from a marine sponge, has been reported to inhibit PLA_2 with an IC_{50} value of 1.8 μM (source of enzyme not indicated) (87).



15 R=c-C_6H_{11}, $PhCH_2$



16a R=$(CH_2)_2NH_2$
16b R=H



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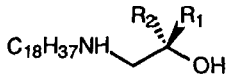

18a R_1=R_2=H: IC_{50}=20μM
18b R_1,R_2=H,Me (racemic monomethyl): IC_{50}=10μM
18c R_1=H, R_2=Me: IC_{50}=10μM
18d R_1=Me, R_2=H: IC_{50}=10μM
18e R_1=R_2=Me: IC_{50}=30μM

Computer Assisted Inhibitor Design - A three-dimensional study of the bovine pancreatic PLA_2 X-ray structure, with the goal of identifying novel compounds that fit both sterically and electronically into the active site, has resulted in the identification of a series of potent *in vitro* inhibitors (88). Examination of fits between 2-arachidonyl phospholipids and the enzyme suggested that a naphthalene ring might mimic the "planar" conformations of the double bonds in the arachidonyl chain and fit in the slot between Leu2 and Tyr69. Bulky hydrophobic groups (benzyl, cyclohexyl) should take advantage of the hydrophobic residues in the active site, and an amine should extend towards the essential calcium and interact with Asp49 or displace the calcium. This analysis led to targets **15**. The compounds synthesized that were closest to the targets, **16a** and **17**, had IC_{50} values of 0.67 and 1.4 μM, respectively. The best compound, **16b**, had an IC_{50} value of 0.2 μM.

Another study of the same X-ray structure has resulted in the synthesis of long chain alkylamine inhibitors (89). A study of the active site revealed only two easily accessible polar amino acids (His48 and Asp49) in the large hydrophobic cavity. These residues are involved in the hydrolysis mechanism and are highly invariant. The inhibitors were designed to interact through hydrogen bonding and electrostatic forces with these residues and with Tyr69 which lies at the edge of the cavity. The most potent compounds (vs. porcine pancreatic PLA_2) were **18a-e**.

Inhibitor-Enzyme X-Ray Structure - Bovine pancreatic PLA_2 is irreversibly inhibited by BPB, and a 2.5 Å resolution crystal structure of the inhibited enzyme has been reported. BPB was covalently bound in the active site to His48, and the conformation of the active site residues was found identical to that in the native enzyme; however, there were some drastic differences in other regions of the enzyme. BPB has hydrophobic interactions with Phe5 and Cys45, and this may explain why it is rather specific for PLA_2. The design of specific inhibitors of PLA_2 based upon the three-dimensional structure of the inhibited enzyme is discussed (90).

Conclusion - Although there has been considerable activity in this field over the last decade, much work remains before a clinical candidate can emerge. The bulk of the literature contains a plethora of *in vitro* assays varying in enzyme type, substrate and mode of analysis. *In vitro* methodology therefore needs to be standardized. Furthermore, the data reported is often represented as inhibitory activity at a single dose as opposed to a dose response generated IC_{50} value making clear comparisons between the potency or mechanism of action difficult. PLA_2 inhibitors are currently assessed in classical inflammatory models, but to set these novel drugs apart from classical NSAIDs, new *in vivo* assays remain to be developed. Many of the PLA_2 inhibitors mentioned in this review are non-specific and have other activities. Of those that were designed, most are phospholipid analogs which were made to answer mechanistic questions and lack any report of notable *in vivo* activity. This deficiency is also true of the compounds designed based upon the enzyme X-ray structure. Therefore, despite the rekindled interest in inhibitors of PLA_2, there is a paucity of information needed to allow *in vitro* to *in vivo* correlations.

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Chapter 18. Recent Advances in the Treatment of Inflammatory Bowel Disease

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Introduction - Crohn's disease (CD) and ulcerative colitis (UC) are chronic forms of inflammatory bowel disease (IBD). Although there are other forms of intestinal mucosal inflammation, such as bacterial induced enteritis and neonatal necrotizing enterocolitis that are clinically important, the present review will be focused on the development and application of pharmacotherapy for CD and UC.

CD is most commonly used to describe ileal inflammation associated with diarrhea (sometimes bloody), increased mucosal permeability, and occasionally granuloma formation (1). CD can occur more proximally and also in the colon. There are many similarities and yet distinct differences in the gross and microscopic pathological picture in CD compared to UC. Especially difficult, because of overlap in the clinical findings, is the differentiation of CD of the colon from UC (2).

IBD occurs worldwide, in developing as well as developed countries. There are some suggestions of racial differences in susceptibility to CD (3). Epidemiologic studies suggest that the incidence of CD may be on the rise (4). Part of this increase, however, may be merely a reflection of better diagnosis and greater recognition over the last 50 years of CD.

Although all age groups are susceptible to IBD, the incidence of CD increases substantially after age 13. Onset is frequently preceded by viral (respiratory) flu, administration of broad spectrum antibiotics or an emotional crisis. The link to emotions is particularly relevant in view of findings that release of mucosal mast cell protease can be induced by Pavlovian conditioning (5). There is approximately equal distribution of the disease among males and females (6), but non-smokers tend to have a higher incidence of UC (7-9). The onset of the disease also seems to be associated with cessation of smoking. It is not known how smoking or cessation of smoking alters mucosal function. The use of oral contraceptives for 12 months or more, has also been associated with CD (10).

Etiology - The etiology of IBD is not known. The fact that patients with UC frequently are noted to have depletion of mucin from colonic goblet cells, and have biochemically altered mucin, has suggested that abnormal mucus production is a factor in UC. Patients with Crohn's colitis also have a deficiency in certain mucin species (11). A defective mucin coating for the epithelial lining which could allow access to the mucosa of luminal factors such as bacterial antigens, leading to the initiation of an inflammatory response, may be an important factor. Increased mucosal permeability associated with a decrease in lysophospholipase activity in the mucosa of CD patients has been reported (12). Absence of this enzyme, which inhibits accumulation of the permeability enhancer lysolecithin, could increase mucosal uptake of microbial or dietary factors capable of inducing pathology.

Other mucosal enzymes are altered as seen in biopsies of patients with IBD. Specifically, acid phosphatase and N-acetyl-β-glucosaminidase were significantly higher in healthy individuals than in those with UC or CD (13). Theoretically, acid hydrolase activity could be reduced by release of lysosomal enzymes with subsequent cellular damage. Several immunological causes of mucosal damage have been postulated. One contested theory states that lymphocytes in the lamina propria of the intestinal mucosa from patients with IBD are cytotoxic to colonic epithelial cells (14). Increased levels of leukotrienes (especially LTB4) compared to normal individuals occur in the mucosa of patients with IBD (15). In addition, decreased levels of interleukin-2 (IL-2) have been reported (16). Active current areas of research into the etiology of IBD are focusing on Mycobacterium paratuberculosis as an initiator in CD and pathogenic E. coli involvement in UC (17,18).

ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASE

Development of pharmacotherapy for IBD must account for the numerous primary and secondary mediators released as a consequence of mucosal inflammation. Current approaches in animal models of human IBD involve inhibiting the release of inflammatory mediators, blocking their receptor sites on effector cells, and preventing the deleterious action of reactive oxygen metabolites on intestinal epithelial cells (19-22). The ideal animal model for IBD should have the same causal factors, pathology and pathophysiology and clinical spectrum as found in human IBD.

Natural Animal Models - Colitis, whose clinical manifestations mimic some of the patterns of human UC and CD has been reported in dog, horse, hamster, pig, mouse, rat (19) and monkey (23). None of the above models of spontaneous colonic inflammation comprises all of the relevant aspects of human IBD.

Experimentally-Induced Colitis - Based upon its neutrophil (PMN) chemotactic properties, colonic instillation of formyl-methionyl-leucyl-phenyl alanine (FLMP) has been used to provide a useful colitis model in rat or rabbit; it caused marked PMN infiltration and edema (24,25). Intracolonic infusion of dilute acetic acid caused an acute colitis with edema, ulceration and PMN infiltration in the rat (26) and guinea pig (27) that has been assessed histologically and by the levels of the PMN marker enzyme, myeloperoxidase (28-30). In addition, the proinflammatory lipid mediators LTB_4 and 12-HETE are present in increased amounts in acetic acid treated colons than in normal rat mucosa, indicating that arachidonic acid metabolism in this model resembles that of human IBD (31).

A chronic irritant colitis model has been described in the rat in which a single colonic instillation of trinitrobenzene sulfonic acid (TNBS) produces ulceration, mediator release and inflammatory cell infiltration for up to 5 weeks (32-34). Delayed hypersensitivity models of colitis in the guinea pig have been described in which 2,4-dinitrochlorobenzene (DNCB) is utilized as a dermal sensitizing agent followed by intracolonic challenge with up to 5% DNCB. This results in edema, erythema and goblet cell depletion in the colon (35-37), thus mimicking IBD. The dietary addition of both natural and degraded carrageenin to induce colitis in mouse and guinea pig has been received with mixed success (38,39). Exposure of rat colons to capsaicin results in an intense focal ulceration accompanied by plasma extravasation lasting four days (40). Immune complex-induced colitis has been described in the rabbit in which 1% buffered formalin is instilled intrarectally followed 2-3 hours later by i.v. infusion of soluble human serum albumin antigen-

antibody complexes; this resulted in inflammatory cell infiltration, crypt abscesses, and mucous cell depletion after 4-5 days (41-43). The surgical formation of an ileal segment "cyst" has been reported as a model of non-specific IBD in the rat (44). Intraperitoneal treatment with clindamycin produces fatal pseudo-membranous colitis in the hamster, secondary to C. difficile overgrowth and enterotoxin expression (45). Another model in mice has been described where manipulation of the intestinal microflora with oral dextran sulfate sodium led to multiple inflammatory changes in the colon (46). A model addressing the blood-to-lumen movement of salt and water, seen as the diarrhea in IBD, has been reported in which egg albumin sensitized, adrenalectomized rats demonstrate upon challenge, colonic infiltration of inflammatory cells, multifocal ulceration, mucosal necrosis and goblet cell depletion (47).

While the disease models described above certainly do not individually represent the ideal, agents that are active in multiple models may well have therapeutic utility in the treatment of human IBD.

MEDIATORS OF THE INFLAMMATORY RESPONSE IN IBD

Soluble mediators of inflammation are thought to be the immediate cause of the functional and histologic changes manifested in IBD. Neutrophil and macrophage infiltration suggest the presence of soluble chemotactic agents that provoke the migration of circulatory neutrophils and monocytes into the mucosa. Characteristic mucosal edema and hyperemia may be attributed to the presence of mediators that induce enhanced vascular permeability and vasodilation (48).

The last few years have witnessed a major growth in the study and characterization of a number of soluble mediators implicated in the inflammatory response associated with IBD.

Eicosanoids - The role of prostaglandins and specifically of PGE_2 as inflammatory mediators in IBD has been downplayed over the last few years. Although levels of PGE_2 have been found to be elevated in UC and CD patients (49,50), therapeutic intervention with cyclooxygenase inhibitors such as flurbiprofen (51) and indomethacin (52) has provided no statistically significant improvement of the disease state. In fact, in animal models of colitis, evidence now exists that prostaglandins are antiinflammatory (53).

Consequently, attention has been focused on the role of leukotrienes in the pathogenesis of the inflammatory response associated with IBD. Levels of the pro-inflammatory eicosanoid LTB_4 (54) in rectal dialysate fluid obtained from UC patients have been shown to correlate with the severity of the colitis; steroid treatment reduced LTB_4 levels with a corresponding marked clinical improvement (55,56). Therapeutic inhibition of lipoxygenase pathways may prove more effective in UC than in CD (50). Inhibition of leukotriene biosynthesis has also been shown to promote healing in animal models of IBD (32-34,57). Inflamed human mucosal tissue has been shown to release significantly more sulfidopeptide leukotrienes. (LTC_4, LTD_4 and LTE_4) than normal colonic mucosa (58). These leukotrienes may contribute to the mucosal edema and hyperemia observed in IBD due to their effects on vascular permeability and resistance.

Platelet Activating Factor (PAF) - The role of PAF as a mediator of the inflammatory response in IBD is currently under investigation in view of its ability to induce both ischemic colitis and ulcerative gastritis after IV administration (59,60). A recent report has asserted that levels of PAF are markedly increased in UC colonic mucosa compared with normal mucosa. In vitro inhibition of PAF production by sulfasalazine and prednisone was also demonstrated (61). The release of PAF and its

role in the TNBS induced colitis model has been the subject of recent studies (62). The data presented supported the conclusion that PAF is unlikely to play an important role in the acute inflammatory response, but may be important in the prolongation of inflammation and ulceration in this model (63).

Cytokines - A number of cytokines have demonstrated biological properties that may implicate them in the pathogenesis of IBD (64-66).

Resected intestines from both CD and UC patients have been shown to have increased concentrations of interleukin 1β (IL-1β) was found to correlate with disease activity (67,68). In addition, peripheral blood mononuclear cells (PBMCs) from Crohn's patients have demonstrated an increased capacity to synthesize IL-1 upon stimulation in vitro with lipopolysaccharide (LPS) or spontaneously in culture (69). In animal models of IBD, IL-1 may be associated with both acute and chronic colitis; also mucosal IL-1 is a more sensitive marker of colonic inflammation than mucosal myeloperoxidase (MPO) activity (70).

The production of tumor necrosis factor (TNF) by peripheral monocytes in IBD patients has been studied. Stimulation of IBD PBMCs with LPS produced significantly more TNF than control. This increased production was inhibited by salazopyrine pretreatment (71).

Peripheral blood T lymphocytes from UC patients have been shown to have a diminished capacity for IL-2 production. This could contribute to abnormal T-cell proliferation and clonal expansion at gut mucosal level, and might induce a defective immune response leading to a chronic inflammatory reaction in UC (72). Involvement of α-interferon (IFN-α) in the inflammatory response observed in chronic IBD appears to be questionable (73) despite contrary earlier reports (74).

Kinins - Recent data indicates that kinins and specifically bradykinin may be involved in the inflammation observed in IBD (75). Inflamed colonic tissues from patients with UC contain abnormally high levels of a bradykinin-releasing enzyme, kallikrein. Plasma and tissue levels of the kinin degrading enzyme peptidyl-dipeptidase are depressed in patients with CD (76). Furthermore, the plasma level of α-macroglobulin, which inhibits kallikrein, is low in CD patients (77). The presence of bradykinin may trigger the liberation of lipoxygenase products thus promoting both the inflammation and diarrhea characteristic of IBD.

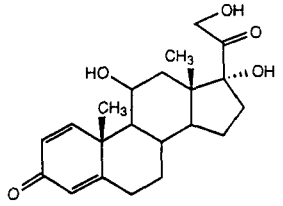
Neuropeptides - Several lines of evidence link the tachykinin neuropeptides with a role in regulating inflammatory and immune responses. In colon tissue obtained from UC and CD patients, very high concentrations (1000 x normal) of Substance P receptor binding sites are expressed by arterioles and venules located in the submucosa, muscularis mucosa and serosa, and also are expressed within the germinal center of lymph nodes (78). In CD, there is an increase in ganglion cells and in the content of vasoactive intestinal peptide (VIP) and the number of VIP-containing nerves in the intestinal wall (79). An overview of neuroendocrine modulation of the immune system and its possible implications for IBD has recently been published (80).

Reactive Oxygen Metabolites (ROM's) - Current developments in the involvement of oxyradicals and other oxidative processes in the inflammatory response of human neutrophils and macrophages have been reviewed with special reference to IBD (81,82). A more recent theory suggests the etiology of UC involves a defect in mucin synthesis and/or degradation (83). ROMs released from neutrophils may degrade mucin, resulting in a breach of this protective barrier, thereby allowing luminal constituents such as bacterial products to enter the lamina propria and initiate an inflammatory response. In this context, it is

interesting to note a report that administration of superoxide dismutase attentuates some of the characteristic tissue damage of CD (84).

DRUG THERAPY

Steroids - For most physicians corticosteroids are the first therapeutic approach for IBD. Even though their mechanism of action in IBD has not been firmly established, the ability to inhibit leukocyte and macrophage migration and stabilize lysosomal membranes plays an important role. Prednisolone 1 reduces levels of PGE_2, $PGF_{2\alpha}$ and LTB4 in colonic tissue (56). The side effects of the oral corticosteroids has prompted the study of poorly absorbed preparations such as budesonide 2 administered by enema which gives good results for diseases in the distal colon (85).



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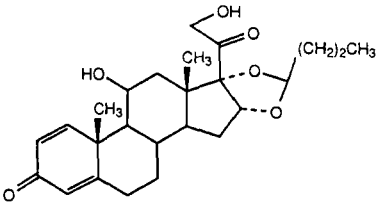

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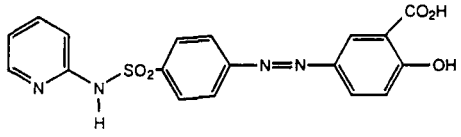
5-Aminosalicylic Acid (5-ASA) and Prodrugs - Sulfasalazine 3 was shown in 1942 to be effective for UC (86), and for forty years was the main-stay therapy for UC. The azo bond is reductively cleaved by the bacterial enzyme, azoreductase, in the colon to sulfapyridine 4 and 5-ASA 5 (87). The identification of 5-ASA as the active component has prompted a number of slow release oral and enema formulations of 5 which show good clinical response (88-91). Other prodrugs were developed the first being olsalazine 6, the dimer of 5-ASA (92,93) which is split by azoreductase to two molecules of 5-ASA (94). 6 is as effective as 5 clinically and is much better tolerated (95-97). 5-ASA has also been bound via the azo bond to a polyethylene polymer (98) and to inert moieties, as in ipsalazide 7 and balsalazide 8. These agents which cleave to 5-ASA are being evaluated clinically (99). Even though 5-ASA is clearly effective in UC, its mechanism of action is not confirmed. A number of theories have been proposed, e.g. alterations of gut flora (100) or reduction in B cell population (101). The current popular mechanism involves inhibition of cyclooxygenase and reduction of leukotriene release from inflamed colonic tissue (102,103).



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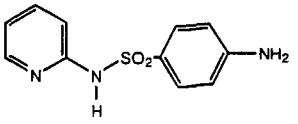

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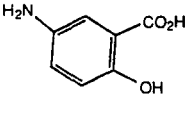

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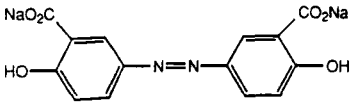

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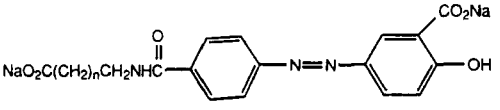

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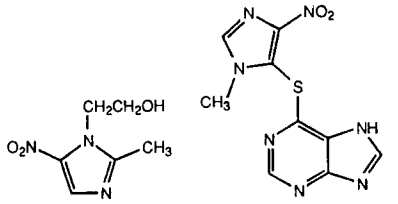
8 n=1

Antibacterial agents - The speculation that anaerobic bacteria are an important component of IBD has prompted the study of antibacterial

agents such as metronidazole 9. A number of studies, have been conducted with mixed results (104), with the most recent study showing intravenous 9 for 5 days was ineffective (105).

Immunosuppressives - The role of immunosuppressive therapy in IBD introduced in the 1960's has been controversial since its inception. Azathioprine 10 and its metabolite 6-mercapto-purine 11 are accepted by many gastroenterologists as having therapeutic value in CD, acting as "steroid sparing agents." Clinical trials in UC have shown mixed results. The immunosuppressive action of these agents require therapy for at least a month which may account for the poor clinical results (104,106). Cyclosporin has been shown to be beneficial for IBD patients who respond poorly to corticosteroids (107) but when added to corticosteroid therapy shows no further improvement (108). Cyclosporin given IV may be more effective than the oral route in severe Crohn's patients (109).



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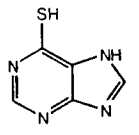

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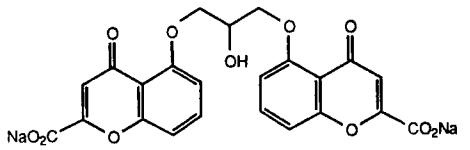

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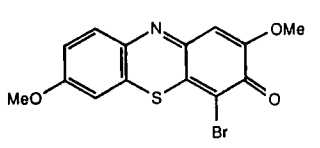

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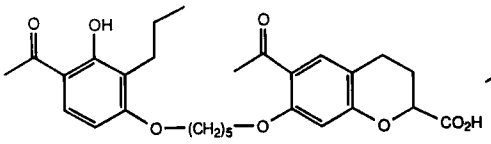

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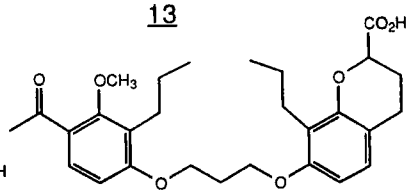

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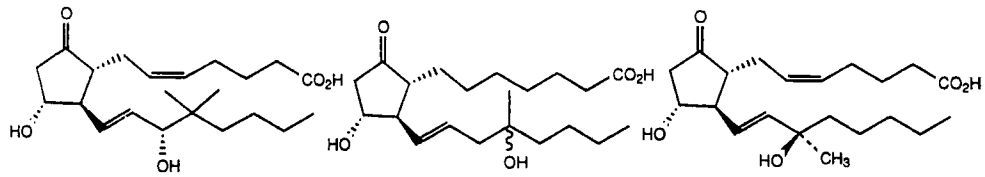
Disodium Chromoglycate - Disodium chromoglycate 12 has been reported to be active clinically in the treatment of UC (110). However, several trials indicate it to be less effective than sulfasalazine 4 or placebo (111).

Newer Therapies - The role of inflammatory mediators in IBD has prompted the introduction of a number of new agents designed to control their action. The 5-lipoxygenase inhibitor, L-651,392 13, which reduced levels of all leukotrienes was effective in the TNBS rat model (33). Similarly the nonselective leukotriene antagonist, ablucast (Ro 23-3544) 14 was effective in the acetic acid rat model (112). The activity of the LTB_4 selective antagonist, SC-41930 15 in the guinea pig model is suggestive of a major role of LTB_4 in IBD (113).

Increased levels of E prostaglandins have been associated with IBD (15,49) and may be related to a hyperemia caused by vasodilation (114) or other factors. It is therefore surprising that several prostaglandin analogs showed activity in colitis models, e.g. 16,16 dimethyl prostaglandin E_2 16 prevented ethanol-induced colitis (115) and misoprostol 17

was effective in the rat-acetic acid model (116). Oral administration of 15-(R)-15 methyl prostaglandin E_2 18 failed to confirm this positive effect clinically (117).



16 17 18

Conclusion - It is clear that a need still exists for more therapeutically efficacious agents for the treatment of IBD, including CD and UC. The clinical evluation of potent and selective 5-LO inhibitors and LTB_4 antagonists in inflammatory disease, will determine whether these newer agents will find a niche in the physicians' therapeutic armamentarium of drugs to alleviate the symptoms associated with these debilitating diseases.

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Chapter 19. Advances in Dermatology

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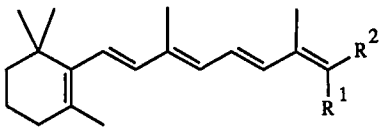
Introduction - Dermatologic research continues to be an exciting and expanding field. Molecular biological tools are better defining markers for dermatoses, and isolated human cell types from normal and disease-states are being utilized to study drug, hormone, and cytokine action. This chapter emphasizes therapeutic advances in acne, psoriasis, and cutaneous inflammatory disorders since the last report on this topic (1). Other new areas of interest include the dermatologic manifestations and therapeutic complications associated with immunocompromised AIDS patients, and the treatment and prevention of photoaged skin (2-4). Other chapters in this volume pertinent to dermatology include ones on alopecia, phospholipase A_2, and wound healing.

ACNE

Known factors in the pathogenesis of acne include desquamation of epithelium blocking sebaceous follicles colonized by *P. acnes*, the action of androgens, and stimulation of sebaceous gland sebum secretion. For milder forms of acne, which may be inflammatory, topical benzoyl peroxide (BP), an antibacterial and oxidizing agent, or topical (erythromycin (EM) or clindamycin phosphate (CP)) or oral (tetracyclines or EM) antibiotics are usually effective (5).

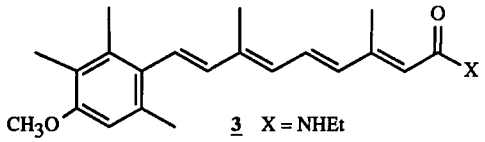
Retinoids - Retinoids continue to be important therapies and synthesis of third generation arotinoids will hopefully lead to effective, safer drugs with expanded indications (6). The first generation isotretinoin (13-*cis*-retinoic acid, **1**) remains the main oral therapy for severe, recalcitrant cystic acne and often results in complete, extended remission. Retinoid side effects continue to be problematic. These include mucocutaneous, opthalmic, musculoskeletal, lipid, hepatic, and teratogenic effects; recommendations for patients, particularly contraception for women of child-bearing age, have been re-emphasized (7,8). In addition to action on sebum secretion and comedogenesis, **1** antagonizes testosterone action on sebaceous glands and inhibits leukocyte chemotactic response, suggesting possible therapeutic effects in other inflammatory or androgen-associated dermatoses (9,10). Topical **1** or tretinoin (all-*trans*-retinoic acid, **2**) are efficacious in moderate acne, **2** acting by increasing turnover and shedding of follicular epithelial cells (11). Combination therapies, such as **2** and EM may be more efficacious than retinoids alone (12). Newly elucidated actions of retinoids include increased inositol phosphate (IP) turnover in cultured epidermal keratinocytes by **1** and **2** (13). Rabbit ear comedone and cultured human sebocyte proliferation assays, effectively assess activity of these retinoids (14-16). Motretinide (**3**) is topically less irritating, but is less efficacious than **2** for acne (17). Temarotene (Ro 15-0778, **4**) suppressed sebum in rodent models, but clinically **4** decreased sebum secretion only at a high dose and was not active in cultured human sebocyte proliferation assay (16,18,19). Arotinoid ethyl ester, Ro 13-6298 (**5**) suppressed sebum secretion in a hamster model but was not clinically effective in acne, and sulfone Ro 15-1570 (**6**) decreased hamster sebaceous gland size (20). Anthracene derivative (**7**) exhibited potent retinoid activity, preventing induction of ornithine decarboxylase (ODC) activity (21). BMY-30047 (**8**) decreased utriculi size in rhino mice and phorbol ester-induced ODC activity with no side-effects (22).

In addition to its action in acne, topical **2** demonstrated both histological improvement (increased skin thickness and granular layer) in animal studies and clinical improvement (decreased fine wrinkles) in photoaged skin (4,23,24).



1 $R^1 = CO_2H$, $R^2 = H$

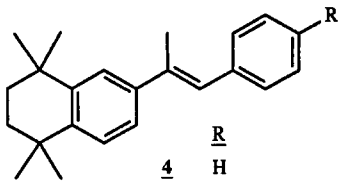
2 $R^1 = H$, $R^2 = CO_2H$



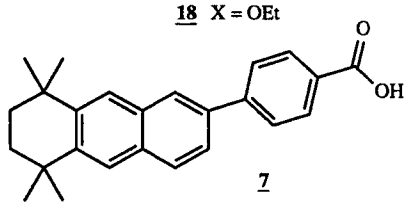
3 X = NHEt

17 X = OH

18 X = OEt



R	
4	H
5	CO_2Et
6	SO_2Et
19	CO_2H



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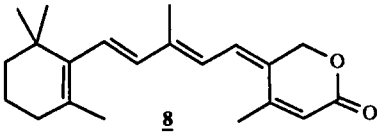

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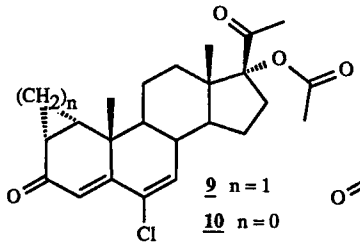
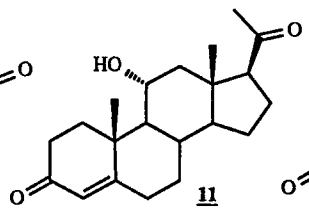
Hormonal/Other - In as much as androgens stimulate sebum secretion and development of sebaceous glands, hormonal anti-acne therapies revolve around inhibitors of androgen receptors, 5α-reductase, or androgen biosynthesis (25). Low concentration synergy between 5α-reductase (progesterone) and androgen receptor (spironolactone (SL); cyproterone acetate, 9) inhibitors was demonstrated in a hamster sebaceous gland model (26). In female antibiotic non-responders, systemic 9, combined 9/ethinyl estradiol, or chlormadinone acetate (10) were effective in the treatment of acne (27,28). Oral SL resulted in significant improvement of acne, but a majority of patients had side-effects (29). Topical 11α-hydroxyprogesterone (11) had some beneficial effects in male acne patients (28). The antiandrogen, 17α-propylmesterolone (12), was shown to be topically active, clinically reducing comedones, sebum secretion rate, and epidermal lipids (30). Epitestosterone (13), a 5α-reductase inhibitor, prevented testosterone and dihydrotestosterone activity in sebaceous glands of hamster flank organ model (31). Luteinizing hormone-releasing hormone analog, buserelin was effective in treatment of acne but had hypoestrogenic effects (32). Azelaic acid and zinc sulfate individually inhibited 5α-reductase activity *in vitro* in human skin, and together their inhibitory effect was additive (33). Clinically, azelaic acid was topically active in the treatment of acne,



9 n = 1

10 n = 0



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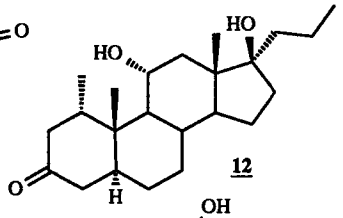

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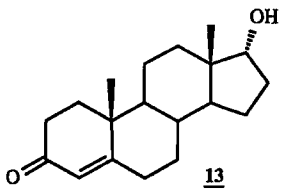

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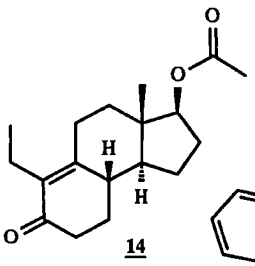

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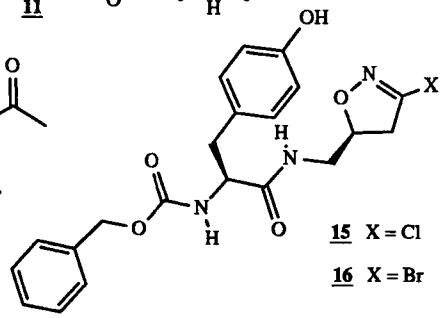


15 X = Cl

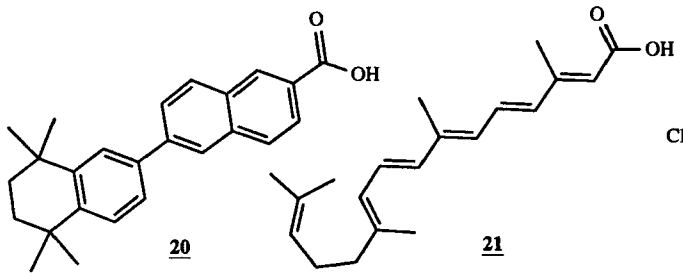
16 X = Br

similar to BP in effectiveness (34). Inocoterone acetate (**14**), a des-A-steroid antiandrogen, was active in rodent and quail uropygial gland models and is presently in clinical trial to assess its effect on sebum secretion (35). Transglutaminase (TG), both membrane and soluble forms present in epidermal layer of skin, may be involved in various dermatoses including acne (36). Haloisoxazoles **15** and **16** potently inhibited epidermal TG and were additive in effect with retinoic acid (36,37).

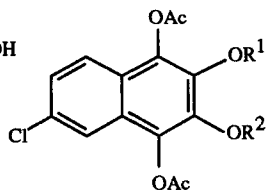
PSORIASIS

Insight into the pathogenesis of psoriasis is evolving towards a better understanding of the major components of the lesion, namely, dermal/epidermal inflammation, acanthosis, altered keratinocyte differentiation and absence of the granular layer (38-40). Dermal infiltration of PMN leukocytes has been linked to numerous chemotactic factors including LTB_4 and 12-HETE (41), IL-1-like proteins and complement derived $C5a_{des\ arg}$ (42,43). IL-1 also induces hyperproliferation (44). Epidermis synthesizes and has receptors for IL-1 (α and β), which in turn enhance granulocyte macrophage colony stimulating factor (GM-CSF) expression suggesting an autocrine role for this cytokine (45). Altered β-adrenergic responsiveness, resulting from a defect of the adenylate cyclase (AC) system at the hormone receptor-G protein interaction, may be linked to hyperproliferation (46). Forskolin normalized response to β-agonists in psoriatic and TPA-dosed skin (47). Enhanced expression of *ras* gene product *ras* p21, which is homologous to GTP-binding protein, provides further support for a link between psoriasis and the AC system (48). Dysfunction in the "skin immune system" is implicated as a major etiological concern (49). Levels of a calpain, localized in human skin, appear to be deficient in psoriasis (50). Evidence implicating protein kinase C (PKC) in psoriasis is mounting (51). Two distinct PKC isozymes, types II(β) and III(γ), were found in psoriatic epidermis (52). Two keratinocyte-derived membrane-associated PKC subspecies are undergoing characterization (53). An activated phospholipase C (PLC)/PKC signal is implicated by a 2-3 fold increase in diacylglycerol (DAG) levels (54). PKC levels are diminished in psoriatic epidermis presumably via down-regulation by excess DAG (55). In contrast, psoriatic dermal fibroblasts possess enhanced PKC activity (56) and increased sensitivity to platelet derived growth factor (PDGF), again suggesting a dermal component in psoriasis (57). PKC regulates terminal differentiation in human keratinocytes (58). Psoriatic lesions contain both increased epidermal transglutaminase (TG) and resultant cornified envelopes (59). Epidermal growth factor (EGF) receptor distribution is altered and [^{125}I]EGF binding is enhanced in upper epidermal layers (60). Growth promoting transforming growth factor-α (TGF-α) is overexpressed in psoriatic skin, while inhibitory TGF-β1 levels are normal, implicating TGF-α in epidermal hyperplasia (61).

Retinoids - Free acid acitretin (**17**), with its much shorter $t_{1/2}$, appears to be a suitable replacement for etretinate (**18**) clinically (62). New indications for **18** in other hyperkeratotic dermatoses (63) and combination therapies for psoriasis, e.g. PUVA with retinoids, are being examined (64). Arotinoid ester **5** demonstrated antipsoriatic efficacy (63). Other arotinoids in clinic include acid Ro 13-7410 (**19**), lacking mucocutaneous toxicity (65), and sulfone **6** (19), lacking skeletal toxicity (66). TTNN (**20**) possesses topical antiinflammatory activity comparable to **19** in guinea pig (gp) UV-erythma, rat



20 **21**

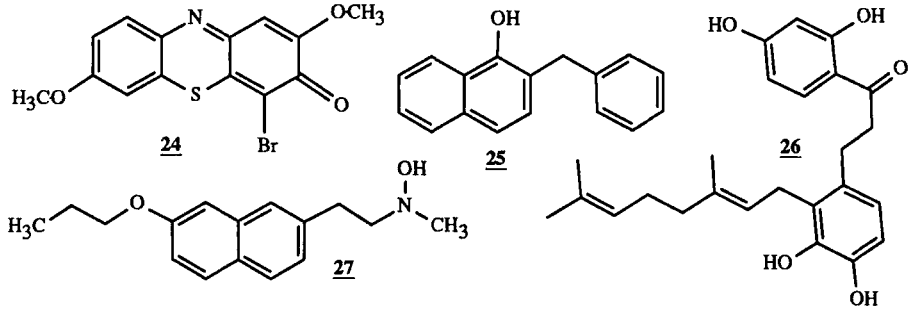


22 $R^1, R^2 = CH_3$

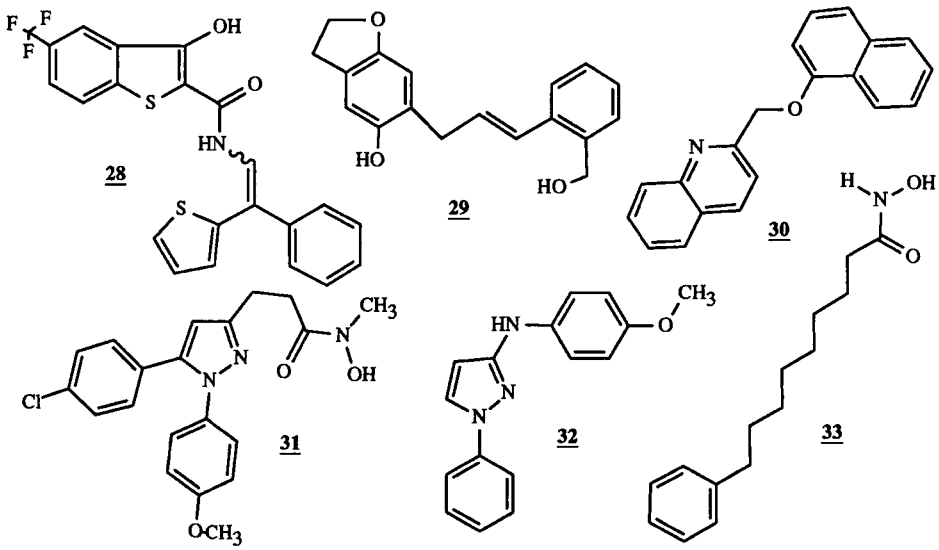
23 $R^1, R^2 = (CH_2)_n$

croton oil-ear and murine arachidonic acid (AA)-ear edema assays (67). E-5166 (21) inhibited EGF or TPA-induced AA release and IP turnover in pig epidermis (68).

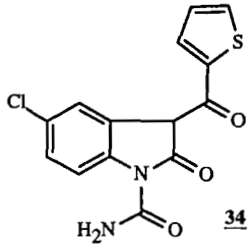
5-LO Inhibitors - Considerable effort is underway to evaluate the therapeutic potential of specific 5-lipoxygenase (LO) inhibitors. Topical lonapalene (22) produced significant reduction in lesional LTB_4 levels in plaque psoriasis without affecting 12-HETE or AA levels (69). Cyclic ether analogues 23 (n=1-3) were less potent in the AA-ear edema assay used to infer clinical potential (70). Topical NDGA attenuated the effects of TPA, i.e. induction of ODC activity, and tumor initiation and promotion, in murine skin (71). Topical NDGA, however, was not effective in stable plaque psoriasis (72). L-651,392 (24) topically inhibited A23187-induced gp ear hyperproliferation (73). Topical DUP-654 (25), which potently inhibited AA-ear edema (11 μg/ear) and moderately attenuated TPA-ear edema (700 μg/ear), is a clinical candidate (74). Natural product AC-5-1 (26) exhibited potent AA-ear edema reduction (75). QA-208,199 (27) inhibits 5-, 12-, and 15-LO in human psoriatic scale homogenate (76).



Dual 5-LO/CO Inhibitors - Oral L-652,343 (28) reduced PGD_2/PGE_2 levels but lacked 5-LO inhibitory effects in stable plaque psoriatics (77). Topical L-651,896 (29), which inhibited 5-LO, CO and hyperproliferation in the A23187-gp ear model (73), failed to abate PG levels or edema in murine oxazolone-hypersensitivity (78). Topical WY-47,288 (30) displayed dermal antiinflammatory properties in several models (AA-ear edema: 0.2 mg/ear) (79). Tepoxalin (31), displaying potent topical activity in both AA-(54 μg/ear) and TPA-ear edema (80 μg/ear), is in clinic (80). Similarly, clinical candidate FPL-62064 (32) topically reduced AA-(41 μg/ear) and TPA-ear edema (250 μg/ear) (81). Topical BMY-30094 (33) has been selected for clinical evaluation in psoriasis (82).



IL-1 Inhibitors - With the recent findings implicating IL-1 involvement in the inflammatory components of the lesion, IL-1 inhibitors, such as CP-66,248 (34) (83,84), SKF 86,002 (35) (85) and E-5110 (36) (86), endogenous (epidermal cell) EC- and serum-contra IL-1 proteins (87) and pentoxifylline (88), offer a new therapeutic approach for the treatment of psoriasis.



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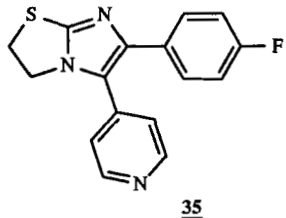

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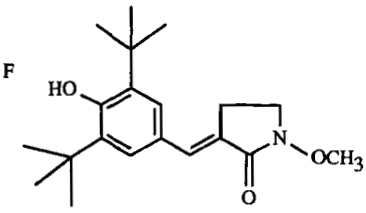
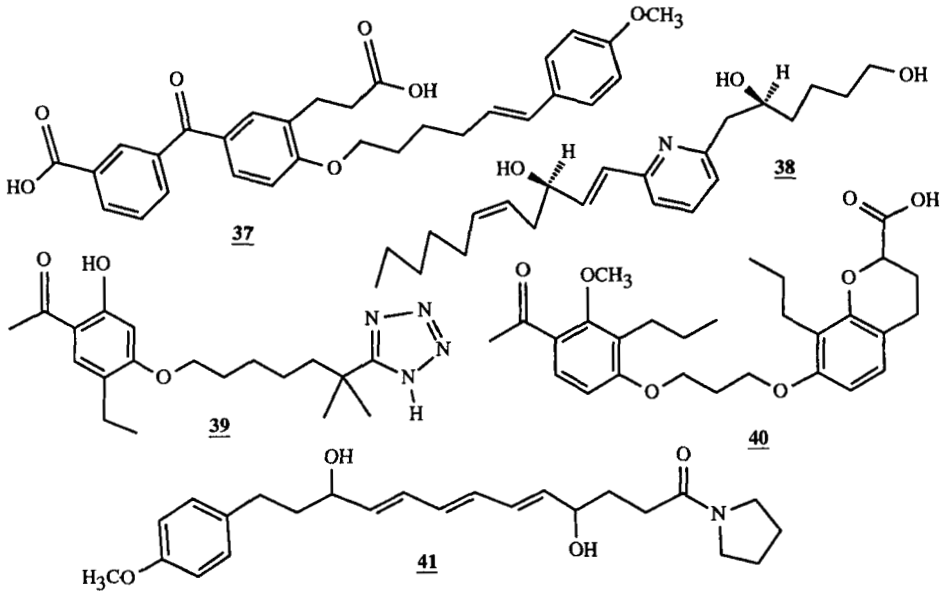


36

LTB_4 Antagonists/15-HETE/12-HETE - LTB_4 antagonists, exemplified by LY-223,982 (37) which has been selected for a clinical study in psoriasis (89), are the latest class of eicosanoid modulators to be evaluated. Other new members of this class include U-75,302 (38) (90), LY-255,283 (39) (91), SC-41930 (40) (92) and SM-9064 (41) (93). The correlation of enhanced 15-HETE levels with decreased LTB_4 levels in human PMNs by 22, NDGA, and 34 further supports the hypothesized endogenous regulatory role of 15-HETE on 5-LO (94). 12(R)-HETE, and not 12(S)-HETE as found in platelets, has been established as the product of 12-LO in psoriatic lesions (95). 12(R)-HETE induced dose dependent chemotaxis of human lymphocytes, while 12(S)-HETE was markedly weaker (96). Neither 12(RS)- nor 12(S)-HETE could stimulate DNA synthesis in human keratinocytes (97). Remarkably, 12(S)- and 12(RS)-HETE, but not 12(R)-HETE, significantly inhibited AA- and TPA-ear edema (98).

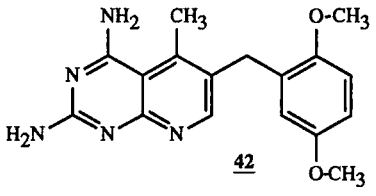


37 38 39 40 41

Folate antagonists/Immunomodulators - Oral methotrexate (MTX) inhibits human C5a-induced inflammatory skin responses in psoriatics (99). Lipid soluble folate antagonist piritrexim (42), with a potential for reduced chronic hepatoxicity over MTX, was effective in a short term trial in severe chronic plaque psoriasis (100). Oral 5-fluorouracil showed good clinical efficacy in refractory subjects (101). Clinical and mechanistic studies with cyclosporin A (CSA) have been reviewed. PMN chemotaxis, TPA-induced inflammation, and ODC gene expression are all inhibited by CSA (102).

Contrary to previous speculation (1), CSA does not appear to be a calmodulin antagonist (103). Unfortunately, topical CSA was ineffective in a pilot study (104). Zidovudine (AZT) therapy improved psoriatic lesions in HIV-positive patients (105).

Calcium Regulators - Calcium plays a major role in regulating epidermal keratinocyte hyperproliferation and terminal differentiation (106). A calcium modulator, a parathyroid hormone-like protein having AC stimulating activity, is encoded in human skin (106). Calmodulin antagonist fendiline (**43**) inhibits human keratinocyte proliferation (107). Metabolite 1α,25-dihydroxyvitamin D_3 (**44**) inhibits proliferation and stimulates terminal differentiation of human keratinocytes (108) and was effective both orally and topically in preliminary trials in psoriasis (109). Topical TV-02 (**45**) appears to be equiactive to **44** clinically (110). Topical MC-903 (**46**), which appears to be non-calciotropic (111), also produced efficacy in a double blind study (112). Both **44** and **46** have an IL-1 inhibitory component (113).



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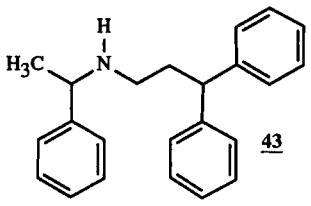

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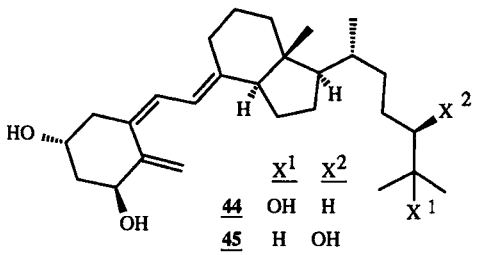
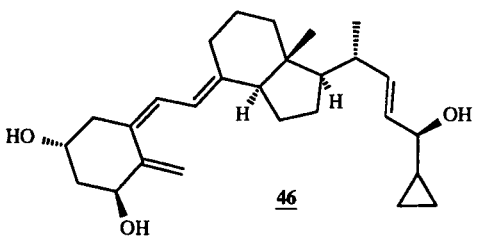
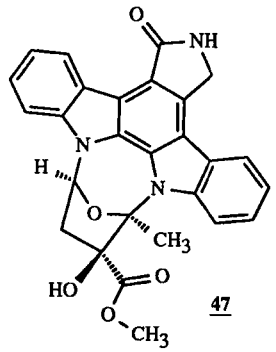


	X^1	X^2
44	OH	H
45	H	OH



46

Protein Kinase-C Inhibitors - PKC inhibitor K-252a (**47**) produces a host of antiinflammatory and antiallergic effects. While having no apparent CO, 5-LO or PLA_2 inhibitory activity, **47** was orally active in PCA, carrageenin-, PAF- and zymosan-induced paw edema, and croton oil-ear edema in rats (114). Sphingosine (**48**) inhibits TPA-induced inflammation, ODC activity and activation of PKC in hairless mice (115). **48** also regulates EGF receptor phosphorylation (116). H-7 (**49**) and staurosporine (**50**) prevent PKC activation and inhibit TG activity in human keratinocytes (58). **50** attenuates tumor promotion and ODC induction but not hyperplasia by TPA in CD-1 mouse skin (117). PKC inhibitors, **50** and quercitin, also displayed antiproliferative effects on Walker carcinoma cells (118).



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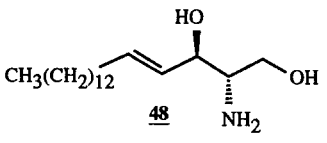

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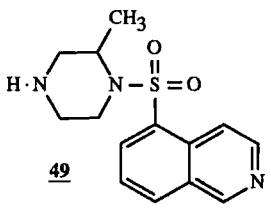

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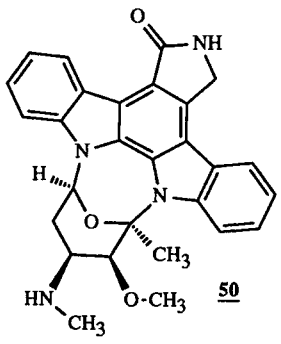


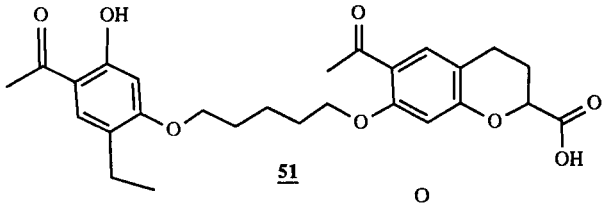
50

ACUTE INFLAMMATION

The search continues for safer topical corticosteroids, the mainstay of therapy for most forms of dermatitis (119). A new hairless mouse model addresses the atrophogenic effects which plague chronic steroidal therapy (120). Several excellent overviews of inflammatory mediators and their role in dermatoses have appeared (121-23). A review of PGD_2 in the skin (124) and a study contrasting the effects of PAF-acether and LTB_4 in human skin (125) are noteworthy.

Contact Dermatitis/Urticaria - Contact dermatitis (CD)(126) and urticaria (127) were reviewed. The dermatologic potential of cimetidine was delineated (128). Topical LTD_4/E_4 antagonist, Ro 23-3544 (**51**), was only modestly effective in murine allergic CD (DNFB-ear) and exacerbated irritant CD (croton oil-ear) (129). Oral PAF-antagonist ginkgolide B (BN-52021) was more effective than **51** in DNFB-ear edema but was inactive in croton oil-ear edema (130). Arotinoid **19** displayed potent antiinflammatory activity in a delayed hypersensitivity model (131).

Atopic Dermatitis (AD) - Three reviews, including one on cyclic nucleotide metabolism in AD, appeared (132-4). Human atopic basophils possess altered histamine "releasability" which is subject to endogenous regulation by PGE_2 (135). Evidence supporting elevated monocyte cAMP-phosphodiesterase (PDE) as a marker of AD was reviewed (136). Elevated cAMP-dependent protein kinase (PKA) and lower PKC activity in AD monocytes are suspected to be linked to this PDE activity via an H_1-histamine-associated desensitization of PKC (137). Sequifenadine (**52**), an H_1-blocker with a serotonin antagonist component, has efficacy in allergic CD, urticaria and AD (138). Topical β_2-agonist salbutamol, although active in the croton oil-ear (rat), was ineffective clinically in AD (139). Chloroquin (140) and CSA (141) were effective in small studies. Modest improvement was associated with dietary eicosapentaenoic acid supplementation (142).



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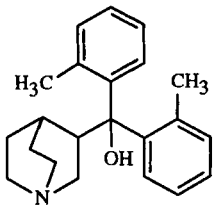

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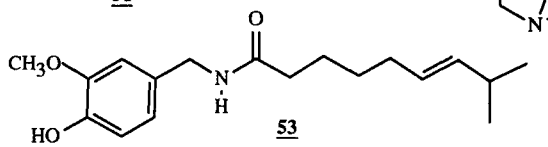

53

Inflammatory Neuronal Link - The neurogenic component, via substance P (SP), neurokinin A and calcitonin gene-related peptide (CGRP), of many dermatoses is being investigated (143). SP depletor capsaicin (**53**) produced some interesting effects on inflammatory reactions in humans (144). Topical **53** has proven effective in both chronic and oral post herpetic neuralgia (145). SP also appears to be associated with histamine release in human skin mast cells (146) and with psoriatic plaque innervation (147).

CONCLUSION

The field of dermatology has witnessed an explosion of information on the role of cytokines and inflammatory mediators in cutaneous inflammation and hyperproliferation. The potential therapeutic use for these factors and new agents which may influence the metabolism or action of endogenous regulators is being vigorously pursued. Future dermatologic research promises more rapid elucidation of the pathogenesis of skin disorders. New biochemical markers and the neuronal aspects of various dermatoses offer new and exciting avenues of medicinal research.

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Chapter 20. Pathogenesis and Treatment of Alopecias

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Introduction - Alopecia (loss of hair) occurs in some form in almost all humans who live to a normal life expectancy. Children as well as adults and females as well as males can suffer hair loss. Alopecia can occur anywhere on the body, with the most visible types involving the scalp, eyebrows, or, in males, the beard. While alopecia is clearly not life threatening, the rapid onset of alopecia of the scalp can be exceedingly traumatic, particularly in children and young adults (1).

Alopecias can be broadly divided into two types: scarring (cicatricial) and nonscarring (noncicatricial). Scarring alopecias are seen as the result of a number of diseases including discoid lupus erythematosus, scleroderma, and lichen planopilaris. These alopecias involve destruction of the hair follicles, and consequently, regrowth of hair is not possible. Scarring alopecias will not be further discussed here, but are reviewed in most dermatology textbooks (2-5).

In contrast to scarring alopecias, nonscarring alopecias generally do not lead to permanent damage to the hair follicle. Hair regrowth is theoretically possible and frequently occurs spontaneously, or can be stimulated by oral or topical administration of a medicinal agent. Some nonscarring alopecias are the result of stress (telogen effluvium), fungal infections (tinea capitis), hair pulling (trichotillomania), or certain hair styles (traction alopecia). In these cases, hair regrowth usually occurs when the identified causes are treated or abated. More detailed discussions of these alopecias are also found in dermatology textbooks (2-5), and they will not be covered further in this review.

Alopecia areata and androgenetic alopecia ("male-pattern baldness") are two common nonscarring alopecias which are due to fundamental changes in the functioning of the hair follicles. These alopecias are not treated as easily as the previous examples, but hair regrowth can be stimulated with drug therapy. The purpose of this review is to summarize the pathogenesis and treatment of alopecia areata and androgenetic alopecia. Since claims of efficacy in hair growth stimulation often tend to be exaggerated or unscientifically documented, a certain degree of cautious skepticism is necessary on the part of anyone attempting to review this area. Therefore, this review will include only those agents for which there is supporting data in scientific, peer-reviewed journals.

Hair Growth Cycle - Both alopecia areata and androgenetic alopecia involve a perturbation of the normal hair growth cycle. The hair growth cycle is composed of a growing phase (anagen), a declining phase (catagen) and a resting phase (telogen) (6-8). In normal human scalp hair, the anagen phase of the cycle generally lasts for at least three years, the catagen phase lasts three weeks, and the telogen phase lasts for three months. Consequently, approximately 84% of scalp hair follicles are in anagen, while 2% are in catagen and 14% are in telogen at any given time (9). Alopecia areata and androgenetic alopecia are characterized

by a much higher percentage of telogen hair follicles, and loss of hair from those follicles. In order for a drug to induce the visible regrowth of hair, it must stimulate entry of the telogen follicles into a sustained anagen phase.

Models of Alopecia and Human Hair Growth - The search for new agents which stimulate hair growth is hampered by the lack of a rapid and reliable assay for this activity. No animal model for alopecia areata has been described in the literature. Scalp skin sections from humans with alopecia areata have been successfully grafted onto nude mice, but these sections do not retain their hair growth defect (10).

The stump-tailed macaque monkey is currently the best available model for human androgenetic alopecia (11,12). Alopecia is seen in both sexes of this monkey beginning at puberty, and histological evidence indicates that balding occurs in a fashion analogous to humans. Unfortunately, the availability of these animals is quite limited, and the study of each experimental drug requires months or years for accurate data collection. Although androgen dependent hair loss in rodents is rare, a strain of mice has been reported in which both males and females show hair loss upon administration of testosterone or dihydrotestosterone (13). Unlike human alopecia however, normal hair growth resumes when androgen treatment is discontinued. Data on the effect of hair growth promoters can be obtained from this model in 8-12 weeks. Hairless mice have been proposed as models for the assay of hair growth promoters, although there appears to be little relationship between this hair growth defect and human androgenetic alopecia (14).

Recent publications have appeared describing the culture of human hair follicle keratinocytes (15) and whole follicles from murine coat hair (16) and vibrissae (17). Normal human scalp skin has been grafted onto nude mice (18), and the associated follicles appear to remain viable. These systems may provide the basis for *in vitro* screens for hair growth activity if their relationships to human hair growth and alopecia can be defined.

Because of the lack of suitable models, the compounds described in this review have largely been evaluated directly in human volunteers.

ALOPECIA AREATA

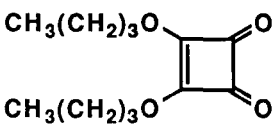
Description - Alopecia areata is characterized by the loss of hair in sharply defined circular or oval patches (7). Hair loss occurs commonly on the scalp, but can occur anywhere on the body. Patchy alopecia areata may progress to total loss of hair on the scalp (alopecia totalis) and even to total loss of all body hair (alopecia universalis). The disease occurs in both adults and children, males and females, and can be short-lived or last for the individual's lifetime. The course of the disease is unpredictable, with frequent spontaneous remissions and hair regrowth followed by new episodes of hair loss.

Upon histologic examination, scalp biopsies from areas of active alopecia areata are seen to contain an unusually high percentage of telogen follicles (19). It appears that in the initial stages of areata, affected follicles rapidly and synchronously cascade into a telogen state. In chronic cases, most of the follicles appear to be in an anagen state, but they contain only small, poorly formed hairs. It has been postulated that these follicles enter early anagen in a normal fashion, but then return to the "safe" telogen state before substantial hair growth can occur (19).

Although the physiologic basis for alopecia areata is poorly understood, an immunologic disturbance is thought to be associated with the disease (7). Histologically, hair follicles in an active area of the disease show dramatic perifollicular infiltration of T-lymphocytes (8). The vast majority of these T-lymphocytes have been shown to be of the helper-inducer phenotype, with the remainder being of the suppressor-cytotoxic phenotype (20,21). Follicles in active areas of the disease also display class I and II human leukocyte antigens (HLA), although there is considerable divergence of experimental data in this area (22,23). Recently it has been shown that HLA expression is secondary to the lymphocyte infiltration, and not apparently the cause of the infiltrate (24).

Treatment - Corticosteroids have frequently been used for the treatment of alopecia areata. Topical application and intralesional injection appear to be the safest and most efficacious routes of administration. The use of corticosteroids has recently been reviewed (25) and will not be covered in detail here. In general, their effectiveness is limited to cases of patchy alopecia areata.

A number of contact allergens have been found to stimulate hair regrowth in alopecia areata. The treatment regimen involves a preliminary sensitization with the allergen, followed by weekly topical applications at a lower dose. Dinitrochlorobenzene (DNCB) was found to stimulate some hair regrowth in a majority of the cases in which it was tested (26). However, the percentage of cases in which hair regrowth was complete or "cosmetically acceptable" was much lower. Since the report that DNCB was mutagenic in the Ames assay (27), its use has been essentially discontinued. Squaric acid dibutyl ester (SADBE, 1) and diphencyprone (DCP, 2) are not mutagenic in the Ames assay, and have been shown to stimulate regrowth of hair in roughly the same percentage of cases as DNCB (26,28-30). DCP has been preferred because it is reported to be more stable than SADBE in acetone solution.



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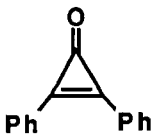

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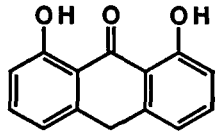

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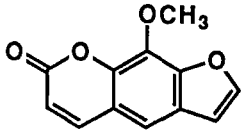
It is thought that these contact allergens exert their effect by the induction of a delayed hypersensitivity reaction, which in turn suppresses the response to another unrelated antigen ("antigenic competition") (31). Topical application of contact allergens in alopecia areata has been found to reduce HLA expression in the epithelium of hair follicles (23) and to increase the fraction of suppressor-cytotoxic T-cells relative to helper-inducer T-cells (31,32). There is some disagreement in this area, since it has also been reported that no changes in T-lymphocyte sub-populations occur upon topical immunotherapy (33).

Contact allergens induce an allergic contact dermatitis as part of their mode of action and the effectiveness of the agent appears to be related to the degree of dermatitis which can be induced and sustained (26). For this reason, contact irritants which are devoid of allergic properties have been investigated as treatments for alopecia areata. Topical anthralin (3, 0.5% to 1% creams) was found to induce cosmetically acceptable hair regrowth in 17 of 68 patients having severe alopecia areata (34). Other contact irritants such as croton oil and sodium lauryl sulphate do not stimulate hair growth (35).

Alopecia areata has also been treated with PUVA therapy which combines administration of topical or oral psoralens with UVA irradiation (320-400 nM). In PUVA studies using 8-methoxypsoralen (8-MOP, 4), hair regrowth was seen in 35-65% of the treated individuals (36). The psoralens have been found to form [2+2] photoadducts with DNA and can cross-link DNA (37). Whether this phenomenon is related to their activity in alopecia areata is unclear. It has been suggested that PUVA therapy causes an increase in the relative numbers of suppressor-cytotoxic lymphocytes present in the perifollicular lymphocytic infiltrate (36). In this respect, PUVA seems to be operating by a mechanism analogous to the contact allergens previously discussed.



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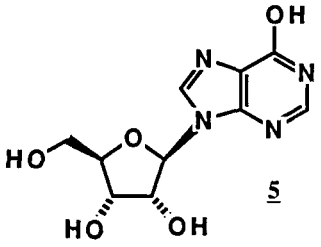

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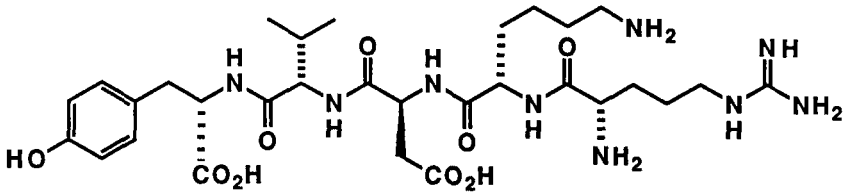
Inosiplex (a complex of inosine, 5, with 1-dimethylamino-2-propanol and 4-acetamidobenzoic acid) and thymopentin (6) are immunostimulants which have been used to treat alopecia areata. Oral inosiplex (50 mg/kg/day) induced hair regrowth in 11 of 25 patients with alopecia totalis (38). Intravenous administration of thymopentin (50 mg, three times per week for three weeks) in patients having severe alopecia stimulated hair growth with an efficacy comparable to that of topical SADBE or DCP (39). Both inosiplex and thymopentin are thought to induce T-cell proliferation and differentiation.



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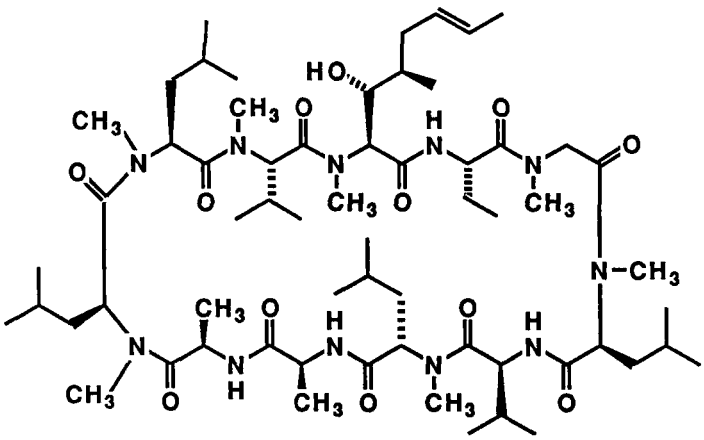

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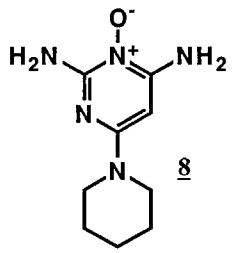
Cyclosporin A (7) is an immunosuppressant which frequently causes hypertrichosis in humans (40) and stimulates hair growth in nude mice (41). While topical cyclosporin A (5% or 10% solutions) stimulated some hair regrowth in patients with severe alopecia areata, it was not considered to be therapeutically useful (42,43). Oral cyclosporin A (6 mg/kg/day) has been reported to stimulate excellent regrowth of hair in one case of patchy alopecia (44). While no side effects were observed in the topical studies, the nephrotoxicity associated with systemic cyclosporin A precludes routine oral administration (45).

Daily topical applications of mechlorethamine (nitrogen mustard, 0.2% aqueous solutions) were found to induce cosmetically acceptable hair regrowth in 7 of 11 alopecia areata patients (46). Mechlorethamine may act as an immunomodulator, although its exact mode of action is unclear. No systemic side effects were observed with this treatment, but two patients developed allergic contact dermatitis. Since mechlorethamine is highly irritating to mucous membranes, its widespread use as a hair growth agent appears unlikely.

Minoxidil (8) has been used with variable success for the treatment of alopecia areata. The use of topical minoxidil in alopecia areata through 1986 has been previously reviewed (47). More recently, topical minoxidil applications (3% and 5% solutions) have been reported to lead to cosmetically acceptable hair regrowth in 13%-45% of cases of extensive alopecia areata, including alopecia totalis and alopecia universalis (48-52). Paradoxically, it has also been reported that 3% topical minoxidil is ineffective in these cases (53). Side effects of topical minoxidil were limited to mild local irritant dermatitis and rare occurrences of allergic contact dermatitis to either minoxidil or its vehicle (47-53). No clinically significant systemic or cardiovascular changes were seen in subjects treated with topical minoxidil. Hair growth response to oral minoxidil (5 mg every 12 hours) in patients with severe alopecia areata was similar to that seen with topical 5% minoxidil, although the time to response was shorter and the degree of response was greater (54). Regardless of the study, minoxidil was found to be more effective in cases of mild alopecia areata involving less than 75% of the scalp.



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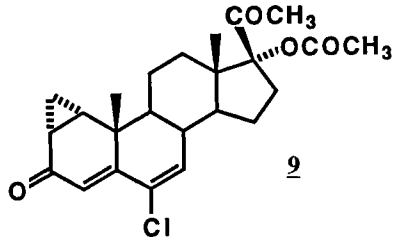

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Histological studies of alopecia areata patients who were responsive to minoxidil showed decreased counts of perifollicular lymphocytes following treatment (55). The changes differed from those seen in spontaneous regrowth and suggested that alopecia areata follicles responsive to minoxidil may be less "attractive" to lymphocytes.

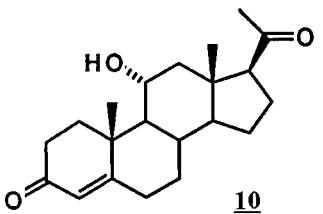
ANDROGENETIC ALOPECIA

Description - Although frequently referred to as male-pattern baldness, androgenetic alopecia occurs commonly in both males and females (8,56). In males, it is characterized by loss of hair in the frontal regions (receding hairline) and on the crown of the head. In females, a diffuse loss of hair occurs on the anterior scalp,

but the hairline usually does not recede (57,58). The presence of systemic androgens is a necessary condition for the occurrence of androgenetic alopecia, but it is obviously not a sufficient condition because not all men go bald (59). No correlation has been found between the quantity of circulating androgens and the degree of alopecia in either males or females (56,60). Androgenetic alopecia apparently involves undefined genetic factors which have been variously hypothesized to be elevated androgen metabolism (61,62), decreased levels of sex hormone binding globulins (63), or increased androgen binding in target tissues (64,65).

Histologically, androgenetic alopecia is characterized by a progressive miniaturization of terminal hair follicles, which is thought to be due to abnormal interruption of the anagen phase of the growth cycle (8). There is no reduction in the number of follicles in recently bald patches of scalp, but a higher than normal percentage are found to be in telogen (66). There seems to be no damage done to follicles early in the condition, although loss of follicles occurs with time. Unlike the spontaneous remissions of alopecia areata, regrowth of hair does not occur in androgenetic alopecia without treatment.

Treatment - Since androgens are implicated in the pathogenesis of alopecia areata, one form of treatment has involved the administration of antiandrogens which interfere in the receptor binding of testosterone or its metabolites. In females, oral spironolactone, cyproterone acetate (**9**), and cimetidine have been reported to result in some hair regrowth, presumably due to the antiandrogenic properties of these compounds (57,67,68). No major side effects were reported with oral cimetidine, but both spironolactone and cyproterone acetate may cause menstrual irregularity. In addition, spironolactone causes transient diuresis and cyproterone acetate can cause malaise, lassitude, and loss of libido (57). Oral antiandrogen therapy is inappropriate in males due to interference with androgen-mediated processes and concomitant side effects including impairment of spermatogenesis, loss of libido, and gynecomastia (69). Topical treatment of bald males with the antiandrogen 11α-hydroxyprogesterone (**10**) has been reported to increase both the number of anagen hair follicles and their mean hair shaft diameters, although none of the reported data were statistically significant (70). No side effects were described in this study.



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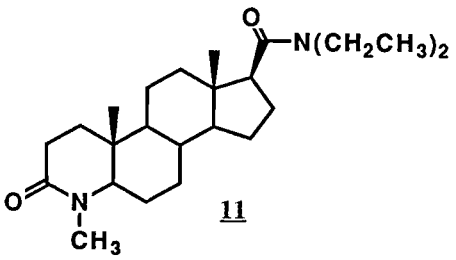
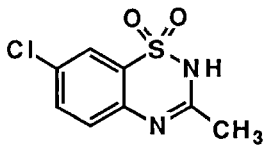


11

In contrast to androgen receptor blockade, interference with androgen metabolism may provide a useful approach to treatment or prevention of androgenetic alopecia in both males and females. Males having a deficiency in 5α-reductase (the enzyme responsible for conversion of testosterone to dihydrotestosterone) show normal postpubertal development in most respects, but they do not develop androgenetic alopecia (56). Topical administration of the 5α-reductase inhibitor 4-MA (**11**) to juvenile macaque monkeys for two years resulted in inhibition of hair loss (71). No studies of **11** in humans have been reported.

Topical application of progesterone (another 5α-reductase inhibitor) to human males has been found to neither stimulate hair growth nor prevent hair loss (56,69). No other data has appeared in the literature to support the use of 5α-reductase inhibitors to treat androgenetic alopecia, although these inhibitors are currently the focus of intense research efforts (72).

Other agents which are not involved with androgenic pathways have shown hair growth stimulating properties in androgenetic alopecia. Diazoxide (**12**), a potent vasodilator, stimulated visible regrowth of hair in 15 of 60 patients without visible side effects (73). Viprostol (**13**), another vasodilator, showed hair growth activity in a placebo-controlled study of 200 patients, but its efficacy was not significantly better than that of the placebo (73). Tretinoin (retinoic acid, **14**) was reported to stimulate hair regrowth, either alone or in combination with minoxidil (74). Topical cyclosporin A was found to be ineffective at promoting hair growth in a group of 11 patients with androgenetic alopecia (73).



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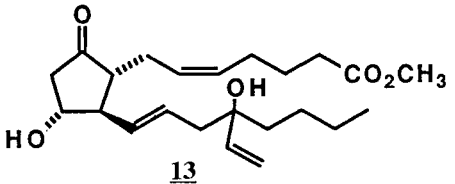

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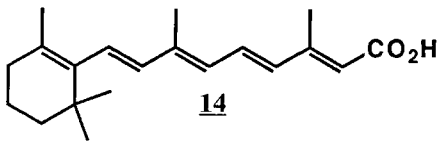
Topical minoxidil has received by far the greatest amount of study for the treatment of androgenetic alopecia, and several recent reviews have appeared (47,75-77) which summarize the results. In a large double blind study involving over 2300 subjects, it was found that twice daily topical applications of minoxidil (2% or 3% solution) for one year led to moderate or dense regrowth of hair in 39% of the participants (78). In general, it was also found that minoxidil treatment appeared to slow the progression of baldness. Side effects were limited to local dermatological effects and no significant differences in clinical evaluations were observed (79). More recently, it has been reported on the basis of echocardiogram data that topical minoxidil treatment may result in slight increases in heart rate, left ventricular mass, and cardiac output (80). In contrast, other studies have reported no changes in echocardiogram data (81). No changes in blood pressure have been observed in any human subjects treated with topical minoxidil.



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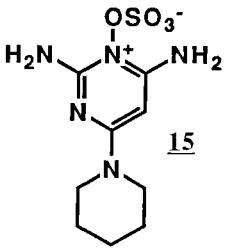

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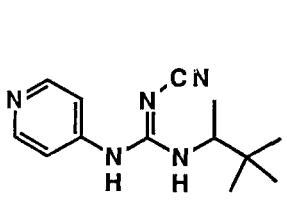
MECHANISM OF HAIR GROWTH STIMULATION

Outside of immunomodulatory or antiandrogenic effects, the mechanism by which hair growth is stimulated is unclear. Minoxidil is currently unique in that it shows hair growth stimulating activity in both alopecia areata and androgenetic alopecia. Although it has been reported that minoxidil exerts a minor suppressive effect on normal human T-cells *in vitro* (82), it shows no systemic immunosuppres-

sive properties (55). Additionally, minoxidil is neither androgenic nor antiandrogenic (83,84). Minoxidil causes follicular hypertrophy (rather than follicular neogenesis) and appears to sustain the anagen phase of the hair cycle (85). Prolongation of anagen could explain minoxidil's effect in both androgenetic alopecia and alopecia areata since hair loss in these conditions is thought to be due to premature anagen termination.

Minoxidil's hair growth stimulating properties were originally discovered because it induced hypertrichosis as a side effect of its action as an antihypertensive agent (86). Although minoxidil and diazoxide are both potent vasodilators, there is currently no consistent evidence in the literature that these compounds stimulate scalp blood flow, or that increased scalp blood flow promotes hair regrowth (87,88). *In vivo*, upon systemic administration, minoxidil is sulfated to afford minoxidil sulfate (**15**) which is a potassium channel activator and is considered to responsible for minoxidil's hypotensive effect (89,90). Diazoxide similarly activates potassium channels (91), as does pinacidil (**16**) which has also been reported to cause hypertrichosis (92,93). Although minoxidil sulfate is apparently produced in hair follicles (94), the role (if any) of minoxidil sulfate in hair growth is unclear. Potassium channel activation may be important to hair growth stimulation, but other potassium channel agonists such as cromakalim (**17**) or nicorandil (**18**) apparently do not cause hypertrichosis upon oral administration (92).



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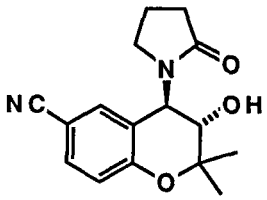

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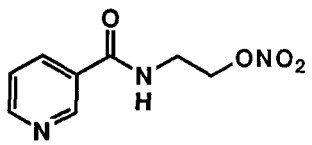

18

Other biological activities have been reported for minoxidil which may or may not be related to its effect on hair growth in humans. Minoxidil has been shown to delay senescence of cultured keratinocytes (95), to suppress production of lysyl hydroxylase (in cultured human skin fibroblasts) (96), and to inhibit prostacyclin synthase (in cultured bovine aorta epithelial cells) (97). Minoxidil stimulates DNA and protein syntheses in cultured mouse vibrissae follicles (17), implying a direct stimulatory effect. Minoxidil has been found to be mitogenic in murine (98) and macaque (94) hair follicles *in vivo*. The molecular mechanism or mechanisms by which minoxidil exerts these effects are currently unknown.

Conclusion - Few effective agents are currently available for stimulation of hair growth. Outside of immunomodulation in alopecia areata, there is no clear mechanism by which hair growth can be induced. Further development of hair growth promoters is critically dependent on the discovery of a rapid and accurate assay system which has some known relationship to alopecia in humans. Future areas of research will most certainly focus on the development of agents with greater efficacy and safety. Separation of hair growth and vasodilator properties in compounds such as minoxidil is an important goal. Further research with 5α-reductase inhibitors may demonstrate their value at preventing hair loss in humans. The serendipitous discovery that a simple organic compound like minoxidil could stimulate hair growth has led to a reawakening of interest in the medicinal chemistry of alopecia.

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Chapter 21. New Horizons in the Treatment of Proliferative Prostatic Disease

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Introduction - Prostate cancer (PC) and benign prostatic hyperplasia (BPH) are major medical problems in the aging human male. PC is the second leading cause of cancer in males with approximately 96,000 cases diagnosed and 26,000 deaths annually in the U.S. (1). Although usually not fatal, BPH is the second leading cause of surgery in the U.S. with over 400,000 prostatectomies performed each year, but this only represents 20-25% of men exhibiting symptoms (2,3). The world-wide market potential for anti-prostatic agents has been estimated at $750-1000 million (4,5).

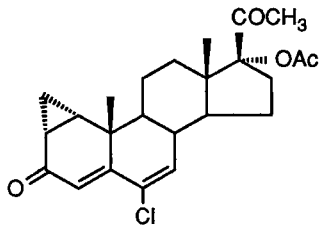
PC and BPH do not appear to be related since these diseases most frequently occur independently and develop in anatomically distinct regions of the prostate gland (6). Although their etiology remains unknown, an endocrine basis is supported since both diseases occur almost exclusively in men with functional testes and regress following castration (3,7-9). The most common form of PC is a malignant transformation of epithelial cells in the peripheral region of the prostate gland (adenocarcinoma) (10). Hormonal treatment is presently limited to metastatic disease and is considered palliative since evidence supports that PC progresses from an androgen-dependent to an androgen-independent phase (11). Castration, estrogens (diethylstibestrol(DES); ethynyl estradiol; estramustine), progestins (megestrol acetate; medroxyprogesterone acetate (MPA); progesterone), androgen receptor (AR) antagonists (cyproterone acetate; flutamide), steroidogenic inhibitors (aminoglutethimide; spironolactone) and LHRH agonists have all been used for the treatment of metastatic PC and have been previously reviewed (12-14).

BPH represents a non-malignant hyperplasia and hypertrophy of both stromal and epithelial elements in the periurethral and central regions of the prostate gland (3,15). The histopathologic state can vary from a "pure" stromal to a "pure" epithelial hyperplasia/hypertrophy (16). Surgery via transurethral resection currently represents the leading treatment for BPH and has a high degree of efficacy and safety (2,17). Although BPH responds to either surgical or chemical castration (8,18), these are not acceptable forms of treatment to a majority of the patient population. Consequently, it is important that any long-term pharmacological treatment for non-life threatening cases of BPH have minimal side-effects.

This review will focus on new endocrine as well as non-endocrine pharmacological agents currently under development for the treatment of PC and/or BPH. In addition, information is provided on compounds previously reviewed (19) that are of continuing interest.

ANDROGEN RECEPTOR ANTAGONISTS

Cyproterone Acetate (CPA) - CPA (**1**) continues to be the most thoroughly studied antiandrogenic agent (19) and has approval for use in PC in several countries. A review (20) and history (21) of **1** as well as recent preclinical studies (22-26) describe the antiandrogenic and antigonadotrophic activity of this agent. Clinical studies with **1** in PC have been reported (27-32). The utility of **1** or androgen deprivation in BPH has been questioned (33).



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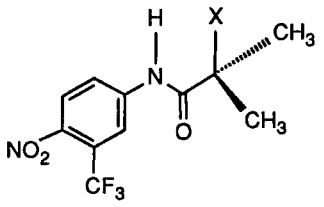
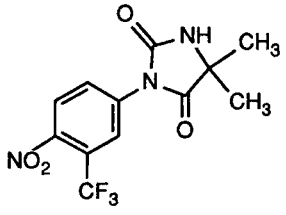
Flutamide (SCH 13521) - Flutamide (2), the prototype non-steroidal pure AR antagonist, has approval for use in PC in 14 countries and is under investigation in 7 others including the U.S. (34). The first studies with 2 were reported in the early 1970's and interest in this drug continues (19). Preclinical studies continue to investigate the mechanism(s) of action of 2 and its active metabolite 2-hydroxyflutamide (3) (35-42).

A study on the clinical pharmacokinetics of 2 in 10 PC patients confirms the extensive first pass metabolism to 3 (43). In addition to peripheral AR antagonism, 2 acts centrally to antagonize the inhibitory effect of T on LH secretion. A study in 6 stage C2 PC patients concluded that oral 2 (250 mg tid) significantly increased LH pulse frequency and consequently plasma T concentration (44). Uncoupling negative feedback at the hypothalamic/pituitary would theoretically limit utility to PC patients undergoing surgical or chemical castration, although early phase II clinical studies report favorable results in patients with advanced PC. A controlled clinical study, comparing 2 to DES, reported objective efficacy comparable to conventional estrogen therapy (45). It is interesting that the investigators reported plasma T levels after 12 months therapy equivalent to pretreatment levels. Unlike the DES group, no effects on libido or potency were observed in the flutamide group.

The effects of 2 on T, 5α-dihydrotestosterone (DHT) and the localization of AR in tissues from BPH patients have been reported (46). Flutamide seemed to shift DHT localization from the nucleus to the cytosol. Two clinical studies on the effectiveness of 1 in BPH report improvement in subjective parameters (hesitancy, nocturnia, urine flow) as well as a reduction in prostate size as determined by ultrasonography (47,48). The most recent study (48) clearly indicates the need to fully evaluate the utility of this agent in BPH.



2 X = H
3 X = OH



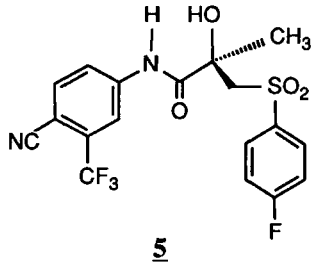
4

Anandron - RU 23908 (4) is a non-steroidal AR antagonist primarily targeted for use in hormone-responsive PC (49). It binds to the rat prostate AR with affinity similar to 3 and does not interact with other steroid hormone receptors (50). Structure-activity studies of this compound class were previously addressed (19). Reviews of preclinical studies have been reported (51-54), as have the clinical pharmacokinetics of 4 (55,56). It acts as an AR antagonist in peripheral tissues, as well as centrally. This effect, again, would limit the clinical utility of this drug to adjuvant therapy in PC patients undergoing surgical (57,58) or chemical castration (LHRH) (59). In combination with buserelin (LHRH agonist) or surgical castration, improvements in subjective and objective parameters were reported in PC patients (53). Concerns over the ocular toxicity of 3 have been

expressed (60,61). It presumably inhibits the effect of adrenal androgens in castrate patients and the effects of LHRH analog-induced initial T surge (flare), which potentially exacerbates both subjective and objective clinical parameters.

ICI 176,334 - ICI 176,334 (**5**) binds to rat prostate AR with an affinity approximately 4x **3**, effectively inhibits T stimulated growth of sex accessory tissues in immature castrate rats and has no androgenic activity. In intact adult male rats, **5** caused dose-related reduction in accessory sex organ weights, had no effect on the testes and did not cause a significant increase in serum LH or T (62). In adult male beagle dogs, **5** suppressed prostate weight at doses as low as 0.1 mg/kg/d (apparent ED_{50}), without affecting the epididymides or testes at this dose (63). Results of clinical trials have yet to be published. The peripheral selectivity of **5** is unique among non-steroidal antiandrogens. Potential explanations for this have been proposed based upon studies indicating that **5** fails to penetrate the hypothalamus as effectively as **2** (63).

Structure-activity relationship investigations in this series have been extensive. Peripherally selective antagonists such as **6** were reported, as well as non-steroidal androgen agonists, **7** (64). Replacement of a methyl group of **3** with fluoromethyl increased receptor affinity up to seven fold. These investigations explored the structural requirements for antiandrogenic activity. Subsequent studies identified **5** as the principal lead (65) and show that activity resides primarily in the R(-) enantiomer with a eudesmic ratio of 60 (66). It is reported to be inactive in the Clauberg assay for progestational activity although, no reports on interaction with the progesterone receptor (PR) have appeared. Related compounds also possess progestational and/or antiprogestational activity (67,68).



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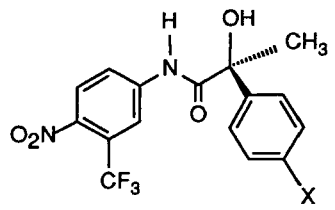
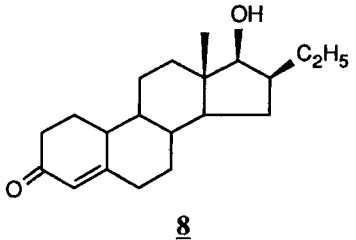


6 X= CH_3SO_2 **7** X= NO_2

Oxendolone (TSAA-291) - A previous review in this series (19) described preclinical and early clinical results on the antiandrogen oxendolone (**8**). It binds to the rat prostate AR (K_i= 320 nM), inhibits 5α-reductase (IC_{50}=1.4 μM), and binds to the PR (K_i=20 nM) (69). The effects of these activities in BPH are unclear; however, human prostatic tissue contains both PRs and 5α-reductase activity. Studies on experimentally induced canine BPH indicated that **8** in combination with MPA worked better than **8** alone (70). Clinical results on the effectiveness of **8** alone or in combination with the α-adrenergic blocker bunazosin have been reported (71). At 12 weeks objective parameters (maximal and mean flow rate) were most improved in the **8** /bunazosin treated group.



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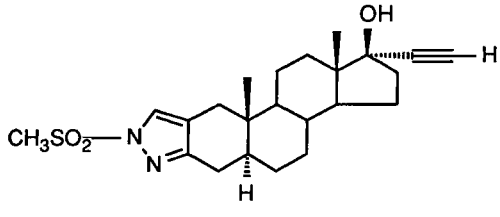


9

WIN 49596 - The dihydroethisterone derivative WIN 49596 (**9**) is reported to be an AR antagonist (72), binding to the AR with a K_i=2.2 μM and a time course similar to **3**. It inhibits sex accessory tissue growth in intact, adult male rats and in T propionate or DHT treated castrated, immature male rats. Treatment of adult male rats for 72 days with doses up to 500 mg/kg/d, p.o., did not significantly inhibit mating performance or fertility. It is devoid of other hormonal activities except for antiprogestational activity in rat and rabbit models and does not inhibit 5α-reductase, aromatase and 3α- or 3β-hydroxysteroid dehydrogenase in vitro. It is reported that **9** inhibits androgen mediated nuclear accumulation of AR in the ventral prostate, in vivo (73).

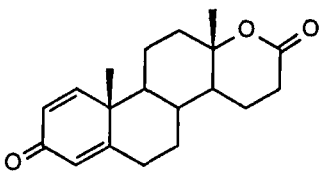
MODULATION OF STEROIDOGENESIS

Ketoconazole - In male subjects, ketoconazole lowers serum T levels to near castrate levels at high doses. Reviews on the hormonal effects have been published (74,75). Clinical studies on the effectiveness in stage D2 PC indicate excellent subjective clinical response and long term efficacy similar to other methods of androgen ablation (76,77). The inability to consistently maintain serum T near castrate levels and pronounced side effects potentially limit its utility as first line therapy of PC (78). A direct cytotoxic effect of ketoconazole on PC cells has been suggested (79). The potential of finding more efficacious agents of this class from the many structural analogs of ketoconazole warrants further investigations (80,81).

Aromatase Inhibitors - The utility of aromatase inhibitors in prostatic disease is not yet fully defined; however, the presence of estrogen receptors in human prostatic tissue (82,83) and the synergism of estrogens with androgens in canine models of BPH suggest a role for estrogens in spontaneous BPH (84-89).

Steroidal and non-steroidal inhibitors of aromatase are well known (90,91). 4-Hydroxyandrostenedione (4-OHA; K_i=30-50 nM), has recently been reviewed (92,93), as has MDL-18,962 (**11**; K_i=3-4 nM) (84). SH-489 (**12**; K_i=80 nM) in combination with **1** synergistically inhibits BPH development in experimentally induced canine BPH (85,94). FCE 24304 (**13**) is reported to inhibit aromatase (K_i= 24 nM) and not to affect 5α-reductase (95). Structural modifications of aminoglutethimide involving N-alkylation of the piperidinedione demonstrated improved activity (96-98). CGS-16949A (**14**; K_i=1.7 nM) (99), was shown to inhibit aromatase activity in vivo. After chronic administration to intact male beagles with enlarged prostates, **14** had no significant effect on prostatic weight or total DNA content vs controls (86,100).

Two limited clinical studies with aromatase inhibitors in BPH have been reported; testolactone (**10**) and aminoglutethimide (K_i≈4 μM and K_i≈40 μM respectively). In a 6 month study with **10**, 7 of 13 patients experienced improvement of objective parameters (flow rate, residual urine, prostate volume by sonography). All thirteen patients showed decreased prostatic volume, while having significantly elevated serum T. The investigators ascribed the effect to an altered T/E2 ratio since E2 (estradiol) levels were unaffected by **10**. In an aminogluthethimide 8 week study no changes in urine flow rate or prostate size were found, although T, FSH and LH levels were elevated while E2 levels were significantly lowered (101,102).



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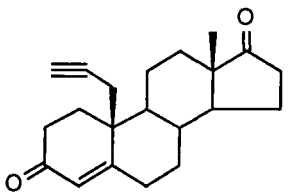

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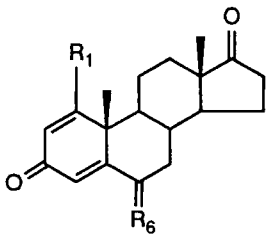
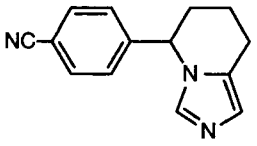


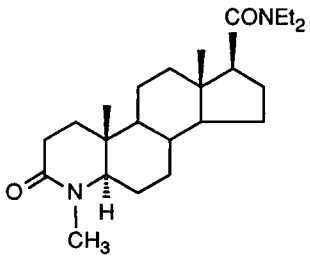
12 $R_1 = CH_3$, $R_6 = H_2$

13 $R_1 = H$, $R_6 = CH_2$



14

5α-Reductase Inhibitors - 5α-reductase inhibitors block the formation of DHT at the cellular level without altering plasma T levels. This offers a clear efficacy advantage over the first generation pure AR antagonists which uncoupled negative-feedback, thereby raising plasma T levels. Several 4-azasteroid inhibitors of 5α-reductase are known (103-105). In vitro and preclinical in vivo studies with 4-MA (**15**) have been reported (106-108). MK-906 (**16**) is a Δ^1, N(4) des-methyl analog of **15** with enhanced in vivo activity and much lower affinity for rat prostate AR (109). Removal of the N(4) methyl reduces cytotoxic effects in rat hepatocytes although the structure-toxicity relationship at this position could not be generalized and was influenced by the C(17) substituent (110). Pharmacokinetic studies with **16** indicate good oral absorption and relatively high plasma levels of intact drug and an active metabolite (111,112). A pilot study shows lowered plasma DHT in healthy male volunteers after oral medication. Phase III clinical trials are underway with an anticipated launch in 1991 (112,113).



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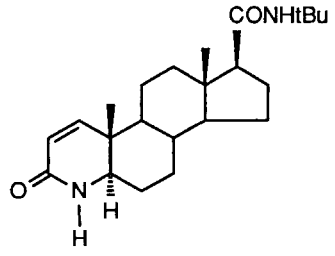
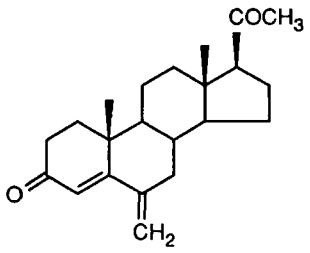


16



17

6-Methlylene-4-pregnene-3,20-dione (6-MP; LY 207320, **17**), a steroidal 5α-reductase inhibitor (Ki=1.25 μM), inhibits the conversion of T to DHT in explants of human PC and BPH explants of rat ventral prostates and cultured human genital skin fibroblasts (114). It does not bind to either the AR or estrogen receptor, but has a relative binding affinity of 16% for PR (R5020 = 100%) (115). Subcutaneous administration of **17** inhibits sex accessory tissue growth in the T stimulated immature castrate rat and inhibits tumor growth in the rat Dunning prostatic tumor model. Doses less than 50 mg/kg have no effect on either serum LH or T, but higher doses inhibit testicular steroidogenesis (115).

LHRH Agonists / Antagonists - LHRH agonists are being developed for the treatment of

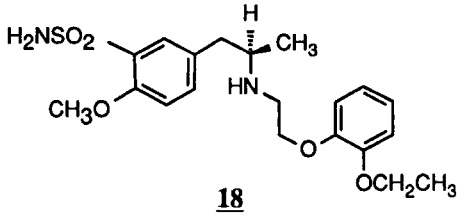
various hormone dependent disorders including endometriosis, breast cancer, PC and BPH (116,117). The physiology, pharmacology and structure-activity relationships of LHRH agonists have been reviewed (117,118). Detailed structure-activity studies of LHRH antagonists have also been published (119,120); however, considerable work remains before LHRH antagonists are commercially available.

Chronic administration of LHRH agonists to males leads to a suppression of testicular steroidogenesis or "chemical castration". Studies indicate that LHRH agonists are as equally effective as orchiectomy or DES in treating PC (116,118). Total androgen ablation using LHRH agonists in combination with either the antiandrogen **2** (121), **4** (122) or **1** (123) failed to demonstrate any benefit in long-term survival over LHRH alone. LHRH agonists approved or in development for the treatment of PC include leuprorelin goserelin, and buserelin. Goserelin is administered monthly as a subcutaneous implant with equal efficacy to daily injections (124,125). Side-effects of LHRH agonists include loss of fertility and libido, flushing and headaches.

Limited clinical studies have been conducted on the efficacy of LHRH agonists in the treatment of BPH. Reversible prostatic regression (18,126) or a reduction in prostatic androgen levels (127,128) occurred following chronic treatment. Despite symptomatic improvements in some patients, not all patients demonstrated improvements in objective measurements of urinary flow (18,126,127). LHRH agonists may be useful in BPH patients who are not surgical candidates.

MISCELLANEOUS

Alpha- Blockers - The symptoms of BPH result from constriction of the urethra by the hyperplastic prostate (129,130) and increased autonomic tone of the prostatic smooth musculature and surrounding structures involved in micturition (131). The prostatic capsule, trigone muscle and bladder neck muscle are rich in alpha-1 adrenergic receptors (132-135). Both human and canine prostatic strips contract in response to the alpha agonist norepinephrine and this contraction can be attenuated by selective alpha-1 antagonists such as prazosin, as well as by mixed alpha-1/alpha-2 antagonists such as phenoxybenzamine (136). Consequently, the use of selective alpha-1 adrenergic antagonists for treatment of urinary retention associated with BPH is being investigated (131,137). Prazosin has recently been approved in England for the treatment of BPH, although clinical results are equivocal (138-140). Terazosin (141,142), alfuzosin (143) and bunazosin (71) are selective alpha-1 blockers undergoing clinical evaluation in BPH. YM-12617 (LY23352, **18**) is an optically active alpha-1 receptor antagonist in early clinical testing (144-146).



18

Natural Products - Prostatic tissue is rich in cholesterol and other related sterols (147). Although their function is unclear, it has been proposed that lipid lowering agents or agents which interfere with lipid metabolism may be effective in treating prostatic disease, primarily BPH (147,148). The majority of these products are phytochemical plant extracts of unknown composition. Many of these products contain ß-sitosterol and claim to be either 5α-reductase inhibitors or AR antagonists (149). Agents currently under clinical investigation include extracts of serenoa repens (150,151), radix urticae (152), pygeum african (153) and the polyene macrolide of Streptomyces aureofaciens,

mepartricin (154). Identification of the active component(s) in these natural products may reveal significant leads for chemical synthesis.

Growth Factors - Growth factors must also regulate neoplastic prostatic growth since both normal and malignant prostatic epithelial cells can proliferate in vitro in the absence of androgens (155). Androgen independent growth has been clearly demonstrated for PC, but has not been studied for BPH (156). Extracts of human prostate tissue contain factors that stimulate both growth and DNA synthesis in a number of fibroblast cell lines (157). The major mitogen in human prostate is both structurally and immunologically related to basic fibroblast growth factor (bFGF) isolated from bovine pituitary and brain (158-160). BPH and PC tissue appear to contain greater levels of this prostatic growth factor than normal tissue (161,162). However, the exact role of this factor in normal and abnormal prostate growth, as well as its relationship to the steroid hormones remain to be determined. Whether growth inhibiting factors are also present in the prostate is also under investigation (163).

SUMMARY

Several new chemical entities with diverse mechanisms of action are being pursued for the treatment of PC or BPH. The discovery and development of a) potent and selective inhibitors of 5α-reductase and b) "pure" AR antagonists that do not uncouple negative-feedback at the hypothalamic/pituitary demonstrates continuing interest in this therapeutic arena. Molecular cloning of a homogeneous AR with appropriate immunological and hormone binding properties (164,165) may facilitate the structure elucidation of specific binding domains. Further research on the prostate will undoubtedly reveal additional new potential approaches (e.g., growth factors) and allow clinicians to select specific agents or a combination of agents based on the underlying pathophysiology. With the increasing longevity of the male population and subsequent increase in proliferative prostatic disease, the need for effective non-surgical treatment of this condition is obvious.

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Section V. Topics in Biology

Editor: Kenneth B. Seamon
Food and Drug Administration
Bethesda MD 20892

Chapter 22. Transgenic Animals and the Evaluation of Therapeutics

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Introduction - Transgenic animals possess foreign DNA which has been stably incorporated into their genome. The addition of the foreign DNA (transgene) is most commonly achieved by micro-injection into the male pronucleus of a fertilized egg which is then transferred back to a recipient female and allowed to develop. The transgene usually becomes incorporated at a single site on one of the host chromosomes, apparently at random. As only one chromosome carries the transgene the founder transgenic animal is heterozygous. Homozygous progeny can usually be bred unless the random integration of the foreign DNA has resulted in an insertional mutation of an essential endogenous gene. DNA incorporation normally occurs prior to the first cell division, in which case all cells carry the transgene. However, if DNA incorporation occurs after the first cell division the resulting animal will be mosaic comprising a mixture of normal and transgenic cells. If some of the germ cells are transgenic, a true transgenic can be derived from the mosaic.

Alternative methods for generating transgenic animals involve either infection of a developing embryo with a viral vector or the genetic transformation of pluripotential embryonic stem (ES) cells in vitro (1).The ES cell technique involves removing the blastocyst cells, which can be maintained in vitro and still retain the ability to generate a normal embryo (2). The blastocytes can be genetically modified in vitro and the desired mutated cells can be selected and implanted to produce chimeric animals. Retroviral and chemical mutagenesis of ES cells has been used to modify the genetic material (3,4). Methods are being developed for targetting mutation to specific endogenous genetic locations (5,6). One recently described selection technique may provide a general method in which the cloned gene of interest is used to disrupt the endogenous gene sequence in ES cells prior to reconstruction of the animal (7). In this way animals with a mutation in any cloned gene of interest can be produced. A factor which inhibits ES cell differentiation has been cloned and identified which may facilitate these approaches (8,9). Recombinant retroviral vectors have also been used to incorporate transgenes (10). Retroviral vectors insert only one copy of the transgene at a specific chromosomal location, but the transgene is limited in size to about 7 kilobases and inevitably surrounded by some vector sequences even in the case of self-inactivating vectors.

Expression of the transgene in the host depends primarily on the regulatory regions present in the transgene. Novel patterns of gene expression can be obtained by combining the structural gene of interest with regulatory elements (promoters/enhancers) from unrelated genes. Thus proteins of interest can be expressed in specific tissues where they are not normally found, or in excessive or diminished amounts, or at inappropriate developmental stages, or in an unregulated manner. For example, the immunoglobulin enhancer Eu can be incorporated into the transgene to direct expression to the B lymphoid cells, the 5' region of the elastase I gene can be used to direct expression to the exocrine pancreas, and the 5' region of the rat insulin II gene can be used specifically to express genes to the B-cells of the endocrine pancreas. The metallothionein regulatory region promotes gene expression in a wide range of tissues and can be induced by metals and glucocorticoids. It has been used in many transgenic constructs, one major example being those which drive growth hormones (1).

Earlier work on transgenic animals and technology has been reviewed (1,11-15). Reviews on the use of transgenic animals in specific areas have appeared, including oncogenesis (16,17), immunology (18-20), pathobiology (21), gene expression (22,23), the nervous system (24), pharmaceutical protein production (25-27), and livestock improvement (28-31). Experimental procedures for producing transgenics have been reviewed (10,32).

Transgenic animals of several species have now been produced including mouse, rat, rabbit, sheep, pig and cow. In the research context, the main species is likely to remain the mouse with the possible extension to the rat which is used more extensively in drug and metabolic studies. It is also possible that other small laboratory animals which have particular experimental value in specific areas (such as hamsters in hypertension) may be needed.

Pharmacological Test Models - Transgenic animals represent a reproducible and renewable source for the generation of models for the testing of pharmacological substances. Because of the developmental stage at which the transgene is introduced there are no restrictions on interspecies transfer and the recipient animals show immunological tolerance to the foreign proteins produced. Thus human proteins can be generated and studied in transgenic mice, rats and other small laboratory animals. A wide variety of changes can be induced in the transgenic animal which can parallel those found in human disease, making transgenic animals valuable new tools for drug evaluation. Transgenic mice have been produced which overexpress the gene for growth hormones leading to dramatically increased growth (1).

Transgenic animals for use as disease models may be engineered by both insertion of new functions and by deletion of endogenous functions. Anti-sense DNA transgenes have been used to block the expression of specific host genes at the intermediate messenger RNA level thus reducing the levels of specific host proteins. For example, this technique has been used to produce a reduction in the synthesis of the myelin basic protein or hypoxanthine guanine phosphoribosyl transferase (HPRT) activity (33,34).

The activity of a multimeric protein may be reduced by using a transgene encoding a mutant subunit producing an inactive multimer. Transgenic mice were made that produce a mutant subunit of collagen, overexpression of which caused a syndrome in newly-born mice equivalent to human congenital ostea imperfecta (35).

Transgenes composed of toxic protein genes encoding for toxic proteins such as ricin or diptheria toxin, attached to tissue specific promoters have been used to achieve tissue ablation (36-38). Disruption of lens tissue was observed in animals carrying a γ-2 crystallin/D-TA gene construct (36) while pancrease ablation was achieved with an elastase I/D-TA transgene (37).

An endogenous function may be removed by disruption of the endogenous gene, by selection of mutation after random mutagenesis (insertional or otherwise) or by specific gene targetting. Specific gene targetting for the mutation of an endogenous gene, although demonstrated in vitro in ES cells, has not yet been demonstrated in the germ line (7). There is no systematic reason why this should not be forthcoming in the near future. Mutagenesis followed by selection has been used successfully to isolate HPRT-ES cells (3,4, see Lesch-Nyhan disease below).

Genetic Disease - Transgenic animals in which specific genes are defective provide models in which replacement therapy, gene therapy and prevention of sequelae can be evaluated. Models of interest include those for Lesch-Nyhan disease caused by a defective hypoxanthine guanine phosphoribosyl transferase (HPRT) enzyme and severe combined immunodeficiency disease (SCID) caused by a defective adenosine deaminase. HPRT defective transgenic mice were generated using the ES cell technique but in contrast to humans were phenotypically normal, suggesting species differences in purine metabolism (3,4,39).

Familial hypercholesterolemia involves a defect in the gene encoding the low-density lipoprotein (LDL) receptor regulating the plasma level LDL, leading to elevated LDL levels and premature coronary atherosclerosis (40). Transgenic mice expressing the human LDL receptor showed increased clearance and reduced plasma levels of LDL (41).

Diabetes - Type 1 (insulin dependent) diabetes may involve an autoimmune attack on the insulin producing B-cells of the pancreas (42). Class I and Class II MHC (major histocompatability complex) molecules were expressed in transgenic mice using the insulin promoter in an attempt to provoke

an immune reaction similar to that of Type 1 diabetes (42). In all cases the MHC molecules were strongly expressed, pancreatic B-cell degeneration occurred, and the animals developed diabetes (43, 44, 45). However, there was no evidence of an autoimmune reaction or T cell involvement (46,47).

Diseases of the Central Nervous System (CNS) - The search for promoters allowing targetting of gene expression to specific neuronal cell types and tissues remains a major goal to defining alternate RNA processing pathways which operate in the CNS and to generating new animal models (48-50). CNS specific proteins such as neurofilament protein and myelin basic protein have been expressed in mice (51,52). Neural tumours have been produced in mice by expression of viral proteins encoded by the human papovavirus JC and by overexpression of the tat protein from the human T-lymphotrophic virus type I (53,54).

The addition of the myelin basic protein (MBP) gene to 'shiverer' mice by transgenesis changed the phenotype of the mice to normal and corrected the deficiency (52). The normal phenotype was converted to 'shiverer' by the addition of MBP anti-sense DNA which blocked the expression of normal levels of MBP (33).

Down's Syndrome is associated with the trisomy of chromosome 21 which bears the gene for superoxide dismutase 1 (SOD1) normally responsible for the removal of oxygen and hydroxyl radicals. Transgenic mice carrying the human SOD1 gene displayed abnormalities in the synapses between the neurones and muscle cells of the tongue similar to those found in Down's patients (55,56). Brains from Alzheimers Disease patients possess increased levels of mRNA for amyloid precursor protein (APP), which is also encoded by a gene on chromosome 21 (57,58). As the genetic basis of CNS diseases, such as Down's Syndrome and Familial Alzheimer's Disease becomes better understood and the genes involved identified, then transgenic animals incorporating these genes can be developed as animal models.

Viral Diseases - Transgenesis allows the introduction of the whole or part of the viral genome into cells, tissues and species not susceptible to normal infection. Transgenic mice therefore provide models for studying human viruses which do not infect common laboratory animal species. Hepatitis B virus has been introduced into transgenic mice resulting in HBV-antigen production in the bloodstream but without any apparent deleterious consequences to the animal (59,60).

The transgenic mouse has been proposed as a model for the human carrier state in which viral DNA has become integrated into the host genome (61). HIV infection in man is restricted to cells bearing the CD4 receptor for the gp120 binding protein of the virus. The generation of the CD4 receptor on the surface of T cells of transgenic mice disappointingly failed to render the mice susceptible to HIV infection (62). However, by expressing parts of the HIV genome in transgenic mice progress is being made in reproducing and understanding the complex symptomatology of AIDS. Introduction of the HIV tat (trans-activator) gene into transgenic mice results in dermal lesions resembling Kaposi's sarcoma seen in man (63).

Cancer - Mutations in specific proto-oncogenes are believed to play a causal role in tumour development (64). The incorporation of oncogenes in transgenic mice can produce novel tissue neoplasia or hypertrophy producing a new animal pathology. Choroid plexus tumours are induced in mice containing SV40 large-T antigen gene expressed from the viral promoter/enhancer (65). Hyperinsulinaemic mice produced by B islet cell hypertrophy are induced by coupling the same SV40 T antigen oncogene to the insulin gene promotor (66). These animals also provide recurrent and reproducible animal tumours and have the great advantage over the tumour transplant animals models that what is seen is a tumour development ab initio in the context of the whole animal.

Oncogenes, which lead to a loss of growth control and an initial hypertrophy such as T and myc, are particularly useful as they provide an animal with a naturally high tumour incidence having already endogenously undergone one of the first steps in the multi-stage process of clinical oncogenesis. These animals will provide a vital model for studies of environmental and other effects upon tumour promotion. They also represent a sensitive indicator for studies of in vivo mutagenesis. The more recent identification of recessive oncogenes, such as that in retinoblastoma where loss of function at both autosomal loci is the cause of tumourigenesis, re-affirms the future importance of specific gene deletion as well as gene insertion in this field (67).

Transgenic mice carrying activated oncogenes provide models for investigating the role of oncogenes in tumour development and evaluating anti-cancer therapies. By combining the

oncogene with enhancer/promoter regulatory elements determining cell/tissue specificity a number of tumour models have been developed (16,17). Oncogenes used include SV40 early region/large-T antigen (68-73), c-myc (72, 74-77), M-ras (72,78,79), c-neu (80) and polyoma middle-T antigen (81,82). Promoter/enhancer sequences used to achieve tissue specificity include insulin for pancreatic B cells (83), elastase 1 for pancreatic acinar cells (68, 79), milk whey protein for mammary gland (77,78), arginine vasopressin for endocrine pancrease and anterior pituitary (69, 84), immunoglobulin heavy chain for B-cells (74,76), crystallin for lens (73,85) albumin for liver (86), and B casein for mammary gland (87). SV40 large-T antigen is a potent tumuorigenic agent for many cell types and is useful for generating tumours in transgenic mice. Although tumourogenesis is usually a multistep process, the rapid development of tumours following expression of a single gene has been reported for c-neu (80) H-ras (79) and polyoma virus middle T antigen (81,82). Some oncogenes may act synergistically to decrease the latency time for tumour development. For example, transgenic mice carrying both MMTV/c-myc and MMTV/v-Ha-ras genes display a dramatic acceleration in the kinetics of tumour formation compared with mice carrying only one of these transgenes (88). Chimeric mice in which oncogene bearing cells and normal cells coexist have been suggested to provide a more valid model of human cancer (82).

Side Effect Models - The potential side-effects of human proteins can be examined in the absence of an immune response by expressing these proteins in transgenic mice. The protein of interest can be generated endogenously or administered intermittently to reproduce the normal therapeutic regimen. Several human therapeutic proteins have been generated in transgenic mice including insulin (89,90), interferons (91,92) GM-CSF (93,94), Factor IX (95), TPA (96), growth hormone (97) and C-reactive protein (98).

Receptors - The cloning of genes encoding receptors permits their under or over expression in transgenic mice. Receptors of pharmacological interest which have been cloned include G protein coupled receptors, such as the α_1, α_2, β_1, β_2 adrenergic, muscarinic family, 5HT1A and 5HT1C serotonergic, dopamine D2,and other receptors such as progesterone (99) and LDL (41). Ligand gated channels, such as the nicotinic-acetylcholine receptor, the $GABA_A$ receptor and the glycine receptor, have also been cloned (100). It may be anticipated that transgenic animal models incorporating these receptors will be developed in the near future.

Conclusion - A fully experimental approach to mammalian genetics is very rapidly becoming a reality through the use both of conventional zygote injection transgenics and of embryonic stem cells. The latter approach allows extensive in vitro genetic manipulation, selection and screening prior to whole animal reconstruction. For practical purposes the mouse is the species of choice for such studies, and one of the most exciting prospects is the construction of animal models of disease for pharmaceutical testing. Imagination, ingenuity of gene design and application is now all that are required.

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Chapter 23. Second Generation Recombinant Therapeutic Proteins

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Introduction - Recombinant DNA techniques have afforded large-scale production of many new therapeutic proteins. Many classes of recombinant proteins such as antibodies, enzymes, enzyme inhibitors, cytokines and hormones have been evaluated clinically. As clinical data has accumulated pertaining to the intrinsic stability, pharmacokinetics and pharmacology of these proteins, it has often become desireable to substantially improve their therapeutic properties. Protein engineering techniques have been applied to these problems, and in this review the second-generation therapeutic proteins that have been generated are surveyed, emphasizing those investigations with the applied goal of improving a clinically-relevant property.

ANTIBODIES and IMMUNOCONJUGATES

The antibodies that mediate the humoral immune response are the immunoglobulins (Figure 1). These tetrameric glycosylated proteins consist of disulfide-linked heavy (H) and light (L) chains . Each chain has a relatively invariant constant region (C_H and C_L). The more variable regions (V_H and V_L) are further divided into the framework (FR) or complementarity-determining hypervariable regions (CDRs). Proteolysis with papain generates the antigen-binding F_{ab} fragment and the F_C fragment, which contains the effector function regions that mediate binding to complement and phagocytic cells.

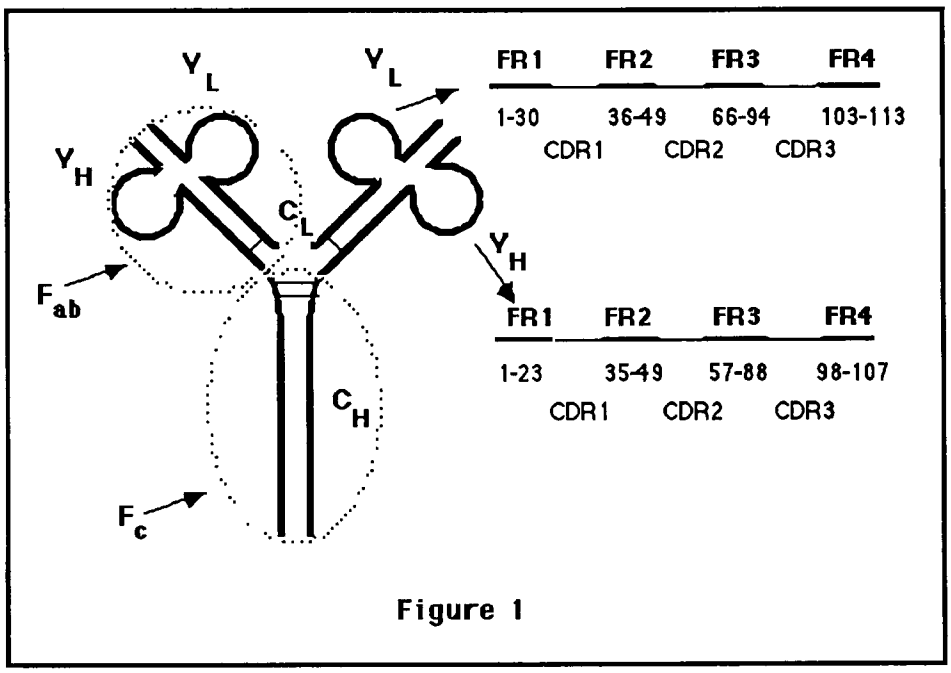

Figure 1

Chimeric Antibodies - Monoclonal antibodies, which bind to a single epitope of an antigen, can be produced by transfection of immunoglobulin genes into lymphoid cells (1). This "transfectoma" technology allows one to alter the immunoglobulin gene and thus the recombinant antibody product. Monoclonal antibodies that recognize specific epitopes on human tumor cells have been derived from rodent hybridomas and developed for tumor cell imaging and treatment of malignancy (2). These immunoglobulins often are rapidly cleared from the circulation, are poor at inducing a cell-mediated response or complement fixation, and may induce an anti-idiotypic response (2). Second-generation antibodies have been constructed by fusing human-sequence constant regions to rodent-sequence variable domains from immunoglobulins that recognize tumor-associated antigens from human colon, breast and lung carcinomas (3-5). These chimeric antibodies have antigen affinities essentially equal to the parent mouse immunoglobulin.

The therapeutic use of antibodies to produce complement-fixation or cell-mediated lysis requires that the antibody have an appropriate heavy-chain subtype (6). Chimeras of a murine anti-colorectal cancer antibody with four human IgG constant regions were produced and evaluated for antitumor activity (7). The IgG1 antibody was superior in antitumor activity in a mouse model. An investigation of dansyl hapten-binding chimeric antibodies with the four different IgG constant regions suggests that the ability to mediate complement-fixation may be related to the segmental flexibility of the immunoglobulin hinge regions (8).

Replacement of the constant region of a rodent antibody with the constant region of a human antibody may not be sufficient to obviate recognition of the protein as foreign by the human immune system. Therefore, human antibodies have been produced where the complementarity determining regions (CDRs) have also been replaced by the CDRs from a tumor specific mouse monoclonal antibody. CDRs from a mouse anti-lysozyme antibody D1.3 were grafted into a human antibody heavy chain (9). The chimeric antibody retained affinity to lysozyme, albeit ten-fold lower than the parent D1.3 monoclonal antibody. This result indicates that despite the large number of intermolecular interactions between the framework region of an antibody and its protein antigen, the CDRs alone can confer substantial affinity to a humanized antibody.

An anti-human lymphocyte antibody which can fix complement was produced by the replacement of the CDRs (10). The cDNA for a rat anti-lymphocyte antibody was cloned and sequenced. The deduced amino acid sequences of the CDRs were used to construct mutagenic oligonucleotides for altering the human antibody genes coding for the four human IgG subtypes. The chimeric antibody had a low affinity for antigen suggesting that there was a defect in the antibody structure. An additional mutation was made to correct a defect in the structure of a loop in the heavy chain variable domain and this chimeric antibody had an affinity for antigen that was only two-fold lower than the parent rat antibody. The chimeric antibody was also more effective in vitro at inducing antibody-dependent cell-mediated cytotoxicity. This chimeric antibody was effective in killing leukemic B-cell lymphocytes from three patients, raising the prospect of anti-cancer use for this highly engineered antibody.

Immunoglobulin Fragments - Fragments of monoclonal antibodies have been produced successfully in E. coli (11-14). An F_V fragment of an anti-phosphorylcholine immunoglobulin, which comprises a V_H and V_L region linked by a 15-amino acid spacer, was produced in native form in the periplasmic space of E. coli (11). A larger F_{ab} fragment of a chimeric anti-human carcinoma antibody was secreted in inactive form from E. coli (12). Secretion of any proteins from E. coli is a rare occurence and suggests that the Erwinia carotovara pectate lyase leader sequence used in this work may be unique in this regard. The recombinant F_{ab} had an affinity for C3347 colon carcinoma cells which was indistinguishable from a conventional papain-derived F_{ab} fragment of the chimeric antibody.

An efficient bacterial synthesis of single chain therapeutic antibodies has been developed which exploits the higher efficiency of intra-molecular folding relative to intermolecular association (13,14). Single-chain antibodies have been designed and produced by fusing the DNA coding sequence of a V_L region to a V_H region. The antigen-binding regions of single-chain antibodies are functional, but they lack the cell-binding activities of conventional immunoglobulins because the constant regions are removed. The affinity of a single chain antibody against bovine growth hormone was only four-fold less potent than the parent immunoglobulin (13). An anti-digoxin single-chain F_V fragment was produced which was only six-fold less potent than the corresponding F_{ab} fragment (14). The ability to synthesize high-affinity immunoglobulin fragments will lead to their evaluation for in vivo diagnosis and cancer treatment using immunotoxin conjugates. They are

certain to have altered pharmacokinetics, and lower non-specific binding to non-antigen presenting cells than conventional antibodies.

Immunotoxin Conjugates - These hybrid molecules are constructed by cross-linking an anti-cancer monoclonal antibody to a protein toxin, such as diptheria and pseudomonas toxins, or ricin (a plant toxin) (15,16). Second-generation immunotoxins have been produced using protein engineering techniques to modify the toxin or antibody moieties. Ricin is a heterodimer, with the A-chain an enzyme capable of irreversible modification of the 60S ribosomal subunit, thereby disrupting protein synthesis. The B chain is a lectin which binds to cell-surface galactosides and triggers endocytotic uptake of the protein. Variants of the ricin B chain with lower affinity for galactoside binding have been generated by site-directed mutagenesis (17). Such variants might be useful for generating immunoconjugates with enhanced specificity, although this has yet to be reported for ricin immunoconjugates.

A similar strategy has been effective with diptheria toxin immunoconjugates (18). Three mutants of the diptheria toxin B chain have been generated which are defective in cell-binding but fully functional in mediating cytosolic entry of the A chain by endocytosis (18). These variants, which have as little as 0.01% of the toxicity of the parent protein, were chemically conjugated to a monoclonal antibody specific for the T3 antigen of human T cells (18). The second-generation immunotoxins were as toxic to target cells as the parent conjugate. The enhanced selectivity of the second-generation conjugates may be as high as 10^5 greater than the parent conjugate (18). Murine T-cell lymphocytes, which are normally resistant to diptheria toxin, are highly sensitive to intoxication by a chemical conjugate of diptheria toxin and an anti-murine Thy1 antibody (19).

SOLUBLE RECEPTORS

Soluble CD4 - Human immunodeficiency virus (HIV-1) attaches to target T4 lymphocytes via interaction of the viral gp120 envelope glycoprotein with the CD4 receptor (20). Soluble variants of the CD4 receptor have been engineered by deletion of the cDNA regions which code for the transmembrane and cytoplasmic domains (21-24). These soluble, secreted proteins are produced in mammalian Chinese Hamster Ovary or S9 cells (21-24). The soluble CD4 protein blocks viral infection of lymphocytes and synctitium formation of HIV-infected lymphocytes (21,22). The K_d of soluble CD4 for binding to gp120 approaches that of the holo cell-surface receptor, and soluble CD4 appears to inhibit viral infection at submicromolar concentrations (21,24). Rhesus monkeys which have been challenged with SIV (a primate virus closely related to HIV-1) appear to be protected by soluble CD4 (25). Surprisingly, soluble CD4 also lowers viral titer in lymphocytes and bone marrow cells isolated from animals with pre-existing SIV infections (25).

Therapeutic proteins with short systemic half-lives are sub-optimal for the long periods of chronic invtravenous administration contemplated for clinical treatment of HIV-infected patients. Soluble CD4 has a 15 minute systemic half-life in rabbits and a 30 to 120 minute half-life in humans (26). A recombinant chimera of the soluble CD4 and the constant region of a human IgG1 heavy chain has been generated (26). One of these "immunoadhesin" constructs retains the ability to bind HIV gp120 and block T-cell infectivity, but has a systemic half-life of 48 hours in rabbits. The immunoadhesin retains the affinity of IgG_1 for F_c cell receptors (K_d of 3 nM), but cannot bind or activate complement, possibly due to insufficient mobility of the C_H region (8).

Another extension of the use of soluble CD4 to block synctitium formation is to use the molecule to target toxins to infected T-lymphocytes. A chimera of the soluble CD4 receptor with pseudomonas exotoxin was capable of killing CV-1 cells expressing HIV envelope protein (27). The effective IC_{50} for the chimera was subnanomolar, with a selectivity of at least 100-fold vs. the same cells infected with vaccinia virus or a control non-infected human T-cell line.

Synthetic peptides derived from the sequence of CD4 have been used to block the interaction between gp120 of HIV and CD4. Dibenzylated peptide derivatives of CD4 residues 83-94 possess antisynctial and antiviral activity at 125 μM concentration (28). Other peptides derived from CD4 sequences both N- and C-terminal to residues 83-94 are inactive in these assays (28).

Detoxification with Soluble Receptors - The principle of blocking cell receptor uptake of ligands with engineered soluble receptors is being extended to other areas of medicine. Engineered antibodies against digoxin are in clinical use for detoxification (29). The cloning of the insulin receptor and expression of a mutant with a transmembrane region may yield an agent for rapidly

depleting high levels of insulin (30). A 17-amino acid sequence of the nicotinic cholinergic receptor is capable of binding α-bungarotoxin ($K_D = 10^{-7}$ M), α-cobratoxin ($K_D = 10^{-6}$ M) and *d*-tubocurarine ($K_D = 10^{-4}$ M) (31). Although these affinities are several orders of magnitude below that of the holoreceptor, further engineering of these "molecular decoys" should lead to agents with long systemic half-lives and higher ligand affinity.

CYTOKINES AND POLYPEPTIDE GROWTH FACTORS

Interleukin 2 - This cytokine is produced by activated T-cells and stimulates proliferation of T-cells, cytotoxic T-cells and natural killer cells. Interleukin 2 (IL2) is effective as an anti-tumor agent alone or in combination with conventional chemotherapeutic agents (32-34). IL-2 has been the subject of structure-function studies by physical and genetic analyses. A high-resolution crystal structure of the protein indicates that four α-helices are present as an anti-parallel bundle (35). The receptor-binding region is apparently formed by residues on helices 1,2 and 4 as deduced from structure-activity relationships determined by deletion analysis and by point mutation (36-39).

IL2 is produced for clinical use in a recombinant process using E. coli as host cell (40,41). The protein forms insoluble inclusion bodies in the bacterium and must be renatured by treatment with the chaotrope guanidinium chloride and the reducing agent glutathione, followed by mild oxidation (41). Variants of IL2 have been engineered which are more stable to these reactions. A variant of human IL-2 in which cysteine^{125} was replaced with serine was generated to prevent formation of a non-native disulfide bond between cysteine^{125} and cysteine^{58} or cysteine^{105} during renaturation (41,42). The latter two cysteine residues form the disulfide bond in native human IL2 that is essential for biological activity (43). The serine^{125} IL2 variant has wild-type biological activity in a mouse T-cell proliferation assay (42). Methionine^{104} which is labile to oxidation during purification and storage of the protein, has been replaced by serine or alanine to yield IL2 variants which are stable to oxidation by chloramine T or H_2O_2 (44).

The T-cell specificity of IL2 has been exploited in the construction of IL2-toxin fusion proteins. Such chimeras have potential as treatments for T-cell-mediated autoimmune diseases such as rheumatoid arthritis and AIDS (45). The gene for a truncated diptheria toxin protein (which lacks 50 residues of the C-terminal region of the B-chain) has been fused with amino acids 2-133 of IL2 (46). This chimera retains the ability to ADP-ribosylate elongation factor (EF2), as well as specific binding to human peripheral blood mononuclear cells (47). The chimera is at least 500-fold more potent in killing T-cells compared to cell lines which do not express IL2 receptor (46). The immunosuppressive activity of this molecule has been demonstrated in mice, where a dose of 50 ng/day could suppress delayed-type hypersensitivity, a T-cell mediated response (48).

A similar chimeric IL2-toxin using the pseudomonas exotoxin A has been tested in a rat arthritis model and found to delay the onset and decrease the magnitude of arthritis as determined by histological and radiologic studies of joints (49,50). Neutralizing titer to the toxin moiety was detectable after three weeks and these antibodies could potentiate the action of this molecule (50).

Interferons - This family of cytokines has been divided into three classes - interferon (IFN) α, ß, and γ. IFNs have antiviral, antiproliferative and immunomodulatory functions and have been used therapeutically for each of these activities (51). Recombinant IFN-ß is produced in E. coli and is labile to oxidation and malfolding . A fully biologically active variant of IFN-ß with a substitution of serine for unpaired cysteine^{17} has been produced (42). Similar substitutions have been made in IFN-α at cysteine^{1} and cysteine^{98}, with both single-mutants and the double mutant having wild-type antiviral activity (52). Replacement of oxidation-sensitive methionine^{62} of IFN-ß with alanine yielded a variant with 13% of the antiviral activity of the parent protein (42). IFN-γ is also produced in E. coli, and its cDNA has been altered by site-directed mutagenesis to remove an N-terminal cysteine-tyrosine-cysteine sequence (53). The resulting IFN-γ variant is expressed at ten-fold higher levels than the parent cytokine by protein analysis and at 500-fold higher levels than the wild-type IFN-γ.

Second-generation IFNs are being engineered which have less pleiotropic effects and may potentially have fewer clinical side-effects than the natural IFNs (51). Although deletions of regions of these molecules can eliminate their bioactivity completely, functionally active chimeras of IFN-α and IFN-ß have been constructed (52). A hybrid INF with amino acids 1-81 and 106-166 derived from IFN-β and residues 82-105 derived from IFN-α has a higher ratio of antiviral to antiproliferative

and immunostimulatory activity than the parent molecules (54). A hybrid of residues 1-41 of IFN-α1 and residues 47-166 of IFN-ß1 was reported to have only antiviral activity (55). IFN-α1/IFN-ß1 hybrids have also been constructed which have no antiviral activity but have potent antiproliferative and natural killer cell stimulating activities (55). A biologically active hybrid of IFN-α2/α1 has been constructed, albeit with antiviral and antiproliferative activities intermediate between those of the parent cytokines (56). Chimeras of mouse and human IFN-α have been constructed and a model of the three-dimensional structure of IFN has been used to propose loop regions which may be important in binding to the high affinity receptors on human cells (57).

Tumor Necrosis Factor (TNF) - This macrophage-derived cytokine has been clinically evaluated for its anti-tumor effects. Recombinant TNF is produced in E. coli (58). Site-directed mutagenesis of the TNF gene has produced biologically active TNF variants in which cysteine^{69} and cysteine^{101} have been replaced with serine residues (59). Nine or ten-amino acid deletions at the N-terminus also yield TNF variants which are biologically active and may be more efficiently processed by the bacterial N-terminal methionine by methionyl aminopeptidase (59, 60). Additionally, variants with arginine residues replaced appear to be more homogeneous and stable than the parent cytokine (60).

Growth Factors - Granulocyte-colony stimulating factor (G-CSF) has five cysteine residues, four of which form disulfide bonds (61). Mutation of the unpaired cysteine^{18} to a serine residue generates a variant with wild-type biological activity (61). Two of four cysteine residues of basic fibroblast growth factor (bFGF) have been mutated to serine (62). The modified bFGFs are fully active and more stable and less heterogeneous under acidic conditions than the parent protein (62). Transforming growth factor-α (TGF-α) has been fused to pseudomonas exotoxin (PE) by a similar strategy as the IL2-PE toxin construction (63). The chimeric TGFα-PE toxin is a potent cytotoxic agent for tumor cells which overexpress the epidermal growth factor receptor and may be useful in treating carcinomas which overexpress this receptor (63).

HORMONES

Growth Hormone (GH) - Native growth hormone is a predominantly α-helical 22kD protein which is found in all vertebrates and regulates growth of the animal (64). The biochemical activities induced by growth hormone include protein synthesis (growth-promotion), increase and decrease in blood glucose levels, and breakdown of storage lipids. Pituitary-derived human growth hormone (hGH) was the only source of this protein prior to the development of the recombinant E. coli-derived hormone (65,66). The first recombinant hGH proteins contained an N-terminal methionyl residue which is removed by proteolysis during the isolation of the pituitary-derived product. The recombinant GH (Met-hGH) was found to be more immunogenic than pituitary hGH (67). However, the immunogenicity of Met-hGH was shown to be due to protein contaminants and not a function of the methionyl N-terminus (68). Recombinant hGH that did not contain a methionine at the N-terminus and Met hGH were indistinguishable in their bioactivity and pharmacokinetics (69,70).

Recombinant bovine growth hormone (bGH) has been produced in E. coli (71) and has identical physical properties and bioactivity as bovine GH (72). Two variants of bGH have been generated with altered N-termini, one with a point mutation (alanine to methionine), the other with a truncation of eight N-terminal residues (73). These variants have been found to be similar to bGH in receptor-binding and in a rat bioassay (73). The truncated variant is expressed at high levels in E. coli, and is a candidate for clinical trials (73).

The apparent tolerance of the N-terminus of GH to mutation contrasts with the sensitivity to mutations in internal segments of the protein. By analysis of GH conserved domains in ten species, four homologous regions of the GH from ten different species have been identified which may be involved in binding to the GH receptor (74). Three of these regions fall in the helical segments of the protein (64). Deletion of the C-terminal region of hGH (residues 139 - 132) results in a variant with insulin-like growth factor (somatomedin-like) activity. An allelic variant of hGH which lacks residues 32-46 (C-terminal to the first helical region) apparently lacks insulin-like activity (75). Second-generation GHs with deletions in this region have been generated. A porcine GH analog lacking residues 32-38 was found to have a 40% higher potency in a rat weight gain assay than porcine GH (76). A bovine GH analog lacking residues 33-39 was found to increase growth rate and milk production in farm animals (77).

Insulin - The success of injected porcine insulin and recombinant human insulin to control blood glucose levels in diabetes has stimulated efforts to develop engineered insulins that more closely mimic normal fluctuations in hormone concentration. Insulin is a dimeric hormone which associates into hexamers in the presence of millimolar Zn^{2+} concentrations. Conventional prolonged-acting insulins are administered as suspensions, which require sufficient agitation to be injectable. Engineering of insulin has been applied to generating long-acting forms which can be injected in soluble form and will subsequently form crystals in the tissue (78,79). Tryptic transpeptidation has been used to substitute alanine^{130} of the B-chain (the C-terminus) of insulin with basic or hydrophobic amino acids (78). Recombinant insulins with the C-terminal alanine replaced with lysine or arginine crystallize at pH 7 and have prolonged action in vivo (78). Recombinant insulin produed with negatively charged amino acid derivatives at the C-terminus did not have prolonged action (78). Insulins produced with hydrophobic residues at the C-terminus remain soluble at pH 7, but exhibit protracted activities, possibly due to interaction with tissue (78). Additional substitutions to alter surface charge can further prolong the in vivo action of insulin analogs (79). A substitution of threonine^{13} of the B-chain with lysine or arginine promotes crystallization, possibly by promoting packing of the Zn^{2+} hexamers. A replacement of glutamic acid^{13} with glutamine also promotes crystallization, possibly by removing a negative charge from the center of the hexamer (79). The stability of insulin variants at acid pH used in their formulation can be increased by replacing asparagine^{21} of the A-chain which can deamidate or form intermolecular covalent dimers (80). Replacing this residue with glycine aspartic acid or histidine generates variants with five to ten-fold lower rates of deamidation or dimerization than the corresponding insulin variant with asparagine^{21} (or than native insulin).

Insulin variants have been produced which are absorbed more readily than native insulin and could be used to mimic the rapid surge of insulin that occurs after a meal. This has been accomplished by mutating neutral amino acids at the monomer-monomer interface to acidic residues (81). These insulin variants become monomeric at acidic and neutral pH. As such, they are absorbed two to three times more rapidly than human insulin after subcutaneous injection. These derivatives are being clinically evaluated in diabetics (81).

ENZYMES AND ENZYME INHIBITORS

Plasminogen Activators - The serine protease enzymes urokinase and tissue-type plasminogen activator (tPA) cleave the zymogen plasminogen to the active enzyme plasmin. They are in clinical use as exogenous thrombolytic (clot-dissolving) agents for acute myocardial infarction and pulmonary embolism. There is intense interest in improving the pharmacokinetics, fibrin-affinity, catalytic activity and lowering side effects of these enzymes. Several recent review articles summarize protein engineering studies of urokinase and tPA (82-84).

Factor VIII - This protein is a non-enzymatic clotting cofactor which participates in the Factor IX_a-catalyzed activation of Factor X to X_a. Hemophilia A, a bleeding disorder, is due to a deficiency of this protein, and can be treated with Factor VIII. Recombinant Factor VIII is a 2351-amino acid protein which is produced in mammalian cell culture (85). The recombinant Factor VIII consists of three types of structural domains. The central "B" domain comprises residues 771-1666 and has been deleted in one variant (86,87). The deletion variant is expressed at 5-fold higher levels than the parent protein (86). This variant appears to be fully active as a procoagulant, in binding to von Willebrand factor, and is activated to a greater extent than the parent protein by thrombin (86).

Antitrypsin - This protein is the physiological inhibitor of neutrophil elastase. Deficiency of a_1-antitrypsin (α_1-AT) can be heriditary or induced by oxidative damage caused by free radical species in cigarette smoke. α_1-AT normally protects lung connective tissue from damage by elastase (pulmonary emphysema), and is being evaluated clinically for treatment of this disease. α_1-AT contains an oxidatively-labile methionine^{358}. This residue has been replaced with several non-polar residues or arginine (87). One α_1-AT variant, leucine^{358}, was found to be refractory to oxidative inactivation by N-chlorosuccinimide (88), and to be a potent inhibitor of elastase and the protease cathepsin G (87). A variant with an arginine^{358} substitution was found to be inactive as an elastase inhibitor, but a potent inhibitor of thrombin, which is the terminal protease of the clotting cascade (87). This variant was discovered in a severe bleeding disorder (89).

Hirudin - A 65 amino acid thrombin inhibitor, hirudin, is secreted from the leech salivary gland. Hirudin has been produced by recombinant methods, and is being evaluated clinically as an

anticoagulant. Several naturally-occurring hirudin variants have been sequenced (90). A point mutation in a hirudin variant HV2 was made which was predicted to bind with higher affinity to the thrombin active site (90). This lysine^{47} HV2 variant is four-fold more potent than the parent protein in vitro, and extends clotting time 20-fold longer in a rabbit model, raising the prospect of using significantly lower therapeutic doses of the hirudin variant.

SUMMARY

The improvements in each of the major classes of therapeutic proteins by site-directed mutagenesis will continue. As new second-generation protein therapeutics are tested in humans, the clinical data generated will guide future protein engineering efforts. In a very short period of time, pre-clinical studies reviewed herein suggest that protein engineering has been successful in improving stability, pharmacokinetics and potency of many protein drugs. Perhaps most exciting have been the creation of novel therapeutics, such as chimeric proteins and soluble receptors, which could not have been generated without these techniques.

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Chapter 24. Exogenous Growth Factors in Dermal Wound Healing

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Introduction - Growth factors can regulate the migration, proliferation and differentiation of a wide variety of cell types that may play a role in tissue repair. These peptides act in a paracrine or autocrine fashion through a variety of mechanisms mediated by cell surface receptors (1-4). Several growth factors are related to cellular oncogenes (2). The clinical goal for growth factors is to accelerate healing in normal wounds, to initiate healing in impaired wounds, or to limit uncontrolled repair. Factors with the ability to recruit or regulate inflammatory cells, to induce proliferation of endothelial cells, fibroblasts or epithelium, to stimulate matrix synthesis or epithelial migration, or to limit the extent of these processes may be useful in wound healing (5). Table 1 summarizes the principal growth factors with relevance to wound healing. For a given factor, the existence of appropriate biological activity in vitro leads to in vivo investigations of preclinical efficacy. Due to the paracrine or autocrine nature of these factors, local application is usually preferred.

Animal Models - Recent compendia illustrate the range of growth factors currently under investigation and the variety of animal models in use (6,7). Each of the several animal models used to assess preclinical efficacy has particular advantages and disadvantages. Relatively uncomplicated subcutaneous models are utilized to evaluate the chemoattractant, mitogenic and stimulatory activities of a factor. The factor may simply be injected by itself or it may be injected into a previously implanted foreign body such as polyvinyl alcohol sponge or a wire mesh chamber. Alternatively, the factor may be formulated with a carrier vehicle such as collagen or carboxymethyl cellulose (CMC) which is then implanted as a composite. Such delivery vehicles make it easier to recover any induced tissue for study, but the foreign body may significantly alter the biological response. Wound contraction and reepithelialization cannot be studied in subcutaneous models.

Factors with the ability to enhance connective tissue may be studied using incisional skin wounds which are surgically closed with suture or staples following treatment. Such models are convenient because they permit direct measurement of wound strength, but they make recovery of tissue samples difficult. Dermal wounds may also be created by surgically excising a portion of dermis and allowing the defect to heal without surgical intervention using wound strength measurements or histological methods to evaluate healing. Wound contraction, however, plays a greater role in the healing of such wounds in animals than it does in human wounds. Wounds may also be created in other organs than skin, for example, in corneal stroma. When interest is in the effect of a factor on epithelial repair, rather than on connective tissue, then very shallow wounds are created using a keratome, trauma or suction to remove epithelium with little or no connective tissue involvement. Burns may also be created using a variety of methods.

In all of these models, healing normally occurs rapidly and without complication. Accordingly, to simulate chronic wound healing, the animals may be treated so that healing is impaired. Experimental diabetes may be induced by administration of streptozotocin. Topical or systemic corticosteroids, or systemic antineoplastic agents may be used to impair healing through multiple mechanisms. Models of impaired wound healing are the most rigorous tests of a factor's therapeutic potential. In addition, models of compromised healing may be useful in defining the specific actions of a factor. No well characterized animal model is widely accepted as a standard model of clinical wound healing in general, or of the chronic, non-healing wound in particular. Ultimately, the real value of any animal model, and of any growth factor, will only be known when clinical trial data eventually become available and it can be shown that the animal model results correlate with clinical utility.

Epidermal Growth Factor (EGF) - EGF and the homologous molecules, transforming growth factor-α (TGF-α) and vaccinia virus growth factor (VVGF), can stimulate the proliferation of a number of cell types which display cell surface EGF receptors including epithelium, fibroblasts, and endothelium (8). EGF is present in secretory glands and in human urine, where it is known as urogastrone (8,9)

EGF is associated with platelet alpha granules and TGF-α is synthesized by keratinocytes both of which may occur naturally at sites of wound healing (10,11). Endogenous EGF is detectable in experimental granulation tissue in rats, and TGF-α is expressed by activated wound macrophages (12,13).

TABLE 1.
PEPTIDE GROWTH FACTORS WITH APPLICATION TO WOUND HEALING[1].

FACTOR	SOURCE	TARGET CELL	ACTIVITIES IN VITRO	EFFECTS IN VIVO	IN VIVO MODELS
EGF / TGF-α	Epithelium Platelets Macrophages	Fibroblasts Epithelium Endothelium Smooth Muscle	Proliferation Contraction	Angiogenesis Fibroplasia Reepithelialization Wound contraction	Subcutaneous Epithelial defects Burns Open wounds Corneal stroma
FGF	Fibroblasts Endothelium Macrophages Smooth muscle	Fibroblasts Endothelium Epithelium Smooth muscle	Proliferation Differentiation Migration Matrix synthesis	Angiogenesis Fibroplasia Reepithelialization Wound contraction	Subcutaneous Incised wounds Open wounds Skin grafts Corneal stroma Epithelial defects
TGF-ß	Platelets Macrophages Lymphocytes Epithelium Fibroblasts	Fibroblasts Epithelium Endothelium Monocytes Lymphocytes	Matrix synthesis Inhibition Chemotaxis Contraction	Angiogenesis Fibroplasia	Subcutaneous Incised wounds Open wounds
PDGF	Platelets Macrophages Endothelium Smooth muscle	Fibroblasts Smooth muscle Monocytes Neutrophils	Proliferation Matrix synthesis Activation Chemotaxis	Fibroplasia	Subcutaneous Incised wounds Open wounds
NGF	Epithelium Fibroblasts Fibroblasts	Neutrophils Monocytes	Chemotaxis	Wound contraction Inflammation	Subcutaneous Open wounds
TNF-α	Macrophages Endothelium	Fibroblasts Macrophages Neutrophils Lymphocytes	Inhibition Activation	Angiogenesis Fibrosis Differentiation	Cornea Incised wounds
IL-2	Lymphocytes	Lymphocytes	Proliferation	Fibrosis	Subcutaneous Incised wounds
INF-γ	Lymphocytes Macrophages	Fibroblasts	Inhibition	Inhibition	Subcutaneous

[1] The activities and effects listed do not correspond directly to cell types on the same line in the table. Target cells may respond in several ways to a given factor. The list is not exhaustive. See text for abbreviations and details.

Natural and recombinant human EGF (rhEGF) stimulate cellular proliferation as well as protein and collagen synthesis in rat subcutaneous models and in transected achilles tendon (14-20). Recombinant human TGF-α was found to be more potent than purified mouse EGF (mEGF) in promoting angiogenesis in the hamster cheek pouch assay, although both factors were equipotent in vitro (21). mEGF had no local effect when injected into the subcutis of neonatal mice, while the same amount of transforming growth factor ß (TGF-ß) stimulated a pronounced connective tissue accumulation (22). Again, mEGF had no effect on the colonization of collagen gels contained in Hunt-Schilling wound chambers in the subcutis of doxorubicin treated rats, but there was a synergistic interaction between EGF, platelet derived growth factor (PDGF) and TGF-ß, but not between EGF and the other factors alone (23). mEGF had no effect on the DNA content or histological appearance of a collagen vehicle contained in tubes implanted subcutaneously in rats, but, in contrast, PDGF, TGF-ß and basic fibroblast growth factor (bFGF) were active (24). EGF did not interact with the other factors. The failure of EGF to produce an effect in these subcutaneous models when applied once may reflect the relatively rapid clearance of the molecule from the implants. Only 10% of radiolabeled EGF could be detected in cellulose sponges 4 hours after implantation (14).

The effect of EGF on both mesenchymal and epidermal repair has been studied in partial and full thickness dermal wounds with inconsistent results. Local and intradermal treatment with EGF accelerated reepithelialization of full thickness wounds in the skin of fetal rabbits (25). Topical application of mEGF to full thickness dermal wounds appeared to enhance wound contraction in normal and sialectomized mice, while systemic delivery from an intraperitoneal osmotic pump was less effective (26). Reepithelialization was not affected by EGF treatment (26). In contrast, EGF, PDGF, and FGF had no effect on the contraction of full thickness wounds in hamster skin (27). EGF accelerated granulation tissue formation and reepithelialization in full thickness rabbit ear skin wounds (28). rhEGF accelerated reepithelialization in split thickness dermatome wounds in domestic pigs when applied in lanolin or cream vehicles in one study but not another (29-31). A single application of EGF in CMC gel enhanced the epithelial healing of partial thickness dermal wounds in pigs but had no effect on connective tissue repair (32). TGF-α combined with insulin-like growth factor-1 (IGF-1) promoted healing in this model (33). Slow release of rEGF from liposomes increased the tensile strength of rat incisional wounds, whereas a single treatment was ineffective (34).

EGF has produced equivocal results on the healing of dermal burn wounds. mEGF had no effect on rat burns when given in silver sulphadiazine cream or delivered intraperitonealy (35,36). Partial thickness burn wounds in domestic pigs were reepithelialized more rapidly when treated with topical rhEGF in silver sulphadiazine cream (30, 37). TGF-α and VVGF were also active in this model (37).

EGF was not as effective in promoting healing in models where damage to non-epidermal structures was minimal even with repeated application at higher doses. EGF had no effect on reepithelialization of suction blister wounds in humans when treated daily or in guinea pigs when treated once using either aqueous or CMC vehicles, or on reepithelialization of tape stripped wounds in rats when treated topically or intraperitonealy (36,38,39). In these models, it is likely that the basement membrane was still intact (40).

A major focus of investigation has been the role of EGF in the repair of both epidermal and stromal defects of the cornea (41). Multiple topical application of EGF accelerated reepithelialization of rabbit corneal wounds created by iodine fumes, alkali burns or surgical denudation even when the preparations were inactive in epidermal wounds or scalds (36,42-47). Fibronectin, which by itself produced a slight stimulation of healing did not enhance the effect of EGF (46). rhEGF significantly accelerated reepithelialization of monkey corneas subjected to experimental epikeratophakia and stimulated elevated late cellularity and collagen content (46). No epithelial downgrowth occurred along suture tracks when full thickness incisions were made in the clear cornea or the corneal-scleral boundary of monkey eyes which were subsequently sutured and treated with EGF (46). Enhanced epithelial proliferation in rabbit corneas was transient, even when EGF treatment was continued (47). Exogenous ^{125}I-EGF rapidly disappeared from tear film in monkeys, with 85% being lost within 10 minutes (46). rhEGF reversed the impairment of corneal reepithelialization by treatment with corticosteroids (48). In another report, however, EGF failed to enhance reepithelialization of rabbit corneas treated with dexamethasone (49). In a further study, EGF did accelerate reepithelialization in the absence of steroid, but a dose effect could not be demonstrated (50). Corneal endothelium displays cell-surface receptors for EGF, and EGF injected into the anterior chambers of rhesus monkeys resulted in elevated numbers of corneal endothelial

cells ten weeks following denudation of the central corneal endothelium during autograft transplantation (51).

Mouse EGF was mildly immunogenic in rabbits when administered by a variety of routes, with topical application producing the least immunogenic response (52). All routes induced cell mediated immunity, but circulating antibodies were produced only in animals treated with EGF systemically in the presence of adjuvant. Only those animals with circulating antibodies responded adversely when challenged by topical corneal application of EGF. The regenerated epithelium showed abnormal morphological changes, and cessation of treatment led to secondary breakdown of the repaired sites. Studies which confirmed accelerated reepithelialization of corneal alkali burns in eyes treated with rhEGF also noted severe inflammation or a transient sloughing of the regenerated epithelium (44, 45).

The therapeutic potential of EGF in the treatment of stromal wounds has been addressed in several preclinical and clinical studies. The amount of an EGF-like substance in tear fluid as well as the amount of EGF in plasma were significantly reduced in sialoadenectomized mice (53). When superficial corneal wounds were induced in these animals, approximately 50% of the subjects developed severe ocular lesions or loss of sight. These changes were prevented or reversed by topical application of EGF (53). The tensile strength of perforating corneal wounds in rabbits was increased by EGF treatment (54). rhEGF also increased the bursting strength of full thickness penetrating incisions through the cornea of monkeys impaired by topical steroid application (48). In the absence of dexamethasone, EGF or insulin significantly enhanced the tensile strength of full thickness incisions in rabbit corneas, but in the presence of dexamethasone, EGF was superior (55). In a similar experiment with penetrating wounds in monkey corneas, rhEGF had no effect on wound strength in the absence of dexamethasone, but did enhance the strength of dexamethasone treated wounds after 4 weeks of treatment (56).

Clinical trials have been initiated to study the ability of EGF to promote epithelial repair. mEGF did not enhance healing of penetrating keratoplasty in a closed label study with 35 patients (57). Two of three patients with long-tern corneal ulceration showed improvement or healing after 33 - 42 days of treatment with mEGF (58). Administration of mEGF to 104 patients with a variety of nondystrophic lesions of the corneal epithelium resulted in significant acceleration of healing of most lesions (59). Unsatisfactory results followed treatment of metaherpetic or stromal keratitis and bullous keratopathy (59). In patients with successful treatment, mild hyperemia was noted, and long term follow up revealed no late complications or relapse. An ophthalmic formulation of EGF is on the market in Europe (60).

Fibroblast Growth Factor(FGF) - Two forms of FGF with differing isoelectric points and 55 - 60% sequence identity are known (61-64). FGF is present in a variety of tissues and receptors for the molecule are present on many cell types; however FGF is only secreted when cells are damaged. (61,62). Both forms of FGF are potent mitogens and differentiation factors for a wide variety of mesodermal derived cell types in vitro , although basic FGF (bFGF) is more potent than acidic FGF (aFGF) (61-64). Both forms interact strongly with heparin (61-64). FGF can stimulate fibroblasts, vascular endothelial cells and keratinocytes to proliferate, and therefore may play a role in wound healing (61-65).

FGF is strongly angiogenic in the chick chorioallantoic membrane assay (66, 67). Both bFGF and aFGF stimulate matrix production and cell proliferation in a variety of rat subcutaneous models (24, 68-74). In polyvinyl sponges, rbFGF was injected into the implants at day 3 following implantation, and the tissue response was seen even though the FGF stimulus was transient, with disappearance of about 90% of the factor by four hours (70). Repeated injection of rbFGF did not further augment the response (70). The response in this model could be partially abrogated by treatment with a slow release formulation of a polyclonal antiserum directed against bFGF (71). Peritoneal placement of a collagen vehicle containing aFGF induced the formation of vascular bridges between isolated organs, and these bridges could serve as host sites for transplanted rat hepatocytes (73).

Injection of recombinant bFGF in surgically closed incisions in rat skin, a model for normal wound healing, on the third day following surgery resulted in elevated wound breaking strength, but no increase in wound collagen was detected biochemically (74). Endothelial cell-derived FGF enhanced vascularization and viability of isologous island skin flaps in rabbit ears when applied at the tissue interface in a collagen vehicle, but not when applied alone (75). Daily topical and subcutaneous application of aFGF in the presence of heparin enhanced the breaking strength of

linear incisional wounds in rat skin and accelerated the closure of full thickness dermal wounds in the skin of mice and rats (76). FGF had no effect on full thickness wounds in hamster skin (27). In a model of impaired wound healing, FGF had no effect on wound strength when applied as drops to steroid-treated penetrating incisions of the corneal stroma, although EGF was effective (55). FGF may also have potential in treating epithelial defects. Both aFGF and bFGF accelerated reepithelialization of denuded rabbit corneas without an increase in vascularity (77). Interestingly, aFGF was more potent than bFGF, whereas bFGF is more potent in most in vitro studies (62, 63). bFGF derived from bovine retina was able to accelerate reepithelialization of guinea pig suction blister wounds when applied in water or CMC gel, where EGF had no effect (39).

Transforming Growth Factor Beta (TGF-ß) - TGF-ß is a multifunctional growth factor with the ability to regulate the migration, growth and differentiation of many of the cell types involved in tissue repair (2,78-80). The two molecular forms of this peptide, TGF-ß1 and TGF-ß2, have about 70 % sequence identity and display similar activities (78). Most cells have receptors for TGF-ß, and many cell types synthesize the factor (78). Large amounts of TGF-ß are present in platelet alpha granules and in wound macrophages, suggesting a natural role for the factor at sites of tissue damage and repair (2, 13, 78-80). TGF-ß appears to enhance the deposition of connective tissue matrix by stimulating the migration of fibroblasts and by inducing synthesis of matrix molecules and protease inhibitors (2,78,79). In addition, TGF-ß may play a role in tissue repair by serving as a potent chemoattractant and regulator of inflammatory cells that secondarily affect the repair process (2). A transient increase in the endogenous TGF-ß present in wire mesh wound chambers subcutaneously implanted in rats accompanied fibroblast colonization and collagen synthesis (81). The effects of TGF-ß on a given cell type may vary dramatically depending on the substrate, cell density and the presence of other growth factors (2).

Exogenous TGF-ß from bovine salivary glands or kidney stimulated increased amounts of protein, collagen and DNA when injected into wire mesh wound chambers implanted subcutaneously in rats in combination with murine EGF (82). However, TGF-ß is active in the absence of other exogenous growth factors. Injection of human platelet TGF-ß into the nuchal subcutaneum of neonatal mice induced an accumulation of vascular connective tissue at the injection site in a dose dependent way, while EGF had no effect (22). The response disappeared in a matter of days when treatment was discontinued. TGF-ß stimulated elevated levels of DNA in a collagen gel used to fill tubes implanted subcutaneously in rats (24). The use of radiolabeled factor showed a rapid loss of 60% of TGF-ß in the first 24 hours, followed by a slower loss to about 10 % over the succeeding nine days (24). Combination of TGF-ß with EGF, PDGF, or aFGF did not enhance the response when compared to the effect of either factor alone (24). TGF-ß was more effective than PDGF or EGF in reversing the doxorubicin-induced impairment of colonization of wound chambers filled with rat tail collagen, and the response was enhanced by PDGF and EGF (23). In the guinea pig subcutis, glycosaminoglycans, and hyaluronate in particular, were specifically elevated in newly formed capsule tissue around osmotic pumps when delivering TGF-ß, but not PDGF (83). Fetal wounds normally undergo repair without inflammation and with only a trace of collagen deposition (84). However, when polyvinyl alcohol sponges containing TGF-ß were implanted subcutaneously in fetal rabbits, the number of inflammatory cells was elevated and a vigorous fibrotic response occurred; but when TGF-ß was absent, the implants contained only glycosaminoglycans (85).

TGF-ß increased collagen and elastin mRNA in incisional wounds in pig skin (86). The breaking strength of linear incisional wounds in rat skin, a normal wound model, was elevated when a single application of TGF-ß formulated in a collagen gel was made at the time of wounding and closure (87). In contrast, in a similar model, a single treatment with EGF was ineffective but slow release of EGF did increase wound strength (34). TGF-ß produced only a very weak response when delivered in a saline vehicle (88). This accelerated wound repair was accompanied by increased numbers of mononuclear cells and fibroblasts (88). When this same model was studied in animals treated with corticosteroids to inhibit wound healing, it was found that TGF-ß could restore wound strength to control levels, while a larger dose of PDGF could not (89). Wounds treated with TGF-ß contained elevated numbers of fibroblasts that stained positively for type I procollagen, while little or no procollagen was seen in wounds treated with PDGF. When this model was studied in rats impaired by treatment with doxorubicin, TGF-ß, but not rEGF, even at a 25 fold greater dose, induced a transient increase in wound strength (90). In mouse excisional wounds, TGF-ß1 and TGF-ß2 partially reversed the inhibition of granulation tissue deposition caused by application of an occlusive film dressing, and these inhibited wounds were also characterized by decreased numbers of activated macrophages (91).

Platelet-Derived Growth Factor (PDGF) - PDGF is a major protein in platelets and is produced by activated macrophages and vascular endothelial cells(92, 93). It is mitogenic and chemotactic for most mesodermal cells including fibroblasts and smooth muscle cells, and is both chemotactic and activating for inflammatory cells (92, 93). PDGF could play a direct role in wound repair by recruiting and stimulating the proliferation of fibroblasts, and by attracting leukocytes which may secondarily influence repair through elaboration of their own regulatory molecules (92,93).

Human PDGF increased colonization and organization of subcutaneous tubes filled with collagen in rats (24). The effect was enhanced when heparin was present in the formulation, but this was not due to a reduction in the rate of release from the implants. Rapid loss of 90 % of PDGF occurred within 24 hours, followed by a slower release over nine days (24). The response was not altered when PDGF was combined with EGF or TGF-ß, but it was enhanced when aFGF was added in the presence of heparin (24). Purified PDGF was inactive in pig partial thickness wounds, but enhanced repair of dermis and epidermis was seen when PDGF was combined with insulin-like growth factor 1 (32, 33).

A series of studies of PDGF and TGF-ß in the same models is important in suggesting the specific actions of each factor in wound healing. PDGF stimulated accelerated influx of connective tissue cells, as well as increased collagen synthesis in subcutaneous wound chambers in normal rats (94). Colonization and matrix synthesis in the chambers was significantly reduced in rats rendered diabetic by treatment with streptozotocin, but this deficit could be restored by addition of PDGF (94). However, when the same experiment was performed in rats impaired by treatment with doxorubicin, PDGF or EGF had no effect on cellular proliferation or on the production of protein and collagen , while TGF-ß alone significantly reversed the doxorubicin-induced deficit (23). A significant enhancement of the TGF-ß effect was observed in the presence of PDGF (23). Since doxorubicin treatment reduces the level of circulating leukocytes (95), it is possible that TGF-ß acted independently while PDGF failed to have an effect in the absence of mediation by leukocytes. In contrast, the leucocyte population in the diabetic animals was probably normal, so that PDGF activity could be detected. A similar conclusion was suggested by experiments using PDGF or TGF-ß to treat surgically closed incisional wounds in the skin of normal and steroid-treated rats (89, 96). In normal animals, 20 µg of either platelet-derived or of recombinant PDGF stimulated increased wound breaking strength when applied in a collagen gel at the time of wounding and closure (96). In contrast, PDGF was not able to reverse the loss of wound strength induced by treatment with methylprednisolone, while TGF-ß could restore strength to control levels (96). Treatment with methylprednisolone could have reduced the leucocyte population producing a decrease in the level of an unidentified cell-mediated activity that is required by PDGF but not TGF-ß (89)

Platelet Releasates - Experiments with purified growth factors suggest that the normal process of wound healing involves specific interactions among at least two of the factors released from platelet alpha granules at sites of tissue damage. Naturally occurring combinations of platelet-derived factors have also been investigated directly. When platelets are isolated and the contents of the alpha granules are released by homogenization or exposure to thrombin, the result is a crude mixture (releasate) of factors normally involved in wound healing, most prominent of which are PDGF and TGF-ß. TGF-ß is normally latent, however, and requires activation by acidification or enzyme activity so that it is difficult to evaluate its role (78). Autologous thrombin-activated platelets stimulated both neovascularization and elevated collagen production in rabbit corneas (97). Daily injections of an homogenate derived from bovine platelets accelerated the repair of rabbit tibia muscles damaged by crushing (98). Partial thickness dermal wounds in pigs did not respond to a single topical treatment with purified PDGF, but both dermal and epithelial healing were enhanced when treated with a crude mixture of platelet derived factors (32). A similar effect was observed when purified PDGF was supplemented with EGF or insulin like growth factor (IGF-1). (32).

The clinical utility of xenogeneic as well as autologous platelet preparations has been studied directly in humans. Five of six patients treated with topical applications of thrombin-activated bovine platelet lysate two or tree times a week showed improvement or healing, and all seven patients treated with autologous serum showed improvement (99). Healing was achieved in 95 wounds in 49 patients who received daily topical application of a thrombin-induced autologous platelet preparation in a non-randomized open study (100). In an extension of this series, 90% of patients achieved 100% healing following an average treatment of 7.4 weeks, whereas the group had previously experienced conventional therapy for an average period of 96 weeks without healing (101). In a subsequent double-blind, randomized, crossover trial with a placebo control group, all of 14 treatment patients achieved 100% reepithelialization in an average of 6 weeks. Only 3 of 12 control patients healed in a twelve week observation period, but all wounds healed within 6.5 weeks

following crossover to releasate treatment (101). In a separate study, releasate treatment also seemed effective in enhancing the salvage of limbs at risk due to nonhealing wounds (101). In all three of these series, treatment with releasate occurred in the context of total therapy, including surgical debridement and antimicrobial therapy.

Other Factors - A number of other peptides with the properties of growth factors have received limited evaluation for their effects on wound healing. Nerve growth factor (NGF) has been detected in mouse granulation tissue and is secreted by fibroblasts in culture (102). Injection of high molecular weight NGF (HMW-NGF) purified from mouse submandibular glands accelerated the influx of inflammatory and fibroblastic cells into air sacs created in mouse skin, an effect similar to that seen with the chemoattractant f-met-leu-phe (103). HMW-NGF did not affect the rate of contraction or reepithelialization of full thickness dermal punch wounds in hamster skin (104). In contrast, HMW-NGF did accelerate the contraction of full-thickness dermal wounds in sialoadenectomized mice (102).

A number of molecules which function as inflammatory mediators have wound healing activity. Tumor necrosis factor (TNF-α) is a polypeptide which can induce the synthesis of factors with potential regulatory roles in wound repair and can stimulate fibroblast proliferation (105). Recombinant human TNF-α (rhTNF-α) inhibited endothelial cell proliferation in vitro, but induced an angiogenic response when applied topically in rabbit corneas in pellets (105). Recombinant mouse TNF-α was also angiogenic in rat cornea (106). The observed angiogenesis may have been a secondary effect of the inflammatory response that resulted from the treatment. Induction of inflammation through chemotactic activity or induction of secondary mediators was observed following a single injection of rhTNF-α into the foot pads of mice (107). When surgically closed incisional wounds in the dermis of rats impaired by treatment with doxorubicin were treated at the time of surgery with rhTNF-α in collagen gel, wound breaking strength and collagen deposition were significantly elevated (108). In rats where subcutaneously implanted osmotic pumps were used to deliver the inflammatory mediator interleukin 2 (IL-2), the strength of dermal incisional wounds was increased and the amount of collagen in implanted polyvinyl alcohol sponges was also elevated (109). Whereas most of the factors discussed above have been investigated for their ability to enhance impaired wound healing, there is also potential utility in factors that may downregulate components of wound repair which may be excessively active, as in keloid formation or scar contracture. Gamma interferon (INF-γ) is a lymphokine that inhibits collagen formation by fibroblasts (110). Murine INF-γ from subcutaneously implanted osmotic pumps in mice significantly reduced the amount of newly deposited collagen capsule around the pumps (110).

Conclusions - Caution must be exercised in interpreting the results of in vivo studies of growth factors in wound healing. Since many of these factors are biologically active in minute concentrations, it may be difficult to rule out other factors as trace contaminants where purified, naturally occurring factors are studied. Isolated instances of negative results may well be attributable to insufficient doses, to inappropriate delivery systems, or to inactivation of the factor by components of formulations used in concurrent therapy. Finally, detailed technical differences among apparently similar models may lead to inconsistent results. Nevertheless, some generalizations are possible.

Each of the growth factors demonstrates in vivo actions consistent with a role in wound healing. In spite of the variety of specific actions displayed by each factor in vitro, the in vivo effect of all the factors is similar, i.e., increased proliferation of vascular and connective tissue or accelerated reepithelialization (Table 1). In part this is due to the relatively crude observations possible in in vivo systems. But in part it is due to the intricate interactions among growth factors so that the effect of the test material can not be isolated (111). All of the factors have activity in subcutaneous models, and most are active in selected wound models. In many cases, treatment merely accelerates repair, but in some cases it may produce a persisting advantage (87, 88). In many cases, a single treatment is as effective as multiple applications (34, 70, 90). But, in other cases, multiple treatment or even slow release devices may enhance the effect (14, 34, 75, 90). In principle, it seems likely that particular combinations of factors with complementary activities may be optimum for particular indications, and in a few experiments, combinations have given better results (23, 32). Only a little evidence has, so far, emerged, however, that any particular factor has an essential role in normal wound healing (71). Finally, it is also becoming clear that the known activity of a factor in in vitro studies may not be predictive of its actions in vivo (105).

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Chapter 25. Regulation of Neutrophil Chemotaxis

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Introduction-Neutrophils provide a first line of host defense by virtue of their ability to rapidly migrate from the peripheral blood through endothelial gaps and tissue matrices to sites where they eventually engulf and destroy microorganisms. Neutrophils exhibit directed migration towards an increasing concentration gradient of chemical signals (chemoattractants), a response referred to as chemotaxis. Potent neutrophil chemoattractants (reviewed in 1) include a cleavage product of the fifth component of serum complement termed C5a, synthetic N-formyl-methionyl peptides which are structurally similar to N-formyl peptides generated by bacteria (2,3), arachidonic acid lipoxygenation metabolites such as leukotriene B_4, and a variety of cytokines such as the recently described neutrophil activating protein (4,5). Chemoattractants bind to specific plasma membrane receptors and elicit a myriad of biochemical and structural changes in neutrophils. Neutrophil chemotaxis requires the adherence of neutrophils to a surface or substratum, the expression of specific chemoattractant receptors on plasma membranes which bind chemoattractant ligands, and the interaction of cytoskeletal proteins resulting in the assembly and disassembly of critical cytoskeletal elements. Neutrophils from patients who have defects in neutrophil adherence, chemoattractant receptors or cytoskeletal proteins have dramatic chemotactic defects in vitro (6-9). The patients have severe recurrent bacterial infections, marked leukocytosis, and are unable to mobilize neutrophils to sites of infection in vivo.

Since the molecular and cellular mechanisms of leukocyte chemotaxis have been extensively reviewed elsewhere (1, 10-13), this article will briefly review structural and biochemical changes that occur during neutrophil chemotaxis and primarily focus upon the pharmacologic manipulation of neutrophil chemotaxis.

STRUCTURAL AND BIOCHEMICAL CHANGES DURING NEUTROPHIL CHEMOTAXIS

Structural Changes in Chemoattractant-Stimulated Neutrophils- Within seconds after adherent neutrophils are exposed to a chemoattractant, plasma membrane ruffling occurs (14,15) and the cells elongate with a broad lamellipodium formed anteriorly and a thin uropod formed posteriorly (Figure 1). Cytoplasmic actin filaments are assembled (polymerized) near the plasma membrane and appear in increased concentrations in the lamellipodium and uropod (16,17), whereas intermediate filaments are found in the core of the uropod and microtubules are concentrated in the body of the cell extending toward, but not into, both poles of the oriented neutrophil (15, 16). The kinetics of actin polymerization and depolymerization correlate closely with neutrophil shape change and are believed to be crutial events in neutrophil chemotaxis (18-20). During neutrophil polarization, chemoattractant receptors are concentrated near the lamellipodium (21), whereas coated pits, clathrin-containing structures that mediate pinocytic uptake, are clustered at the uropod (15). Chemoattractant ligand is internalized under physiologic temperature conditions of 37 °C and the ligand transiently associates with the neutrophil cytoskeleton (22). Adherent, chemoattractant-stimulated neutrophils also exocytose specific (secondary) granules which contain an intracellular pool of N-formyl peptide chemoattractant binding sites (23, 24). It has been proposed that chemoattractant receptor replenishment occurs at the lamellipodia of locomoting neutrophils by either the recycling of previously internalized receptors or by the translocation of a receptor pool associated with neutrophil specific granules or other organelles which are released during chemotaxis (23). Neutrophil receptors appear to move in a consistent pattern from the lamellipodium toward the uropod (15) as the neutrophils exhibit amoeboid-like locomotion in a relatively straight line up the concentration gradient (25). Neutrophil exposure to relatively high concentrations of chemoattractants also activates the extrusion of lysosomal enzyme-containing

granules (degranulation) and increased oxygen consumption resulting in oxygen radical generation (1,26). Although these events do not appear to be involved in the initiation of chemotaxis, they may modulate the chemotactic response and enhance the microbicidal activity of neutrophils mobilized to critical sites in vivo. The dramatic alterations in neutrophil plasma membranes as well as the rapid changes in cytoskeletal architecture which accompany neutrophil exocytosis, pinocytosis, and polarization all appear to be essential morphologic rearrangements that occur during chemotaxis. Agents that affect neutrophil plasma membranes, chemoattractant receptors, or critical cytoskeletal protein interactions generally alter neutrophil motility.

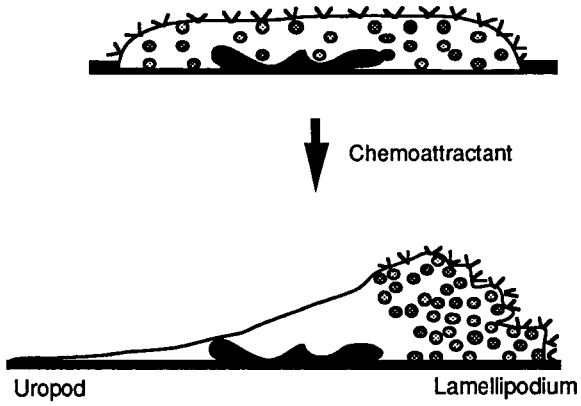


Figure 1. Neutrophil structural changes accompanying chemoattractant exposure. Unstimulated neutrophils dramatically change their shape from a rounded morphology to an elongated, polarized morphology with a broad lamellipodium formed anteriorly and a thin uropod formed posteriorly to the chemoattractant concentration gradient. Chemoattractant receptors (represented as v) initially accumulate at the lamellipodium during polarization.

Neutrophil Motility Assays- Three types of neutrophil motility (random, chemotactic, and chemokinetic) have been categorized on the basis of in vitro migration assays. Random migration is defined as cell movement in the absence of a stimulus, whereas chemotactic migration is directed movement towards an increasing concentration gradient of a stimulus, and chemokinetic motility is the activated random movement induced by a stimulus without a concentration gradient. A variety of in vitro assays have been developed to evaluate the properties of neutrophil motility and the effects of pharmacologic agents on migration. They include quantification of cell migration through porous membranes, migration under agarose, and direct visualization of locomoting cells by phase contrast microscopy. The specific details of these assays and the advantages and disadvantages of each technique have been extensively reviewed (27-30). Although the relative importance of random, chemokinetic, and chemotactic migration in vivo has not been determined, it is likely that all three types of motility are involved in the mobilization of neutrophils to an inflammatory locus.

Biochemical Changes in Chemoattractant-Stimulated Neutrophils- Biochemical events that occur within seconds after neutrophil exposure to chemoattractant include transient elevations in intracellular Ca^{2+}, Na^{2+} and cyclic AMP levels, as well as membrane potential changes (1, 10-13,26). Maximal changes in these events occur less than sixty seconds after exposure to chemoattractant and thereafter return to basal levels (26). Chemoattractant binding to neutrophil receptors activates phospholipase C-mediated hydrolysis of polyphosphoinositol 4,5-bisphosphate (13,31). Two products of this reaction, inositol 1,4,5-trisphosphate (IP_3) and 1,2 sn-diacylglycerol (DAG), activate calcium mobilization (via IP_3) and protein kinase C (via DAG). Calcium and protein kinase C appear to be involved in neutrophil cytoskeletal protein modifications. Intracellular free Ca^{2+} is increased in the lamellipodia of migrating neutrophils (32) and affects actin polymerization in chemoattractant-stimulated neutrophils (31, 33). Other cytoskeletal protein reorganizations may occur via DAG which stimulates protein kinase C to phosphorylate cytoskeletal proteins (31). An inhibitor of protein kinase C and kinase A inhibits neutrophil chemotaxis to several chemoattractants (34, 35). This finding and others demonstrating the phosphorylation of cytoskeletal proteins in chemoattractant-stimulated neutrophils (18, 36) suggest that cytoskeletal protein phosphorylation is involved in neutrophil chemotaxis.

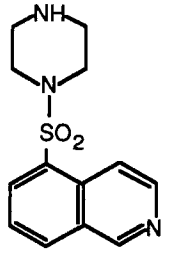
Chemoattractant binding to receptor activates phospholipase C via guanine nucleotide binding-proteins (G-proteins; 12, 13, 31). A G-protein is tightly associated with a solubilized partially purified N-formyl chemoattractant receptor complex (37). It has been proposed that products formed during neutrophil activation can feed back to attenuate chemoattractant receptor-mediated stimulation of phospholipase C by uncoupling the receptor--G-protein--phospholipase C interaction (13). The coupling and uncoupling of G-protein with a chemoattractant receptor may occur via modifications to the G-protein and/or the receptor protein. Protein carboxyl methylation may be involved in the modifications of these proteins. Rapid, transient protein carboxyl methylation has been observed in chemoattractant-stimulated neutrophils (38) Several macrophage membrane proteins are carboxyl methylated by S-adenosylmethionine in an enzymatic reaction that is greatly stimulated by the presence of guanine nucleotides (39). Macrophage chemotaxis is suppressed by bacterial toxin inhibitors of G-proteins and by methylation inhibitors strongly suggesting that carboxyl methylations may regulate the function of some membrane G-proteins (39-42).

The receptors for several different chemoattractants have been reported to exist in two affinity states (12, 13) and guanine nucleotides regulate the receptor affinity (43). One model, which assumes a static population of high- and low-affinity receptors, has proposed that the high affinity receptors transduce chemotaxis, while the low-affinity receptors transduce oxygen radical production (44). A second model, which takes into account interconverting receptor states rather than static populations of receptors, postulates that all chemoattractant receptors may participate in all neutrophil responses coupled via G-proteins; the responses with low 50% effective doses (ED_{50}s) require only a few occupied receptors, whereas the responses with high ED_{50}s require many receptors to initiate and sustain the response (12, 31).

Chemoattractant binding to neutrophils activates phospholipase A_2 and results in arachidonic acid release. Free arachidonic acid is converted by cyclooxygenase to thromboxanes and prostaglandins and by lipoxygenation to a variety of hydroxyeicosatetraenoic acids including leukotrienes (reviewed in 1 and 45). Peak intracellular concentrations of these metabolites occur within minutes after chemoattractant stimulation. The precise roles of these products are unknown, however, they may regulate neutrophil responsiveness to chemoattractants and participate in alterations of neutrophil plasma membranes during activation.

PHARMACOLOGIC MANIPULATION OF NEUTROPHIL CHEMOTAXIS

Second Messenger Regulation - A variety of agents modify neutrophil chemotaxis in vitro (Table 1). Compounds that elevate cyclic AMP such as ß-adrenergic receptor agonists (46, 47), prostaglandin E_1 (46, 47), and histamine (47-49), inhibit neutrophil chemotaxis. In contrast, cholinergic agents (50), α-adrenergic agonists (47) and ß-adrenergic antagonists (51), elevate cyclic GMP and augment neutrophil chemotaxis. Although neither cyclic AMP nor cyclic GMP alone fully meets the requirements of a second messenger in neutrophil activation, experimental evidence indicates that cyclic AMP modulates neutrophil chemotaxis (12, 26). One potential regulatory role for cyclic AMP is its participation in cyclic AMP-dependent phosphorylations of neutrophil proteins whose activities are related to their extent of phosphorylation (12). Isoquinolinesulfonamides such as 1-(5-isoquinolinesulfonyl)-piperazine (1) inhibit protein kinase A and protein kinase C by competitively binding to the ATP substrate site, and reversibly inhibit neutrophil chemotaxis to a variety of chemoattractants, suggesting that protein phosphorylation plays a role in mediating neutrophil chemotaxis (34,35) The cytoskeletal proteins, mysosin and vimentin are phosphorylated in chemoattractant stimulated stimulated neutrophils, providing further evidence for the potential role of protein phosphorylation in chemotaxis (18,36)



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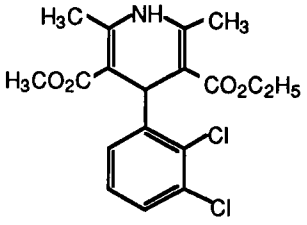

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Table 1.

AGENTS THAT MODIFY IN VITRO NEUTROPHIL CHEMOTAXIS

Inhibitory Agents	Augmentive Agents
Antibiotics	Aliphatic alcohols (58)
Rifampin (61)	
Amphotericin B (58)	**Alpha-adrenergic agonists:**
Nystatin (58)	Phenylephrine (47)
Erythromycin (84)	
Doxycycline (83)	**Amino peptidase inhibitors:**
	Actinoin (79)
Anti-inflammatory Agents	Amastatin (79)
Indomethacin (65)	Bestatin (80)
Isoxicam (75)	
Phenylbutazone (66)	**ß-adrenergic antagonists:**
	Alprenolol (51)
Bacterial toxins	Propranolol (51)
Botulinum C2 toxin (69)	
Cholera toxin (46, 47)	**Cholinergic agents**
Pertussis toxin (68)	Acetylcholine (50)
	Carbamylcholine (50)
Calcium antagonists	
Felodipine (52)	**Other agents**
Verapamil (53, 54)	Pentoxifylline (59, 87)
Cytochalasin B (73)	
Glucocorticoids (60)	
Histamine (48, 49, 50)	
Lipoxygenase inhibitors	
Eicosatetraynoic acid (63, 78)	
Timegadine (76)	
Piriprost (74, 78)	
Prostaglandin E_1 (46, 47)	
Sulfonamides:	
Sulfapyridine (67)	
Sulfasalazine (62)	
Sulfones	
Dapsone (67)	
Sulfoxone sodium (67)	
Other agents	
Ambroxol (81)	
Disodium cromoglycate (82)	
Hexachlorohexanes (56)	
Mepacrine (85)	
Morphine (86)	
Pentoxifylline (88)	

Note: References are indicated in parentheses.

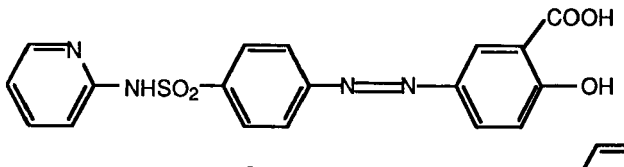
Chemoattractant-stimulated neutrophils exhibit marked changes in plasma membrane permeability to calcium. Calcium influx and efflux both occur rapidly after chemoattractant stimulation, indicating that neutrophils release internal pools of calcium (31). The calcium antagonists, felodipine (2) and verapamil inhibit neutrophil chemotaxis in vitro (52-54). Initially these compounds were proposed to inhibit chemotaxis by a mechanism related to their inhibitory effects on calcium mobilization; however, the inhibitory effects of these drugs on neutrophil chemotaxis do not appear to involve calcium-mediated mechanisms (52, 54). Relatively high

concentrations of these compounds are required to inhibit neutrophil chemotaxis when compared with concentrations inducing calcium antagonistic effects in myocardial and smooth cells. Felodipine (2) interacts with calmodulin and inhibits myosin P-light chain phosphorylation in smooth muscle cells at the concentrations required to inhibit neutrophil chemotaxis (52). Verapamil induces a blockade of potassium and sodium channels in leukocytes (54). The effects of felodipine (2) and verapamil on neutrophil chemotaxis may be related to these others sites of action.

Hexachlorohexanes are structurally similar to inositol and they dramatically alter calcium homeostasis and stimulate the release and metabolism of arachidonic acid via the lipoxygenase pathway (55). Although they are potent stimuli for neutrophil oxidative metabolism and intracellular calcium mobilization (55), hexachlorohexanes inhibit neutrophil chemotaxis, polarization, and actin filament assembly (56). The precise mechanism of action of hexachlorohexanes is unknown. It has been proposed that the effects are related to poorly regulated and dysfunctional changes in intracellular calcium (56).

Membrane Active Agents - Several classes of pharmacologic agents interact with neutrophil plasma membranes to alter membrane composition or bind to chemoattractant receptors. The aliphatic alcohols, butanol and pentanol, are membrane fluidizing agents that increase chemoattractant receptor affinity and augment neutrophil chemotactic responsiveness (57). In contrast, polyene antibiotics such as amphotericin B and nystatin complex with cholesterol, decrease chemoattractant binding and depress chemotactic responsiveness (58). These in vitro studies indicate that membrane fluidity affects the affinity of neutrophil chemoattractant receptors and modifies the chemotactic response. The local anesthetics, bupivacaine and chloroprocaine, administered in vivo do not significantly alter anesthetics may not significantly alter neutrophil membrane fluidity in vivo.

Glucocorticoids (60), rifampin (61), sulfasalazine (3,62), eicosatetraynoic acid (63), indomethacin (64, 65) and phenylbutazone (66) specifically inhibit neutrophil chemotaxis to N-formyl peptide chemoattractant by inhibiting its binding to chemoattractant receptors. Other neutrophil responses which occur subsquent to the binding of N-formyl peptide, including degranulation and oxygen radical formation, are also inhibited by these agents. Sulfapyridine and the sulfones, dapsone (4) and sulfoxone sodium (5), selectively inhibit neutrophil chemotaxis to N-formyl peptide chemoattractant without affecting oxygen radical formation to the stimulus (67). Therapeutic concentrations of these agents selectively inhibit neutrophil chemotaxis to N-formyl peptide, but do not affect neutrophil chemotaxis to complement-derived C5a or leukocyte-derived chemotactic factor. Radiolabeled dapsone (4) binds reversibly and nonspecifically to neutrophils and inhibits N-formyl peptide binding to only a subset of receptors (67). These findings suggest that sulfones bind to a subset of N-formyl peptide receptors that trigger chemotaxis or that they bind to a component of the N-formyl peptide receptor-chemotaxis transduction machinery and thereby interfere with the translation of chemoattractant binding into the events of directed movement.



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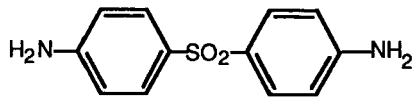

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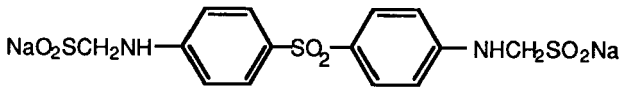

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Several correlations exist between the in vitro effects of these sulfones and their therapeutic effectiveness in neutrophilic dermatoses in vivo (67). Most striking is the observation that dapsone (4), sulfoxone sodium (5) and sulfapyridine, which are the most therapeutically effective drugs in neutrophilic dermatoses, were all effective in selectively inhibiting neutrophil chemotaxis to N-formyl peptide. In contrast, two structurally related sulfonamides (sulfisoxazole and sulfamethoxazole), which are not therapeutically effective in these skin disorders, do not inhibit neutrophil chemotaxis

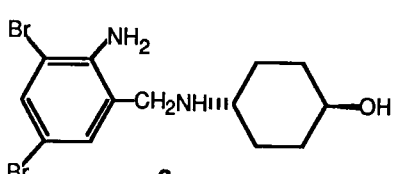
to N-formyl peptide. Significant inhibitory effects of sulfapyridine and sulfones on neutrophil chemotaxis occur at drug concentrations of 5-10 μg/ ml, which are within therapeutic concentration range (67). These striking correlations between the in vitro effects of sulfones and their therapeutic effectiveness suggest that the sulfones exert an inhibitory effect on neutrophil chemotaxis to selected chemoattractants in vivo.

Bacterial Toxins - Neutrophil chemotaxis is inhibited by agents that modify cytoskeletal and signal transduction proteins. Bacterial toxins including cholera (46, 47), pertussis (68), and botulinum C2 toxin (69) are ADP-ribosyltransferases which covalently modify proteins involved in neutrophil chemotaxis. Cholera and pertussis toxins ribosylate the α-subunits of G proteins, some of which appear to couple chemoattractant receptors to phospholipase C (12, 13, 31), and inhibit neutrophil activation. Botulinum C2 toxin ribosylates actin monomers (G-actin) but not actin filaments (F-actin) and as a result, the ribosylated actin monomers can not polymerize into actin filaments (70, 71).

The low molecular weight fungal metabolite, cytochalasin B, binds to high affinity sites on actin filaments and blocks elongation from the barbed end of actin filaments (72). Both botulinum C2 toxin and cytochalasin B dramatically inhibit neutrophil chemotaxis and random migration (69, 73), demonstrating the critical role of actin filament assembly in neutrophil migration.

Enzyme Inhibitors - Agents that inhibit selected neutrophil enzymes have diverse effects on neutrophil chemotaxis. Pharmacologic agents such as piriprost (U-60,257), an inhibitor of leukotriene formation (74); isoxicam, an inhibitor of prostaglandin synthesis (75); and timegadine, an inhibitor of phospholipase A_2, cyclooxygenase, and 5-lipoxygenase (76), have all been reported to inhibit neutrophil chemotaxis in vitro. Leukotrienes, derived from lipoxygenation of intracellular arachidonic acid, are closely associated with neutrophil activation and appear to function as secondary mediators of activation (77). Leukotrienes appear to act in conjunction with calcium and protein kinase C to elicit complete responses in neutrophils (77). Depletion of intracellular levels of lipoxygenase and cyclooxygenase products results in diminished neutrophil responses to several stimuli (63, 74, 76). When lipoxygenase inhibitors were compared for their effects on neutrophil responses to a variety of stimuli, the inhibitory effects on neutrophil chemotaxis and leukotriene production were not closely correlated (78). These differences may be attributed to the relative importance of lipoxygenase products in specific neutrophil responses (e. g. chemotaxis) or they may be due to the effects of these inhibitors on other enzymes. Evidence that some agents inhibit neutrophil function via mechanisms other than lipoxygenase has come from a study in which eicosatetraynoic acid was found to inhibit neutrophil binding of N-formyl peptide (63). The extent to which the products of 5-lipoxygenase contribute to neutrophil activation by specific chemotactic stimuli remains to be determined.

Actinoin, amastatin, and bestatin are low molecular weight amino peptidase inhibitors that augment neutrophil chemotaxis (79, 80). It is not known whether these agents enhance chemotaxis through their inhibitory effects on cell membrane-associated aminopeptidases or by unrelated mechanisms.



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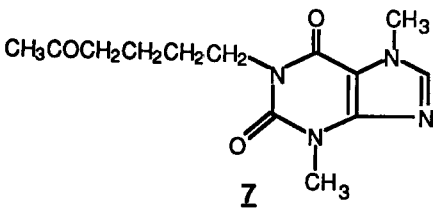

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Other Agents - Numerous pharmacologic agents have been described that inhibit chemotaxis in vitro; however, the mechanisms by which they inhibit chemotaxis remain unknown (Table 1). They include: ambroxol (6), a mucolytic agent used for treating chronic bronchitis (81); disodium cromoglycate, a mast cell-stablilizing drug used for treating allergic asthma (82); doxycycline and erythromycin, antibiotics that alter neutrophil cell membrane properties (83, 84); mepacrine, an experimental agent that salvages pulmonary function in animal models of respiratory distress syndrome (85); and the opioid, morphine (86). Pentoxifylline (7), a methylxanthine derivative that has been therapeutically administered for the treatment of peripheral arterial insufficiency, has

recently been studied for its effects on neutrophil motility. Some studies have indicated that pentoxifylline (7) augments neutrophil motility (87) and partially corrects a developmental defect in neonatal neutrophil motility in vitro (59). Other studies have shown that pentoxifylline (7) inhibits neutrophil chemotaxis (88) and inhibits the action of inflammatory cytokines on neutrophil chemotaxis (89). These divergent results may be attributed to the diverse effects of pentoxifylline (7) as an agent affecting both membrane deformability and phosphodiesterase inhibition. A major problem with pharmacologic agents and the interpretation of their effects on neutrophil function has been the lack of specificity of the agents in an intact cell system.

The pharmacologic agents discussed in this review have been limited to in vitro studies. Recent reports have appeared in the clinical dermatology literature which have evaluated in vivo neutrophil migration. Psoriasis patients receiving cyclosporine A therapy were found to have depressed neutrophil migration to skin chambers during therapy, which correlated with clinical improvement (90). Patients with cystic acne receiving isotretinoin also demonstrated depressed neutrophil motility to skin chambers during therapy (91). These studies provide a unique opportunity to directly compare the effects of pharmacologic agents on neutrophil chemotaxis in vivo and in vitro.

Conclusions- Evaluation of the effects of pharmacologic agents on neutrophil function provides useful information about the activation of neutrophil chemotaxis. Characterization of the critical steps involved in chemotaxis may also be approached by nonpharmacologic means. One approach is to make specific chemical modifications to chemoattractant ligands which alter their biological activity. For example, oxidative modifications of the methionine residue in the N-formyl chemoattractant, N-formyl-methionyl-leucyl-phenylalanine, results in the loss of chemoattractant activity of the molecule for neutrophils (92, 93); however, the oxidized derivatives bind to chemoattractant receptors and activate neutrophil oxygen radical production (93). Comparative studies of oxidized and nonoxidized N-formyl peptides provide a specific means of probing events that are required for chemotactic responsiveness.

Neutrophils are heterogeneous in their in vitro responsiveness to chemoattractants despite their homogeneous expression of chemoattractant receptors (94, 95). A technique has been developed to physically isolate the subpopulations of responsive and nonresponsive neutrophils to facilitate comparative biological and biochemical studies of these subpopulations (94). It will now be possible to test active agents on responsive and unresponsive neutrophils.

Other techniques such as the development of monoclonal antibodies to chemoattractant receptors and transducing proteins are underway in numerous laboratories to find specific probes for the isolation and cloning of critical neutrophil proteins involved in chemotaxis. All of these approaches have provided insights into some of the essential requirements for neutrophil chemotaxis.

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Section VI - Topics In Chemistry and Drug Design

Editor: Fredric J. Vinick, Pfizer, Inc., Central Research, Groton, CT 06340

Chapter 26. Approaches to the Discovery of Non-Peptide Ligands for Peptide Receptors and Peptidases

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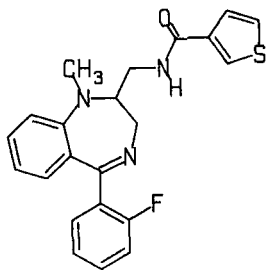
Introduction -The development of rational approaches to the discovery of peptide mimetics is currently a major goal of medicinal chemistry. A brief comparison of the Journal of Medicinal Chemistry in 1988 and 1983 reveals a two- to three-fold increase in research dealing with the design and evaluation of peptides and peptide mimetics. Methods for the sequencing and conformational analysis of peptides and proteins have advanced during the last decade. Thus, medicinal chemists have a plethora of new peptidic targets to challenge their ingenuity. It is ironic that, with the exception of angiotensin converting enzyme (ACE) inhibitors (1) and CCK antagonists (2), medicinal chemists have been unable to harness the profound pharmacologic effects of peptides by the discovery of bioavailable analogues. That is not to say, however, that progress toward this goal has not been achieved. The objective of this review is to outline recent advances in the various strategies available for the discovery of peptide mimetics.

Peptide mimetics can be defined as structures which serve as appropriate substitutes for peptides in interactions with receptors and enzymes. The mimetic must possess not only affinity, but also efficacy or substrate function. Antagonists do not interact with receptors in the same way as agonists. Consequently, structural hypotheses based on correspondence or overlay of agonist with antagonist may not be valid. However, as the major thrust of peptide mimicry has been directed towards receptor antagonists or enzyme inhibitors (as opposed to agonists or substrates), for the purpose of this review all these classes -agonists and antagonists, substrates and inhibitors - are regarded as valid targets for mimicry using the more general term "ligand."

As non-peptide ligands (≡ peptide mimetics) may be discovered by two basic strategies the body of this review has been arranged in two sections: approaches which have resulted in the serendipitous discovery of a novel lead, and progress in the design of peptide mimetics, including strategies which vary in complexity from the simple replacement of single atoms in the amide bond to the considerably more ambitious undertaking of constructing mimics of peptidic secondary structural elements such as turns, helices and sheets.

EMPIRICAL DISCOVERY OF PEPTIDE MIMETICS

Empirical approaches to the discovery of peptide ligands have involved the screening of pure compounds and complex mixtures in biological assays. The screening of pure entities (from the large compound collections assembled by pharmaceutical or chemical research houses) has generally afforded little reported success, with the exception of ligands for the promiscuous μ-opioid receptor (3). However, tifluadom, **1**, is worthy of mention due to its benzodiazepine structure (vide infra), potency (IC_{50}=1.7nM, electrically stimulated guinea-pig ileum assay), and selectivity for K opioid receptors (4).



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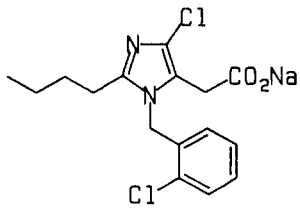
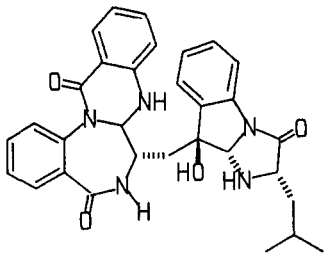


2

Recently the imidazole S-8307, 2 and related analogues, have been described as weak but selective antagonists at the angiotensin II (AII) receptor (pA_2=5.49 vs. AII in rabbit aorta) (5,6).

With regard to complex mixtures, natural products have provided a particularly useful source of leads for the discovery and development of peptidase inhibitors. Examples include the prototypic ACE inhibitor, teprotide, from the venom of *Bothrops jaraca* (7). In a similar way, the characterization of pepstatin (8) provided the key structure for the development of "transition-state" inhibitors for proteases such as renin (9).

The discovery of receptor ligands has proved to be more elusive. Apart from a report of a structurally uncharacterized bradykinin antagonist from *Mandevilla relutina* (10), the discovery of the CCK antagonist asperlicin, 3, remains as the single significant success (11).



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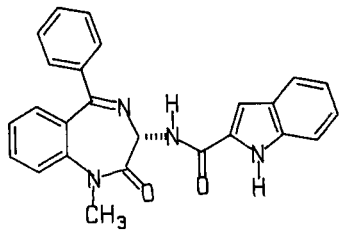
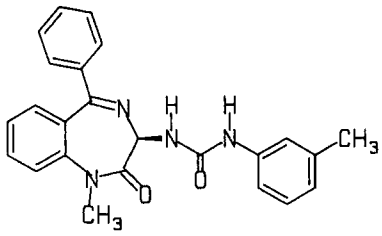


4

Recognition of a 1,4-benzodiazepine as the key structural feature of asperlicin and optimization of the 3-substituent resulted in the discovery of the orally active agent L-364,718, 4. This compound has affinity at the CCK-A receptor comparable to CCK itself ($IC_{50}=10^{-10}M$) and high selectivity (>1000 fold) with respect to the central CCK receptor (12). Very recently this series has been extended to yield the selective gastrin antagonist, L-365,260, 5. This compound has high potency ($IC_{50}=10^{-9}M$) for gastrin and central CCK-B receptors, and good selectivity (>100 fold) with respect to the peripheral CCK-A receptor (13). In addition, 5 is an orally effective antagonist of gastrin-stimulated acid secretion in various animal models (14). Remarkably, inversion of the 3R-urea

substituent of **5** to give **6** also reverses the selectivity, yielding a selective CCK-A ligand!



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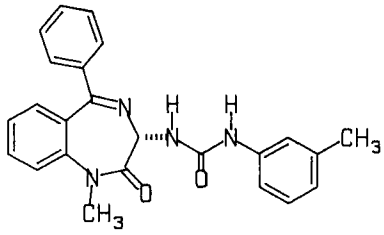
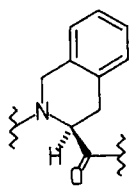


6

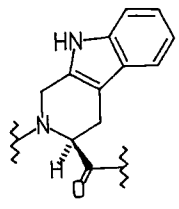
The discovery of asperlicin and the development of benzodiazepines, **4** - **6**, present a challenge to proponents of computer-assisted molecular design, in that it may be possible to relate their structures to pharmacologically relevant conformations of CCK and its analogues. A comparison of **4**, the CCK-carboxy terminal tetrapeptide (CCK-4), and ergotamine, based on an "extensive Dreiding model-based computer-assisted search procedure," has been reported (15). In another study, analysis of multiple conformations of CCK-7, CCK-4, the C-terminal heptapeptide of cerulein and **4**, has led to the conclusion that β-turn containing conformations of CCK-7 resemble the benzodiazepine **4**, and that this may represent the structural basis for interaction with the peripheral CCK-A receptor (16). However, the importance of experimental data to support the mode of matching in such modeling studies has been emphasized (17). With this in mind, a series of cyclic analogues of CCK has been designed and synthesized yielding *cyclo* [Phe-Met-Gly-D-Trp-Met-D-Asn(Bzl)], the most potent cyclic peptide antagonist of CCK (IC_{50}=0.21μM). Solution conformation and modeling studies on this series are in progress (18).

PEPTIDE MIMETICS BY DESIGN

Amino Acid Manipulations - Several new applications of conformationally restricted amino acids have been described. The tetrahydroisoquinoline (Tic) moiety, **7**, has been substituted into peptide inhibitors of rat brain μ-opioid receptors (19,20). The D-Tic analogue, **7b**, was an order of magnitude more potent than the corresponding D-Phe peptide, **7a**.



7



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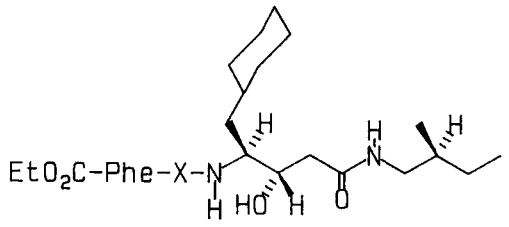
X-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2

7a	X = D-Phe	pA_2	7.10
7b	X = D-Tic	pA_2	8.10

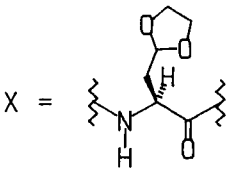
Modified peptides of angiotensin II containing the mimic, **7**, at Phe^8 have also been described (21). Interestingly, the L-Tic peptide is an agonist (ED_{50}=2.8nM, isolated rat uterus) whereas the D-Tic peptide is an antagonist in the same tissue (IC_{50}=0.64nM vs. AII). The related tryptophan mimic (Tpi), **8**, has been recently reported as a key N - terminal amino acid residue in the first pentapeptide tachykinin antagonist (pA_2 vs. SP at NK-1 receptor = 6.4) (22).

A desire to decrease first-pass clearance by biliary excretion of histidine-containing peptide inhibitors of renin has prompted the investigation of a histidine isostere; replacement of this residue in renin inhibitor **9** (X=His) with amino acid **10** caused a four-fold increase in inhibition potency, but increased bioavailability was not described (23).



X = His

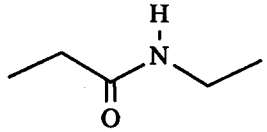
9



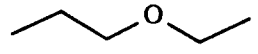
10

Peptide Backbone Modifications - Analogues containing amide bond surrogates have frequently been utilized to investigate aspects of peptide structure and function, including rotational freedom in the backbone, intra- and intermolecular hydrogen-bond patterns, modifications of local and total polarity and hydrophobicity, and oral bioavailability. Several of the isosteric amide bond mimics which have been introduced into biologically active peptides (e.g., $\psi[CH_2S]$, $\psi[CH_2NH]$, $\psi[CSNH_2]$, ψ[NHCO], $\psi[COCH_2]$ and ψ[(E) or (Z) CH=CH]), have been exhaustively reviewed (24). However, some new ideas as to their function and improvements in their synthesis warrant mention. Investigation of the use of the thiomethylene ether as an amide bond mimic has resulted in incorporation of this functionality into cyclic analogues of somatostatin (25,26) and enkephalin (27-30). The cyclic pseudopeptide analogue of somatostatin proved to have approximately 25% of the potency of somatostatin in inhibition of growth hormone release (25). More success was achieved in enkephalin cyclic pseudopeptides, the best compound exhibiting potency comparable to Tyr-*cyclo* (D-Lys-Gly-Phe-Leu) on the electrically-stimulated guinea-pig ileum (27,28).

Arguments based on geometry suggest that the oxymethylene ether linkage may be more useful than a thiomethylene moiety as a *trans* amide bond replacement (31). This oxymethylene ether peptide modification at the Pro^7-Phe^8 position of renin inhibitors afforded potent inhibitors resistant to chymotrypsin degradation (32).

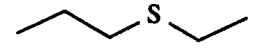


Amide bond
Cα ⇒ Cα
3.8 Å



Oxymethylene

3.7Å



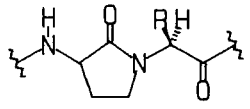
Thiomethylene

4.2 Å

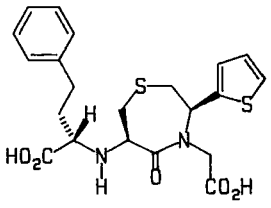
Recently several improved syntheses of (E)-alkene peptide mimetics have appeared in which good stereochemical control of appropriate peptidic side chains was achieved (33-36).

A consequence of the major commitment of resources to the design of inhibitors of peptidases has been the discovery of structures which mimic the tetrahedral transition state associated with hydrolysis of the substrate peptide bond (37,38). Thus, the use of the hydroxymethylene (39) and fluoroketone (40) moieties have yielded effective inhibitors of renin. The application of phosphoramidate transition state mimics to metalloprotease inhibition has recently been reviewed (41).

Dipeptide Mimetics - Ways of achieving conformational constraint in biologically active peptides continue to be explored. Considerable use is being made of lactams such as **11a**, originally introduced by Freidinger (42). Its incorporation into angiotensin II receptor antagonists has resulted in an extremely potent (pA_2=8.3) analogue of Sar^1-AII containing **11b** at the 7-8 position; its potency matches that of saralasin (pA_2=8.6),(43). The δ-lactam replacement has also been successfully used in renin inhibitors, affording compounds which are potent (IC_{50}=6.5nM) and resistant to chymotrypsin degradation (44). The peptide BOC-Ala-(-**11a**-)-Trp-Leu-Asp-Phe-NH_2 was found to be as active as pentagastrin in terms of its ability to induce acid secretion in the rat when administered at a concentration of 4nM (45). A similar strategy has been employed to prepare CCK-4 analogues (46): however, no biological data was reported. Finally, introduction of the lactam dipeptide mimetic **11c** into tachykinin antagonist analogues has yielded pseudopeptides with a variety of interesting selectivities for the four neurokinin receptors (47,48). A related approach has been taken in the design of conformationally restricted analogues, **12** (49) [IC_{50}=2.8nM], **13** (50) [Ki=12pM], and **14** (51), of enalaprilat. The stereospecific synthesis of benzazepinones **15** as Gly-Phe and Phe-Gly dipeptide isosteres has been reported but biological evaluation of pseudopeptides with this substitution has not yet been described (52).



11a R=H
11b R=benzyl
11c R=isobutyl



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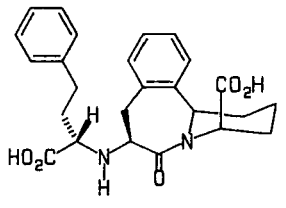

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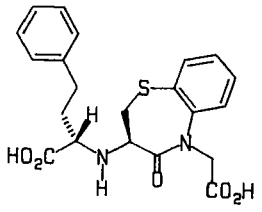

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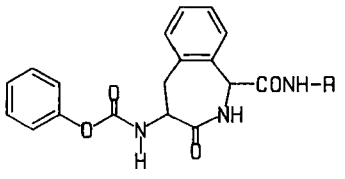
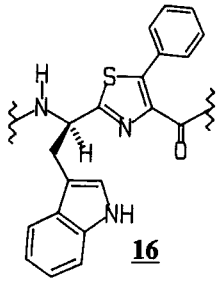


15

The design, synthesis, and pharmacology of novel dipeptide mimetics in which the oxygen and carbon of residue *n* and the nitrogen, C_α and C_β of residue *n+1* have been confined into a planar thiazole moiety **16** has recently been described (53). These thiazole

dipeptides have been incorporated in the tachykinin antagonist Pro^6 D-Trp^{7,9} SP_{6-11}at the 8-9 (Phe -D-Trp) position, yielding the most potent antagonist yet described at the NK-1 receptor (pA_2 = 7.3 vs. SP-induced guinea-pig ileum contractions). Similar use of a thiazole as a dipeptide mimic in an AII receptor antagonist was not effective (21).



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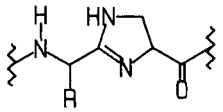

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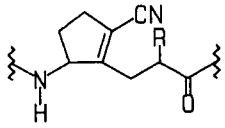

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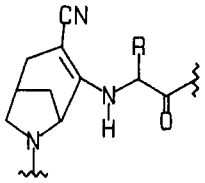
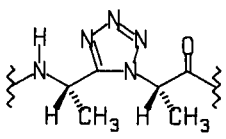


19

Unlike the above planar thiazoles, where the chirality at C_α in residue *n+1* has been removed, a report has appeared recently in which imidazolines **17** have been suggested as dipeptide surrogates (54). These structures have been inserted into analogues of ACE inhibitors and leucine enkephalin; no biological data was reported. A novel dipeptide replacement has been described in which a vinyl nitrile was substituted for the *trans*-amide bond (55,56). Structures **18** and **19** were synthesized but no biological validation of the hypothesis was described. It was noted that compound **19** may act as a γ-turn template.

All of the preceding dipeptide mimics seek to duplicate the configuration of the *trans*-amide bond. Success in mimicking the *cis* amide bond by utilizing the 1,5-disubstituted tetrazole ring (ψ[CN_4]), **20**, has been described using a synthesis which preserved the chiral integrity of the starting dipeptide (57). Moreover, the crystal structure of the tetrazole ring clearly demonstrated its strong geometric similarity to a *cis* amide bond. This tetrazole dipeptide mimic **20** has been substituted into three bradykinin analogues, but no activity was detected in the isolated rat uterus assay (58).



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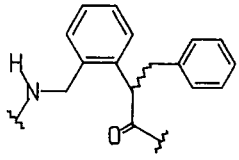
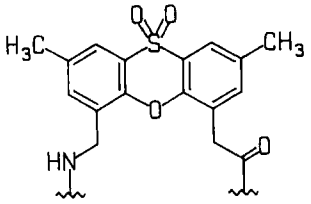


21

Extensive conformational studies have been performed on somatostatin analogues containing the proposed *cis*-amide bond mimic **21** (59-62). While devoid of any activity *in vitro*, one of the two diastereoisomers significantly inhibited growth hormone release *in vivo* (IC_{50} (i.v.)=52μg/kg vs. 62μg/kg for somatostatin) (59).

Mimics of Peptide Secondary Structure - An intriguing challenge of peptide mimetic design is the discovery of structures which can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins: turns, helices, and sheets. Several turn mimics have been synthesized, and although crucial biological evaluation is unavailable in many instances, physical data have been obtained to attest to the desired structures. The phenoxathiin ring system **22** has been shown to mimic a β-turn (63). A gramacidin S (GS) analogue containing the bicyclic β-turn dipeptide (BTD), **23**, retained equipotent antibacterial activity versus GS (64). However, only weak activity was observed when BTD was substituted into potent cyclic hexapeptide analogues of somatostatin (65). The γ-turn tripeptide mimic **24** has been incorporated into analogues of leucine enkephalin (66). These pseudopeptides showed some affinity in receptor binding assays (~1%); however, they were virtually inactive in functional delta receptor preparations such as the electrically-stimulated mouse vas deferens.



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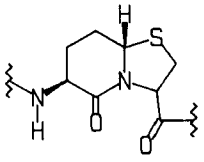

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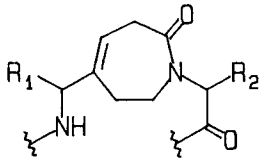

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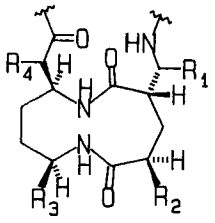

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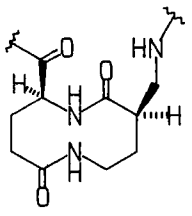
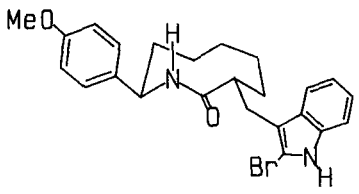


26

Another strategy has utilized the construction of 9- and 10-membered moderately constrained rings to mimic the β-turn structure. Both Kahn (67) and Kemp (68) have designed and synthesized similar cyclic molecules, **25** and **26**, and have obtained physical evidence to support their hypotheses. Similarly, construction of a mimetic of the novel metabolite, jaspamide, has involved transformation of the β-turn portion of this natural product into a 9-membered cyclic amide **27** (69). Preliminary results indicate some activity versus *H. virescens*. Physical evidence has also shown that bicyclic structure **28** contains the β-turn moiety (70). Another novel β-turn mimetic containing the interesting spirocyclic unit **29** has been incorporated into an immunodominant nonapeptide sequence to give a conformationally locked analogue (71).

The design of β-sheet and α-helix mimetics has also been addressed: the sheet mimic **30** is obtained by insertion of the epindolidione structure into a peptide sequence (72,73); the design, synthesis, and conformational analysis of an α-helix inducing template **31** has been described (74,75).

Conclusion-The goal of peptide mimicry is the discovery of structural leads, or the development of strategies for molecular design, which result in bioavailable peptide analogues. Although it may not be necessary to remove all peptide-like character to achieve this objective, reduction of molecular weight and resistance to peptidase action



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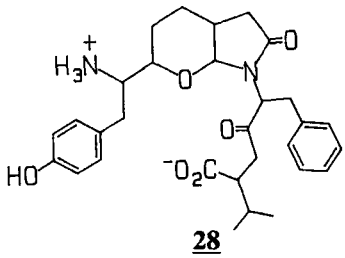

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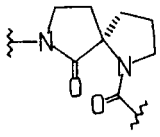

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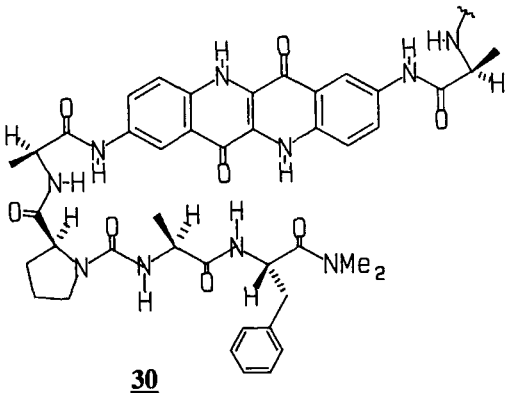

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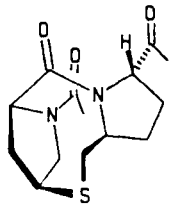

31

will be required. From the preceding analysis, it is clear that the most successful strategy for the discovery of peptide mimetics has been that of lead identification via biological screening of natural products, followed by structural optimization by conventional medicinal chemistry techniques: captopril and the benzodiazepines **1**, **4**, **5**, and **6** were derived in this way.

The ability to predict molecular interactions using computer-based searching of a database of structures could be an efficient alternative to chemical synthesis and biological assay. Distance-geometry based methods for searching for "common pharmacophores" in small molecules (76) or algorithms for exploring the interaction of flexible ligands with receptors of known geometry (77) have been developed, but have not yet shown a great measure of success. Application of methods of this type to peptides is further complicated by the conformational flexibility of these molecules (78).

Medicinal chemists will learn to relate compounds like asperlicin to CCK, and apply this knowledge to improve their conceptualization of molecular mimicry beyond the literal atom-for-atom or bond-for-bond matching strategies outlined above. Hopefully, in this way the discovery of peptide mimetics will not be solely dependent on the vagarious pathways of secondary metabolism in microorganisms and plants!

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Chapter 27. Recent Developments in the Mass Spectrometry of Peptides and Proteins.

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Introduction - The enormous current interest in both natural and synthetic peptides and proteins is placing increasing demands on the analytical methods used to characterize these materials. While many widely used techniques are very useful, they all suffer from specific drawbacks. Thus, for example, amino acid analysis cannot easily be applied to mixtures of peptides, Edman sequence analysis cannot be made on amino terminally blocked peptides and proteins, and electrophoretic molecular weight determination has limited accuracy (usually >5%). Mass spectrometry has long held the potential for overcoming many of the limitations of other methods. A number of revolutionary developments have recently been made which have converted this potential into reality, namely: (a) The discovery of several new ionization techniques for the production of intact gas phase ions from underivatized high molecular weight peptides and proteins; (b) The construction of mass analyzers capable of measuring high molecular weight compounds with good precision and sensitivity; (c) The development of tandem mass spectrometry or mass spectrometry-mass spectrometry (MS-MS), which allows the sequence of a peptide to be determined even when it is a component of a relatively complex mixture; (d) The direct coupling of liquid chromatographs (LC) to mass spectrometers.

In this review we will discuss some of these more recent developments and provide a selection of applications which illustrate the power of mass spectrometry in the analysis of peptides and proteins.

METHODS OF GAS PHASE ION PRODUCTIONS

An absolute requirement for the successful mass spectrometric analysis of any compound is the production of ions of the molecule in the gas phase. The transfer of peptides and proteins from the condensed phase into the gas phase presents special difficulties because these molecules are massive, polar and therefore highly non-volatile. Prior to the advent of the newer desorption/ionization techniques, mass spectrometric analysis of peptides was not possible without chemical derivatizations of the peptides designed to decrease their polarity and thus enhance their volatility (1). Chemical derivatizations of peptides are laborious, and require relatively large quantities of sample. In 1969 the technique of field desorption mass spectrometry (FDMS) was introduced which permitted the measurement of underivatized peptides (2). In this method, the compound of interest is ionized and desorbed from a surface by the application of a strong electric field. Although peptides of molecular weight up to ca.1500 Da have been studied by FDMS (3,4), the technique has been largely supplanted by the more recently developed ionization methods discussed below.

^{252}Cf Plasma Desorption (PD) Ionization - In this technique, peptides and proteins, in the form of a thin solid layer, are desorbed and ionized by the passage of highly energetic fission fragments through the sample (5). The fission fragments are produced from the spontaneous radioactive decay of ^{252}Cf and have energies of ca.100 MeV and masses of ca.100 Da. Samples are generally prepared for mass analysis by adsorption of monolayer amounts (10^{-11}-10^{-9} mole) of the peptide or protein onto a thin layer of nitrocellulose (6,7). The masses of the desorbed ions are determined by time-of-flight mass analysis (see later). PD mass spectrometry has been used extensively for measuring molecular weights (MW's) of peptides and proteins up to 10,000 Da and, less frequently, up to 35,000 Da (8,9). Because of the unavailability of commercial ^{252}Cf PD mass spectrometers, the use of this technique has, until recently, been limited to a relatively few laboratories. This situation has now been remedied by the introduction of a commercial instrument (10).

KeV Ion or Atom Bombardment-Induced Ionization - In this method, peptides and proteins introduced into the mass spectrometer in the condensed phase are desorbed and ionized by bombardment with ions or atoms (e.g., Cs^+, Ar, Xe) with energies in the range 5-30 keV. The sample is introduced into the mass spectrometer either as a solid (11-16) or dissolved in a low volatility liquid such as glycerol (17-19). When the sample is introduced as a solid, the technique is known as static secondary ionization mass spectrometry (SIMS), because low ion fluxes are used in order to avoid rapid sample destruction. When the sample is introduced in solution, the technique is known as liquid secondary ionization (LSI) or fast atom bombardment (FAB). The liquid sample matrix allows the use of intense bombarding ion or atom fluxes because the surface is continuously replenished with sample from the bulk of the solution. LSI is currently the most used ionization technique because of its ready compatibility with commercially available high performance magnetic deflection mass analyzers and quadrupole instruments. LSIMS has been widely used to analyze nanomole amounts of peptides and proteins up to MW 6,000 Da (20) and, less frequently, up to 24,000 Da (21). Recently, a promising method for directly coupling microbore HPLC to LSI sources has been demonstrated (18,19).

Laser Desorption and Ionization - Intense pulses of laser light can also effect the volatilization and ionization of underivatized peptides and proteins from condensed phase samples. Although the first use of laser pulses for this purpose was reported more than a decade ago (22), laser desorption has not been widely applied in this area. Three recent developments have resulted in a strong resurgence of interest in laser ionization: 1) it has been demonstrated that selected peptides desorbed by infrared laser radiation from a surface can be entrained in and cooled by a supersonic gas jet and thence ionized by multiphoton absorption of UV light from a second laser (23-25). Performing the desorption and ionization in these two separate steps provides selectivity in ionization and control over the fragmentation of the ionized molecule(s). At present the sensitivity of this method appears to be too low for many practical applications; 2) a method has been developed whereby proteins with MW's of up to 34,500 Da have been desorbed and ionized in a single step by pulsed laser irradiation (λ = 337 nm) (26). Nanomole amounts of the protein of interest are dissolved in glycerol and mixed with ultrafine metal powder. The fine powder is rapidly heated by the laser pulse causing desorption of the ionized protein. MW's were determined with a precision of ca.1%; 3) a UV laser bombardment technique has been developed in which proteins with MW's in excess of 100,000

Da can be efficiently desorbed, ionized and measured (27,28). Samples are produced by mixing picomole amounts of protein with a large molar excess of nicotinic acid in solution and then allowed to dry. Irradiation of this sample mixture with 266 nm wavelength laser pulses (a wavelength close to the absorption maximum of nicotinic acid) produces copious amounts of protein ions which are measured in a time-of-flight mass analyzer.

Thermospray Ionization - In thermospray ionization, peptides in an electrolytic solution are introduced directly into the mass spectrometer vacuum through a heated capillary tube in the form of a fine mist of charged droplets (29-31). The charge is produced by statistical fluctuations in the distribution of positive and negative ions in the droplets. Isolated peptide ions are ultimately formed in the gas phase from these charged droplets, and are subsequently mass analyzed. Special features of this mode of ionization are its ability to produce ionized peptides with no apparent fragmentation, and its natural suitability for coupling with on-line liquid chromatographic systems (30-32). Thermospray ionization has primarily been used with quadrupole mass analyzers of limited mass range (less than m/z = 2000). The availability of quadrupole mass analyzers with extended mass range (33) and the recent successful use of thermospray ionization on high performance magnetic deflection mass spectrometers (34) promise to extend the range of masses of peptides which can be analyzed with this method.

Electrospray Ionization - In electrospray ionization, peptide ions are again produced from small highly charged droplets (35-39). The droplets, in this case, are generated by applying a high electric field at the tip of a capillary through which the sample solution can flow. This procedure is carried out at atmospheric pressure and the peptide ions thus produced are sampled into the mass spectrometer through a small orifice for mass analysis. A characteristic feature of electrospray ionization is the almost exclusive formation of intact multiply protonated or natriated peptide or protein ions as shown in Fig. 1 for cytochrome c. The high average charge on the ions produced by electrospray yields mass-to-charge ratios that are sufficiently low to allow their determination by relatively modest mass range quadrupole mass analyzers. Proteins with MW's up to 40,000 have been measured with this technique (36). Sensitivities in the picomole range appear to be attainable (39) and it appears feasible to directly couple an electrospray source to a capillary zone electrophoresis separation system (37).

METHODS OF MASS ANALYSIS OF PEPTIDE IONS

Several different types of mass analyzers are used to determine the mass-to-charge ratio (m/z) of ions produced from peptides and proteins. These include the double focussing electric and magnetic deflection mass analyzer (MA), the quadrupole MA, the time-of-flight MA, and the Fourier transform ion cyclotron resonance MA. The reader is referred to standard mass spectrometry texts for details of the principles of operation of these instruments (40,41). Table 1 shows a comparison of the important properties of these various mass analyzers. The table gives the highest mass measured to date for each combination of ionization method and analyzer type. However, in most cases the quality of the mass spectral data falls off rapidly with an increase in mass. Several of the techniques given in the table are quite new and in the development stage, so that they have not been fully commercialized. Thus, for example, FAB on a Fourier transform ion cyclotron resonance mass spectro-

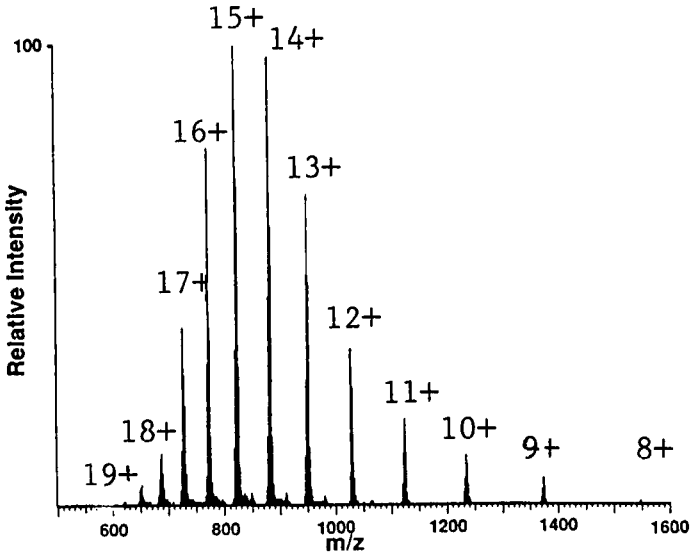

Fig. 1 Electrospray ionization positive ion mass spectrum of horse-heart cytochrome c. A gaussian distribution of multiply protonated intact cytochrome c ions is observed. The numbers above the peaks denote the number of protons attached to the molecule. Adapted from Ref. 37 with permission.

meter has only been extensively utilized for the analysis of peptides by a single research group, albeit with good success (42-44). Similarly, the use of UV-laser desorption time-of-flight mass spectrometry of large proteins has so far been limited (26-28).

TYPES OF INFORMATION AND APPLICATIONS

In this section the types of information that can be obtained from mass spectrometric analyses of peptides and proteins will be discussed, and a selection of recent biomedical applications reviewed.

Information deduced from molecular weight determinations - The most important single piece of information obtained with mass spectrometry which helps to characterize a peptide or protein is its molecular weight. The ionization techniques discussed earlier generally produce protonated positive molecule ions $(M+H)^+$ or deprotonated negative molecule ions $(M-H)^-$, where M denotes the intact molecule. In several of the techniques, multiply charged ions (e.g., $(M+nH)^{n+}$, where n is an integer) are also formed. In general, the determination of the mass-to-charge ratio of these ion species then provides the molecular weight of M. The mass spectrometric determination of peptide or protein molecular weight, alone or in conjunction with classical biochemical analytical techniques such as amino acid analysis, Edman sequencing, DNA base sequencing, etc., can provide solutions to a wide variety of biological and biochemical problems. Examples of such applications are given below:

Synthetic peptides - In recent years it has become feasible to produce routinely and rapidly by stepwise solid phase methods, synthetic peptides containing up to 50 amino acid residues (45). In response to

TABLE 1

Analyzer Type	Ionization Method	Minimum Sample Quantity Required (nmol)	Mass Range Achieved (Da)	Mass Accuracy (Da)
Deflection	FAB	0.01-5[a]	24,000	±0.3 (mass<6000)[c]
	FD	0.5-1[b]	1,500	±0.01%(6000<mass<12000)
	Thermospray	1-10	8,000	±0.3-0.7%(at mass 24,000)
Quadrupole	FAB	0.5-10	3,500	±0.3
	Thermospray	1-10	3,500	
	Electrospray	0·001-1	40,000	±1 (up to mass 17600)[d]
Time-of-flight	PD	0·002-1	35,000	±0.5 (mass<6000)
	SIMS	0·0001-.05	15,000	±2 (5000<mass<10,000) ±0.2% (above mass 10,000)
	LD	0·0001-.01	200,000	±0.1%
Fourier Transform Ion Cyclotron Resonance	FAB	0·01-0.05	13,000[e]	±0.2 (up to mass 2000
	PD	<1	2,000	
	LD	<25	1,100	±0.2 (up to mass 1100)

[a]When an array detector is used the sample quantity required is reduced by a factor 10-100; [b]ref. 4; [c]ref. 21; [d]ref. 39; [e]ref. 44.

the strong demand from the biological community, synthetic peptides are now being produced in very large numbers. These complex biomolecules are produced by carrying out a very large number of sequential chemical operations. There are thus many opportunities for errors and modifications to occur both during and after synthesis. It is therefore imperative to have available effective means for rapidly verifying the correctness of the covalent structures of these materials. A good verification of the correctness of the structure is obtained when the measured MW agrees with the calculated MW. Conversely, any disagreement indicates the occurrence of a synthetic error or modification (46-48). Further, ion peaks in the mass spectrum in addition to those corresponding to the desired product reveal the presence of impurities which have co-purified with the product (46). The differences in MW between the desired product and undesired by-products provide important clues as to the nature and origin of the by-products.

Peptide mapping - Mass spectrometric peptide mapping has become an established and powerful structural tool for the analysis of proteins. The general strategy involves chemical or enzymatic degradation of the protein, followed by mass spectrometric MW determination of the resulting peptides (49-51). When necessary, further detailed structural information is obtained by subjecting the peptides to chemical procedures such as Edman sequencing, or to mass spectrometric sequence analysis (see later). Mass spectrometric peptide mapping has been used for the verification and correction of primary structures of proteins deduced from their DNA sequences (51-54), the structural definition of recombinant-DNA protein products (50,55-58), the identification of sites of variation in mutant and modified proteins (59-65), the determination of carbohydrate attachment sites in proteins (66-68), and the determination of peptide and protein structures (55-57, 69-76), especially in cases where the protein is modified posttranslationally.

Posttranslational modifications - One of the most important applications of mass spectrometry for protein analysis and one which has significant advantages over many classical biochemical methods is the detection and definition of posttranslational modifications. Frequently, post-translational modifications are initially detected by MW determination of chemically or enzymatically generated peptide fragments of the protein. When the MW of a given peptide fragment cannot be rationalized in terms of the sequence of the unmodified protein, the presence of such a modification is indicated. The nature and location of the modification can then be deduced, either from the mass difference between the modified and unmodified peptides, or from a further mass spectrometric structural analysis of the peptide. Examples of such mass spectrometric determinations include the detection and identification of blocked or modified amino and carboxy-termini (52,70,73-83), glycosylation sites (66-68, 84-86), phosphorylation sites (87), and others (88).

Disulfide pairing - Although only used to a limited extent, mass spectrometry appears to be of great potential utility for disulfide mapping of proteins. The mapping is generally carried out by selectively cleaving the protein under conditions where the disulfide bonds remain intact and mass spectrometrically determining the molecular weights of disulfide-containing peptide fragments. If necessary, further information concerning the identity of these peptide fragments can be obtained by amino acid analysis and Edman sequencing, and/or reducing the disulfide bond and subjecting the resultant peptides to additional mass spectrometric analysis. Mass spectrometry proved pivotal in the mapping of the seven S-S pairs in neurophysin (89-91). The task of establishing which of the 135,135 possible ways of pairing the 14 half-cystine residues in this protein was made more difficult by the necessity of using non-specific enzymes to induce release of disulfide containing peptide fragments. Mass spectrometry has also been used to locate the disulfide bonds in a series of model proteins (92), recombinant human growth hormones (93), human insulin like growth factors (94), and Paim I (95).

Miscellaneous - Mass spectrometry has also been used to provide information on the carboxytermini of proteins (78,96-98), the structure of the peptide network of pneumococcal peptidoglycans (99,100), iodination sites in cytochrome C (101) and the correct MW of proteins in cases where SDS gel electrophoresis or gel filtration gave ambiguous or incorrect results (102,103). An example of the latter application concerns the MW of the mating pheromone, Er-1, of the ciliate Euplotes raikovi (102). In urea/sodium dodecyl sulfate electrophoresis, reduced Er-1 migrated as a broad band corresponding to a MW of 29,000, a value considered to be anomalous. MW determinations of native Er-1 samples on gel filtration, which gave values of 9,000-12,000 were thought to be more reliable. The actual MW as determined by mass spectrometry was subsequently shown to be 4411.

Information deduced from mass spectrometric fragmentation - During the production of peptide ions by several of the ionization processes discussed above, large amounts of energy are deposited in the ionized molecule. A fraction of the resulting highly excited peptide ions may then undergo rapid fragmentation in the ion source of the mass spectrometer. The masses of these fragment ions can be determined and provide a partial or complete sequence of the peptide as well as information on the location and nature of any modifications present (1,72,104,105). Obtaining sequence information from these mass spectrometric fragmentation data can often be difficult because of

incomplete or weak fragmentation and interference from matrix or impurity generated ions. Tandem mass spectrometry overcomes many of these limitations and is becoming widely used for peptide structure analysis.

The technique of tandem mass spectrometry involves three sequential steps: 1) selection and separation of an ion of interest; 2) induced fragmentation of this ion; and 3) subsequent analysis of the resulting fragment ions (106). Fragmentation of the peptide ion of interest is induced by energetic collisions with neutral gas molecules or by bombardment with photons. Since a single selected ion is subjected to fragmentation, it is feasible to obtain structural information of a given peptide in a complex mixture. The total sequence can often be determined even when the amino terminus is blocked or when the peptide contains modified amino acid residues. The amount of peptide sample required for a tandem MS analysis depends on the nature of the sample as well as the instrumentation used, and varies from ca.1 nmole for two double-focussing deflection mass spectrometers operated in series (47) to ca. tens of pmoles for a tandem quadrupole Fourier transform instrument (107). It has recently been demonstrated that the sensitivity of the former instrument can be increased by a factor of 100 or more by incorporation of a so-called multichannel array detection system (108,109).

The tremendous utility of tandem mass spectrometry for providing information on the detailed structure of peptides is well documented (1,47,52,110). The technique has been especially useful for locating and defining posttranslational modifications of proteins (47,110) and for providing sequence information on proteins and peptides with blocked amino-termini, such as human lipocortin (47), type II calmodulin-dependent protein kinase (76), yeast cytoplasmic valyl-tRNA synthetase (47,53,54), cellular retinaldehyde binding protein (75), prostatic spermine binding protein (52), and prostatropin, a prostate cell growth factor (111,112). Tandem MS has also been used to determine the complete primary structures of four different thioredoxins (69,113-115), 90% of the primary structures of purple acid phosphatase and uteroferrin (116), the presence of s-farnesyl cysteine as a structural component of *Saccharomyces cerevisiae* mating hormone a-factor (117), the structures of cysteine-rich metal-binding polypeptides in cadmium-resistant tomato cells (118), and the structure of the polyglutamate chain attached to the folates of *E. coli* (119).

The tandem MS studies listed above were all carried out by inducing fragmentation of the peptide ions through collisions with neutral gas molecules. Fragmentation of a selected peptide ion can also be induced by a pulse of UV laser radiation incident on ions captured in a Fourier transform ion cyclotron resonance mass spectrometer, although such analyses have only been performed by a single research group (73,107,116,119-121). These photodissociation tandem MS analyses have yielded the complete sequence of a 10 pmole sample of a 15 residue tryptic peptide from beef spleen, purple acid phosphatase (116), have revealed that three photosystem II proteins of spinach chloroplasts contain N-acetyl-O-phosphothreonine at their amino-termini (73), have allowed the characterization of a benzyladenine binding-site peptide isolated from a wheat cytokinin-binding protein (120), and have assisted in the complete MS sequence analysis of an amino-terminally blocked, 78-residue protein (calbindin-D_{9k}) involved in calcium transport in mouse (121).

THE FUTURE

Relatively straightforward extrapolations of the present revolution in mass spectrometry lead us to predict that, in the not too distant future, the majority of peptide and protein-subunit molecular weights will be readily determinable by mass spectrometry, and that these determinations will be made rapidly (in a few minutes) on subpicomole amounts of material. Similarly, we believe that the ease and sensitivity for obtaining tandem MS structural data from peptides will also continue to increase rapidly. Thus, mass spectrometry is likely to become an increasingly important analytical tool for the biomedical scientist who works with peptides and proteins (122).

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122. This work was supported in part by grants (RO1-GM38274 and RR 00862) from the National Institutes of Health, USA.

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Chapter 28. Contrast Media For Magnetic Resonance Imaging

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Introduction - MRI diagnostic agents have been previously reviewed in this series, (1) and elsewhere (2,3). In vivo MR spectroscopy (4) is evolving as a separate field and is not covered in this review. Contrast MRI is a new and expanding science from the point of view of both technology and medicinal chemistry. Several recent reviews of advances in MRI technology have also appeared (5-7).

The basic principle of this technology is the differential signal arising from differences in T_1 (spin-lattice) and T_2 (spin-spin) relaxation times (8), concentrations and flux of protons of different physiological pools of water and tissue molecules (9, Table 1). T_1 values are strongly dependent on B_O (frequency) and increase in diseased tissue due to increased water content. T_2 values change little with B_O and only slightly from normal to diseased tissue (see Table 1).

Table 1: **Representative Soft Tissue Relaxation Times for Human Tissues (9)**

Tissue	T_1 (msec) (1.7 MHz)	T_1 (msec) (8.5 MHz)	T_2 (msec) (8.5 MHz)	T_1 (msec) (6.5 MHz)
Kidney	300-340	670 ± 60	50 ± 10	
Carcinoma	400-450			
Liver	140-170	380 ± 20	40 ± 20	
Abscess		1180	100	
Metastases	300-450	570 ± 190	40 ± 10	
Pancreas	180-200	290 ± 20	60 ± 40	
Carcinoma	275-400	840	40	
Prostate	250-325	--	--	
Carcinoma	350-450	--	--	
Brain				
Cerebrospinal fluid				1500
white matter				290
gray matter				525
glioma				750-1520

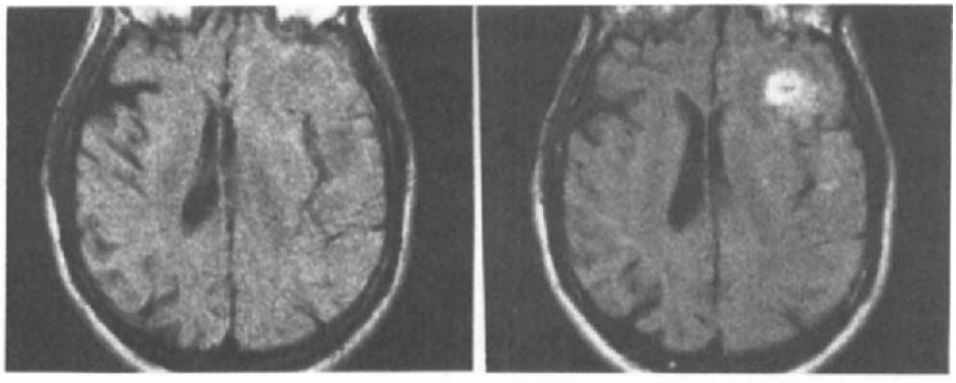
Contrast Media: Rationale for Application - Diagnosis by MRI of certain pathological conditions such as edema associated with tumor growth, inflammation and infarct is certainly possible without contrast agents based on tissue differences in T_1 and T_2 (Table 1). Enhanced resolution of abnormal tissue from normal tissue and global edema obtainable with contrast agents favors their use in surgical planning or following drug therapy in brain tumor patients. Figure 1

(10) shows pre- and post-contrast (with the gadolinium complex of diethylenetriamine pentacetic acid, DTPA) MRI's of a human glioma. While an abnormality (darker area) is seen in the unenhanced scan, the lesion (whiter area) is more clearly visible after application of the contrast medium. Imaging times may also be significantly reduced by use of contrast media allowing for increased patient throughput, although this possibility has not yet been realized in clinical practice.

Types of Contrast Media

Paramagnetic Ions - Paramagnetic compounds may be of two types: complexed (or uncomplexed) metal ions and stable free radicals. The relaxing effect of paramagnetic ions on water protons *in vivo* depends on the square of the effective magnetic moment of the paramagnetic species and its tissue concentration. Lanthanides and transition metal ions are the most important species and Gd^{+3}, with spin 7/2, is the theoretically most relaxive paramagnetic ion (11). The X-ray structure of the disodium salt of GdDTPA (gadopentetic acid) has been reported (11).

The relaxivity of metal ions depends on rapid exchange of tissue water with the metal coordination spheres. Thus, chelation and binding to tissue ligands influences relaxivity. In pure water $GdCl_3$ was more relaxive than GdDTPA at all concentrations (12). However, chelation does not always reduce and indeed may increase relaxivity if the chelate can concentrate in target tissues (see below). The tolerance and elimination pharmacokinetics in rats (13) and *in vivo* imaging results with GdDTPA (14) have been reported.



A B

Caption Figure 1

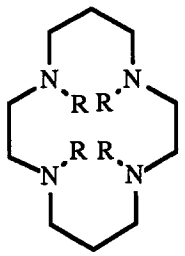
A) T_1-weighted unenhanced MR scan of the brain.

B) T_1-weighted contrast enhanced (with gadopentetate dimeglumine) MR scan of the brain showing improved delineation of the glioma.

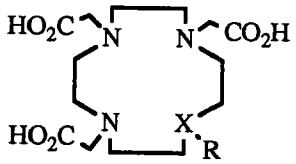
It is necessary to determine whether "uptake" of chelates represents exchange of the metal with tissue ligands in order to fully understand the source of contrast enhancement. This can be done by comparing the clearance rates of radiolabelled chelating agent with the rate of contrast diminution. Biodistribution of metal chelates can be studied by whole body autoradiography using isotopically labelled agents. In the case of Gd [^{14}C] DTPA the chelate is rapidly eliminated by glomerular filtration with minor uptake into renal, placental and intestinal tissue (15). Only a small fraction of the dose of the chelate passes the intact blood brain or placental barriers in pregnant rats. Transfer to fetal circulation does, however, occur to a sufficient degree in rhesus monkeys to allow imaging of the fetal urine duct (16). In dogs ^{54}Mn DTPA showed much greater tissue uptake (liver, bile, pancreas, bowel, kidney) than ^{153} GdDTPA (17).

Biodistribution can be altered by modifying the ligand. Diamides of DTPA form neutral (low osmolar) chelates with gadolinium (18, 25). Complexation of paramagnetic ions with p-tolyl EDTA enhanced urinary excretion and hepatobiliary excretion suggesting increased hepatic uptake due to the lipophilic substituent (19).

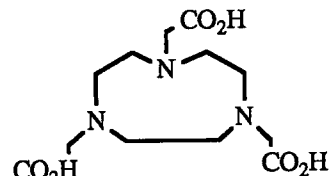
Complexes of manganese and gadolinium with cyclam derived ligands (1a,b) are claimed to have enhanced relaxivity but may suffer from decreased stability and increased toxicity (20).



1a R = H
b R = CH_3



2a DOTA (XR=NCH_2CO_2H)
b DO3A (XR-NH)
c DOXA (XR-O)



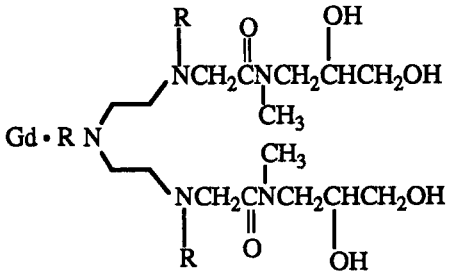
NOTA
3

Cyclic polyazapolycarboxylic acids offer the potential for chelates of increased stability (21). A much studied example, GdDOTA, is more stable than GdDTPA and has similar relaxivity in saline and liver tissue. This complex, however, has a therapeutic ratio approximately 2X GdDTPA and has a similar biodistribution and elimination profile in rats (22, 23).

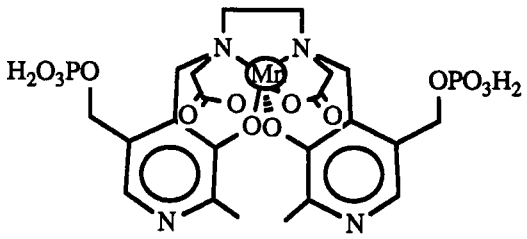
Studies of DOTA (2a) and NOTA (3) complexes of Gd^{+3} and Mn^{+2} showed that $1/T_1$ of aqueous solutions decreases with increasing field strength by a factor of two (24). This study also demonstrated the expected dependence of relaxivity on the temperature, pH, water coordination number and ligand field. Some of these factors may be rationally manipulated

in the design and application of chelated contrast agents.

In a study of complexes of gadolinium with nitrilotriacetic acid (NTA), EDTA, DOXA (2c), DTPA, DOTA (3a) and TTHA (6) the molar relaxivity of the complex increased with increasing number of uncoordinated metal sites. For complexes of DTPA, DTPA diamide (4), and DTPA 'oligomers', relaxivity decreased with substitution of acetic acid residues by amide functions and increased up to three fold for a DTPA-polymer complex of MW ~ 80,000 (25). This effect of molecular weight is due largely to slowing of molecular rotation rates in large molecules to values approaching the Larmor frequency (ω_O) at which $1/T_1$ is maximal. $Mn(DPDP)_2$ (5) has a molar relaxivity ca. 60% that of GdDTPA in vitro, but shows more effective hepatic uptake contrast (rabbits, 13-25 μM/kg) and comparable brain contrast at ca. 1/10 its LD_{50} (in rats ~2mM/kg) (26).



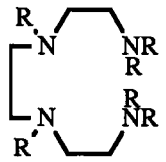
4 $R=CH_2CO_2H$



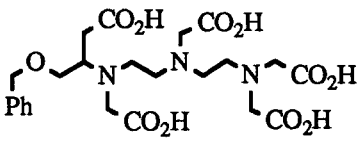
Mn (DPDP)$_2$

5

Gd(DO3A), (Gd•2b), is a new non-ionic (low osmolar) paramagnetic complex with about the same molar relaxivity and stability constant as GdDTPA (27). The Gd complex of 7, B-19036, was eliminated by balanced biliary/renal excretion (55/40% of dose) after 6 hrs in anesthetized rats and produced intense enhancement of liver signal in a field of 0.5T for a duration of 2-4 hrs (28).



6 (TTHA); $R=CH_2CO_2H$



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Thus, in vivo efficacy seems also to depend on accessibility of water molecules to the outer coordination sphere of the metal (89). The exchange of inner sphere water molecules may be much slower than outer sphere water molecules, especially for transition metal ions. For lanthanides these exchange rates are comparable and for ligands such as the aminoacetic acid type (e.g., DOTA) which "recruit" outer sphere water molecules the total outer sphere relaxivity has been shown to be comparable to or greater than

the inner sphere contribution (24, 89).

Porphyrins are a class of macrocyclic aza-ligands. A comparison of Fe^{+3}, Gd^{+3} and Mn^{+2} complexes of porphyrins, especially TPPS (tetrakis(4-sulfonatophenyl)porphyrin), showed the Mn^{+3} complex to have a superior profile of stability and relaxivity relative to porphyrin complexes of other metals (29). The manganese TPPS complex showed contrast enhancement of human colon carcinoma transplanted into athymic mice (30). Manganese protoporphyrin showed promise for selective enhancement of T_1 images of rat liver (31).

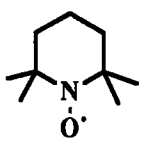
Liposomes are potential carriers for intravenous paramagnetic contrast agents (32). The key factors for liposome performance have been outlined (33). Leakage out of liposomes can be controlled by binding the metal ion to serum proteins (34). Liposomes are theoretically ideal targeting agents for liver imaging (35). Manganese citrate encapsulated in phosphatidylcholine was more effective than the free salt in enhancing signal from rabbit liver, spleen, heart, and kidney (36).

A preferred approach is to chemically bind the chelating agent to soluble protein carriers such as by acylation of albumin with DTPA bis-anhydride. A 4-fold enhancement of relaxivity *in vitro* over that obtained with free GdDTPA has been obtained with such an albumin chelate (37). Albumin-bound GdDTPA showed prolonged contrast enhancement of infarcted vs. normal cardiac tissues in rats compared to GdDTPA (37). The same albumin bound chelate showed prolonged contrast enhancement in blood, lung, heart, spleen, kidney and brain tissue samples from i.v. treated rats (38).

Monoclonal antibodies (MAbs) can be labelled with up to 50 chelated metals per protein molecule without significant loss of *in vitro* antigen binding capacity. Higher relaxivity is achieved if the chelating agent is conjugated to a polymer which is then coupled to the MAb (39). GdDTPA-linked bovine serum albumin coupled to murine monoclonal anti-human T cell antibodies (10 DTPA residues per molecule) provided effective contrast for human but not bovine T cells implanted in canine brains with temporarily disrupted (by mannitol, i.v.) blood brain barriers (40). GdDTPA labelled MAbs to human colon adenocarcinoma containing 25 chelating sites per molecule (administered i.v. 24 hours prior to excision of tumor) provided increased *in vitro* relaxation in excised tumors generated by implantation of the SW 948 cell line in nude mice (41). Extensive further studies are required to demonstrate effective *in vivo* imaging at tolerable doses of such agents.

Stable Free Radicals - The most studied stable free radicals are nitroxyls. The main problems with such agents, exemplified by tetramethylpiperidine-1-oxyl (8) is low

relaxivity. The modest relaxivity achieved with such agents may be enhanced slightly by binding them to macromolecules such as albumin (42). Metabolic reduction in erythrocytes to non-paramagnetic species (43) is not considered to be a major problem at this time (44). In vivo tissue distribution of these free radicals may be altered by forming water soluble sugar derivatives such as the glucopyranose (9) (45).



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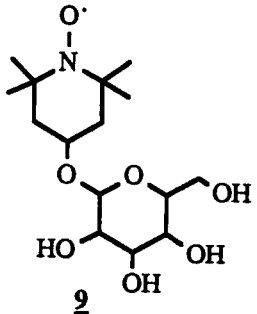

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Particulate Agents - Insoluble agents for intravenous or gastrointestinal imaging can be derived from paramagnetic ions or from ferrimagnetic double oxides of the formula $MO \cdot M_2O_3$ (e.g., Fe_3O_4, magnetite). These substances may be coated with protein or encapsulated in liposomes to target tissues such as the reticuloendothelial system of the liver.

Paramagnetic species such as (insoluble) gadolinium oxalate have been shown to opacify the upper GI tract after oral administration and the colon after rectal administration in rabbits (46). The MRI application of ferrimagnetic particles has been reviewed (47). Albumin coated magnetites have been discussed as potential contrast agents for liver, GI tract and genitourinary tract (48).

Toxicity

The lanthanides are essentially absent in normal mammalian tissues. Acute i.v. toxicity of unchelated paramagnetic salts may be a problem even at useful concentrations for contrast. Specific toxic effects of such metal ions are poorly understood at present. Gadolinium^{+3} at very high concentration (200 μM) causes frank reduction in dopamine β-hydroxylase levels and vasopressin in cardiac tissue homogenates (49). Hemodynamic effects of intravenous $MnCl_2$ and GdDTPA have been deemed to be minor (50). The low toxicity of GdDTPA as a contrast agent has been attributed to its high stability and rapid elimination (51). Thus, increased toxicity may be encountered with new transition metal chelates which are cleared slowly from tissues. Possible effects of R_f pulsing on tissues (mainly local heating) is a minor concern (52) as field strength is increased.

Clinical Progress with MRI Agents

Gadopentetate dimeglumine ($GdDTPAM_2$) is the only

contrast agent approved in the U.S. for use in adult patients undergoing MRI to provide contrast enhancement (CE) in those intracranial lesions with abnormal vascularity or those thought to cause an abnormal blood-brain barrier. This contrast agent has been shown to facilitate visualization of lesions including, but not limited to brain tumors (53). In countries outside the U.S., current registration permits use throughout the central nervous system including the spinal region.

$GdDTPAM_2$ has been studied in both neoplastic and non-neoplastic disease of the brain and spine. The unenhanced MRI may be normal or abnormal, but after the injection of gadopentetate dimeglumine, lesions not detected or unsuspected on the unenhanced MRI can be seen (See Fig. 1). Damage to the blood-brain barrier permits the contrast agent to leak into a brain metastasis or glioma (53). MRI of the brain after $GdDTPAM_2$ injection has demonstrated gliomas (54, 55), metastases (54, 55, 56), lymphomas (54), hemangioblastomas (55), neurofibromas (55, 57), meningiomas (55, 57, 58, 59) and acoustic neuromas (57, 58). In the pituitary and parasellar region, it has been possible to enhance visualization of microadenomas and craniopharyngiomas. In the case of pituitary microadenomas, visualization is due to reverse CE (i.e., the pituitary [background] normally enhances due to its vascularity, and the microadenoma [target] appears not to enhance)(60). In multiple sclerosis, the enhancement of MS plaques has been reported to be associated with active lesions (61). CEMRI may allow discrimination of multiple cerebral infarctions on the basis of age (62).

CE produced by $GdDTPAM_2$ in the spine and associated tissues has helped detect tumors, (63) recurrent or persistent post-operative back pain (failed-back surgery syndrome), (64) and degenerative disc disease (65). CE permitted the detection of a tumor within a syrinx which was not visualized on the unenhanced MRI scan (66). In the detection of neoplastic lesions, intramedullary and intradural-extramedullary lesions appear to visualize well. Extradural tumors (osseous metatases of the spine) may lose conspicuity on the MRI scan following the injection of $GdDTPAM_2$ (67). In the patient with failed-back surgery syndrome, the early enhancement of the remaining disc has been associated with epidural fibrosis (scar), where delayed or absence of enhancement has been associated with recurrent disc disease. The differentiation of these two entities is important since the appropriate therapy is different. In the "virgin" (previously not operated) spine, CE MRI may be of utility in evaluating disc herniation, particularly in the cervical spine (68). CEMRI has also been studied to assess the extent of head and neck disease (69).

New dynamic pulsing sequences have given encouraging results for imaging liver tumors, hemangiomas and focal nodular hyperplasia (70). The reticuloendothelial system (RES) has been imaged using ferrite particles (71). Since the ferrite, a superparamagnetic compound, is taken up by the

normally functioning RES, the signal from the normal liver and spleen (background) is suppressed by the overwhelming T_2 effects of the compound leaving the lesion visualized. Clinical trials of ferrite in liver metastases have been reported (72).

Distinguishing loops of bowel from structures in the abdomen and pelvis may be difficult. Ferric ammonium citrate appears to image the upper GI tract, better than the lower GI tract (73). $GdDTPAM_2$ in an oral preparation (including mannitol and sodium citrate) has shown opacification of the GI tract and detected pathology in adjoining structures such as pancreatic tumors (74). Perfluorooctylbromide (PFOB) has been shown to produce negative bowel contrast (75). Negative bowel contrast has also been demonstrated with an aqueous suspension of kaolin and bentonite (76).

$GdDTPAM_2$ has been detected by CEMRI during excretion from the kidneys (77). In addition, functional imaging of the kidneys has recently been demonstrated (78). The results suggest the possibility of evaluating within the nephron the location of diminished renal function and assessing the nephrotoxicity of drugs. A ferrioxamine complex (ferrioxamine methanesulfonate; S-FDF) also enhances the contrast of urine and may increase information about the bladder, kidney and ureters (79).

In the male pelvis, $GdDTPAM_2$ gives a variable pattern on CEMRI in lesions such as benign prostatic hypertrophy and prostate carcinoma. In the female pelvis, a variable pattern is observed in cervical carcinoma and leiomyomas in part because of normal enhancement of the surrounding structures (80).

In the heart, CEMRI with $GdDTPAM_2$ has detected acute myocardial infarctions (81). The degree of enhancement appears to be a time-dependent function. As the myocardial insult ages, the intensity of the image is diminished.

CE of the breast has been studied using $GdDTPAM_2$ (82). There appears to be improved differentiation of carcinoma and scarring, and poor separation of carcinoma and fibroadenoma.

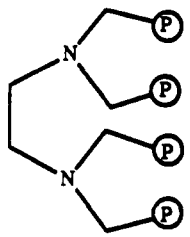
In the musculoskeleton, CEMRI has detected tumors and infarctions of the soft tissue and bone. Examples of these include chondrosarcoma (83) and tuberculous spondylitis (84), respectively. A CE lesion may be useful for selecting a biopsy site. MRI arthrography has been proposed using a dilute preparation of $GdDTPAM_2$. Cadaveric studies have shown meniscal tears and small cartilage defects (85).

$GdDTPAM_2$ is well tolerated (86). The most commonly associated adverse effects are: headaches, injection site coldness, and nausea (87). Transitory changes in serum iron and bilirubin levels have been reported in patients with normal and abnormal liver function (86).

Future Directions - The key advancements in the field of magnetic resonance imaging are expected to be in application of oral and tissue selective compounds, development of instruments and signal enhancement techniques which reduce acquisition time or optimize images (88). The next advancements should come in hepatic and gastrointestinal imaging. Design of more stable chelates (89) especially polymeric chelates may also be an important area.

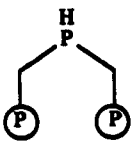
Uncontrasted images can resolve atherosclerotic occlusions (90), but quantitation of plaque may require paramagnetic contrast. Gated cardiac imaging could benefit from paramagnetic contrast enhancement (91, 92) but the current clinical view is that anatomical abnormalities of the heart (e.g., infarction) are not clarified by application of paramagnetic contrast (93). Acute ischemia (without infarction) requires contrast agents, probably because myocardial edema is often manifest only hours after the ischemic event (94).

Newer complexes showing promise for cardiac contrast enhancement are 7 , with maintained contrast up to 1 hr after acute ischemia in rats, and the manganese complex of EDTP (10). The gadolinium complexes with $(BDP)_2$ (11) and PMP (12) have shown some promise in preclinical studies of infarcts (94).



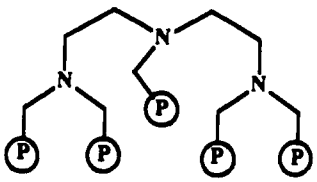
- EDTP (Ⓟ = $PO(OH)_2$)

10



- BDP (Ⓟ = $PO(OH)_2$)

11



- PMP (Ⓟ = $PO(OH)_2$)

12

Conclusions - MRI contrast is effectively achieved with lanthanide complexes such as gadopentetate dimeglumine which detect disruptions in the vascular barrier (e.g., blood-brain barrier). New agents which target specific tissues (e.g., tumor, liver) or which demarcate gastrointestinal motion artifacts are needed. Agents which improve contrast of images based on relaxation of nuclei other than protons (e.g., ^{23}Na) are still many years from clinical development.

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Chapter 29. New Directions in Positron Emission Tomography

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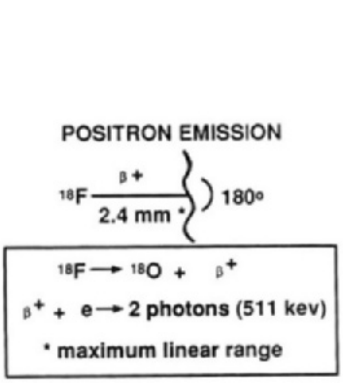
Introduction - Positron Emission Tomography (PET) is an *in vivo* imaging method which uses short-lived positron emitting radiotracers to track biochemical processes in humans and animals (1). Its primary use as a scientific and clinical research tool has recently been expanded to clinical practice to detect disease-related biochemical changes prior to the appearance of anatomical changes which can be visualized with conventional medical imaging modalities (2-6).

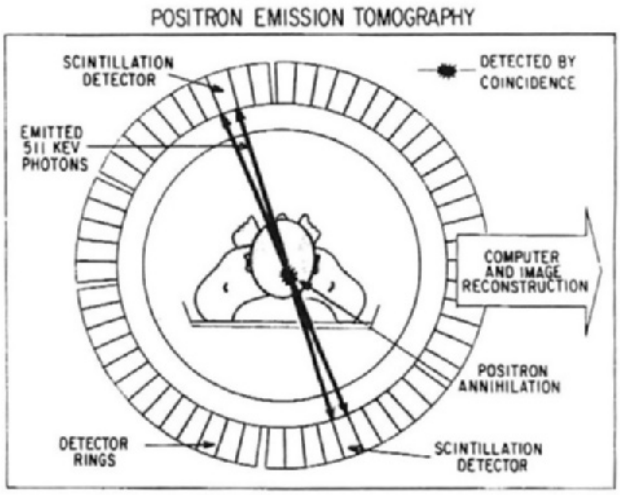
The short half-lives of positron emitting isotopes (ca. 1 minute to 110 minutes) have dominated the development and applications of PET. For this reason, PET research is commonly carried out at a Cyclotron-PET Center where high technology instrumentation (cyclotron for isotope production and positron emission tomograph for radioisotope detection) accommodates research within a restricted time frame.

This chapter, which is the first of two parts, will describe the design and synthesis of radiotracers labeled with the short lived cyclotron produced nuclides, and the application of PET to the study of brain, heart and tumor metabolism. The second part, to appear next year, will describe the use of PET in drug research and development with particular emphasis on clinical research and applications.

Description of the PET Method - In terms of information content, the PET scan is quite different from X-ray CT (Computed Tomography), NMRI (Nuclear Magnetic Resonance Imaging) or SPECT (Single Photon Emission Computed Tomography) (7). X-Ray CT provides anatomical information based on the differential absorption of X-rays by tissue. NMRI uses orthogonal magnetic and radiofrequency fields to afford anatomical information based on the proton relaxation properties and proton density of tissue. SPECT measures the relative concentrations of radioactivity in tissue after the injection of a chemical compound labeled with a single photon-emitting isotope such as iodine-123 or technetium-99m.

With PET, various radiotracers are used to visualize and quantitate different biochemical processes. In a PET study, a radiotracer labeled with a short-lived positron emitting isotope is administered either by intravenous injection or inhalation. The spatial distribution of radioactivity and temporal changes in total radioactivity concentration are quantitatively measured using a positron emission tomograph which coincidently detects the two energetic, body-penetrating photons (511 keV, 180° apart) resulting from positron decay. While both PET and SPECT detect radiotracer distribution, it is the chemical versatility of the positron emitters (see next section), the ability to measure their concentration quantitatively with relatively little attenuation by tissue, the greater sensitivity (more detected events per unit of radioactivity in tissue) and the superior resolution of PET which differentiates these two methods.





Since the commonly used positron emitters have half-lives which range from about 1 minute to just under two hours and decay to a stable ground state, the time during which radioactivity is present in the body is quite short. The radiation dose from a PET study is comparable to other medical diagnostic procedures.

Radiotracers for PET - The four major positron emitters used to label organic molecules are shown in Table 1. There are also two commonly used positron emitters which are available from generators. These do not require an on-site accelerator but are limited in terms of their chemical flexibility. ^{11}C, ^{18}F, ^{13}N and ^{15}O are most commonly produced using a small medical cyclotron which accelerates protons or deuterons in the 0-17 meV range to effect the required nuclear reaction. Three of these isotopes (^{11}C, ^{13}N, ^{15}O) can be substituted for the corresponding stable elements in organic molecules to produce a radiotracer which retains the properties of the parent compound. A fourth (^{18}F) can also be used to produce a radiotracer which mimics the parent molecule.

Table 1. Physical properties of the commonly used positron emitters.

Isotope	Half-Life	Specific Activity[a]	Nuclear Reactions
Carbon-11	20.4 min	9.22×10^6	$^{14}N(p,\alpha)^{11}C$
Fluorine-18	109.8 min	1.71×10^6	$^{18}O(p,n)^{18}F$; $^{20}Ne(d,\alpha)^{18}F$
Nitrogen-13	10.0 min	1.89×10^7	$^{13}C(p,n)^{13}N$; $^{16}O(p,\alpha)^{13}N$
Oxygen-15	2.0 min	9.08×10^7	$^{15}N(p,n)^{15}O$; $^{14}N(d,n)^{15}O$
Generator Produced (proton energies > 17mev required)			
Gallium-68	68.0 min		$^{68}Ge \rightarrow {}^{68}Ga$
Rubidium-82	75.0 sec		$^{82}Sr \rightarrow {}^{82}Rb$

[a] Curies/mmol (theoretical); in reality the measured specific activities of ^{11}C, ^{18}F, ^{13}N and ^{15}O are ca. 10-10,000 times lower because of unavoidable dilution with the stable element.

The cyclotron-produced radioisotopes are obtained as small precursor molecules (Table 2) which must be rapidly incorporated into organic molecules. Considering the limited number of precursors, and typical synthesis times of 30-50 minutes for carbon-11 labeled radiotracers and 1-2 hours for fluorine-18 labeled molecules, the challenge of developing a rapid synthetic scheme is significant. Furthermore, to enable use in human subjects, the resulting radiotracer must be chemically and radiochemically pure, sterile and pyrogen-free, and suitable for intravenous injection. Since the short-lived positron emitters are all of very high specific activity (see Table 1), starting quantities of the labeled precursors are typically in the submicromole range, requiring microscale techniques.

Table 2. Common ^{11}C and ^{18}F precursors and synthetic methods.

Isotope	Precursors	Common Synthetic Methods
Carbon-11	$[^{11}C]O_2$,	Carboxylation; Alkylation via $[^{11}C]H_3I$ Reductive Carboxylation
	$H[^{11}C]N$	Nucleophilic Substitution; Hydrocyanation; Michael Addition
Fluorine-18	$H[^{18}F]$	Nucleophilic Substitution (Aromatic and Aliphatic); Schiemann Reaction;Triazene Decomposition
	$[^{18}F]F_2$	Electrophilic Addition and Substitution

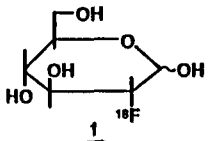
General Approaches to Radiotracer Design and Validation - Although rapid methods have been developed for introducing carbon-11 and fluorine-18 into several hundred different organic molecules (8), only a small percentage of these labeled compounds possess the necessary selectivity in vivo to provide a record of a specific biochemical process. While in vitro systems offer some degree of control over the variables and sampling the system is possible, radiotracer localization in vivo is influenced by many factors including transport into the tissue of interest, metabolism, binding to plasma proteins, non-specific binding and entry into metabolic pathways other than the one of interest. Thus, the selection of a chemical compound which will participate in a defineable biochemical process and the verification of its metabolic fate in small animal studies where tissue sampling is possible is the single most challenging aspect of radiotracer development. Mechanistic information can also be obtained from in vivo PET studies in animals where the stereoselectivity of the localization process (9,10), pharmacological intervention with unlabeled compounds of known pharmacological specificity (11), and kinetic isotope effects (12) and pharmacokinetics can be determined.

Tracer Kinetic Models - At least two measurements are usually made in a PET study: the concentration of labeled compound in a volume element of tissue and the arterial input function (i.e., concentration of radioactivity (or unchanged tracer) in arterial plasma samples over the time course of the study). Once the biochemical basis for the PET image is delineated, the extraction of quantitative information from these measurements requires the application of a tracer kinetic model to convert radioactivity concentration into metabolic rates or other physiologically relevant information. Tracer kinetic models for the PET measurement of regional glucose metabolism (13), brain blood flow (14), protein synthesis (15) and some of the properties of neurotransmitter receptors (16,17) have been described.

RADIOTRACERS FOR BRAIN METABOLISM

Up to this point in time, PET research, including the development of new radiopharmaceuticals, has had its major focus on studies of the brain. The current growth of the PET field and its widespread application to problems in biology and medicine was stimulated by the development of a method for measuring regional brain glucose metabolism with 2-deoxy-2-[^{18}F]fluoro-D-glucose (^{18}FDG, 1) in the mid 1970's (18,19). Today PET methods for the measurement of brain glucose metabolism, blood flow and neurotransmitter activity have been widely applied to problems in neurology (epilepsy and Parkinson's disease), psychiatry (Alzhemier's disease and schizophrenia) and cerebral malignancy.

Brain Glucose Metabolism and Blood Flow - 2-Deoxy-2-fluoro-D-glucose is a derivative of 2-deoxy-D-glucose in which a fluorine atom replaces a hydrogen atom at C-2. ^{18}FDG was the first radiotracer to be widely employed in PET research and is currently used in virtually every PET center in the world. Its primary application is in the measurement of regional glucose metabolism in normal and diseased brain although it has also been used to measure the effects of drugs and substances of abuse, cognitive processing and somatosensory stimulation on brain glucose metabolism (20). The ^{18}FDG method is based on the metabolic trapping of ^{18}FDG-6-phosphate, the product of hexokinase catalyzed phosphorylation of ^{18}FDG. Since glucose derivatives missing the hydroxyl group on C-2 do not undergo further glycolysis, the radioactivity in tissue after the injection consists only of free ^{18}FDG and ^{18}FDG-6-phosphate, allowing the measurement of glucose metabolism via a kinetic model. Carbon-11-labeled 2-deoxy-D-glucose (2) has been used in a number of protocols, since its short half-life permits serial studies to be carried out on a single subject on the same day (21). Although carbon-11 labeled glucose has also been developed along with a tracer kinetic model, it has neither been widely applied nor validated for the measurement of regional brain glucose metabolism (22). Brain blood flow is most conveniently measured using oxygen-15 labeled water and this tracer has been used in a number of elegant studies of cognitive processes (23).



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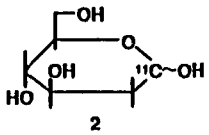

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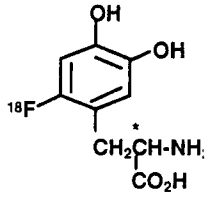
Neurotransmitter Studies - The observation of disease-associated abnormalities in neurotransmitter properties in postmortem human brain tissue has provided an impetus for the development of radiotracers for *in vivo* studies. By far, the most effort has been directed to the study of dopamine and dopamine receptors in the brain, stimulated by the importance of dopamine in Parkinson's disease and schizophrenia (24,25).

Since dopamine does not cross the blood-brain barrier, the investigation of brain dopamine metabolism with PET has required a fluorine-18 labeled derivative of DOPA, 6-[^{18}F]fluoro-DOPA (3). 6-[^{18}F]Fluoro-DOPA crosses the blood-brain barrier and is converted into 6-[^{18}F]fluorodopamine which then concentrates in dopamine-rich areas such as the striatum (26). Dopamine (D_2) receptor activity has also

been examined with PET using positron emitter labeled antagonists of the D_2 receptor. A key development in the application of PET to neurotransmitter studies has been the discovery of synthetic methods for producing high specific activity tracers which also have high affinity and selectivity for the receptor. High specific activity, where the dilution of radioisotope by the naturally occurring element can be in the range of factors of 10 to 10,000, is required in order to avoid saturation and measureable physiological reactions to the labeled compound. For example, N-methylspiroperidol has been labeled with carbon-11 (4) and fluorine-18 (5), and used to study dopamine receptors in normal and diseased brain and to probe dopamine receptor occupancy by antipsychotic drugs (25,27-29). The benzamide, [^{11}C]raclopride, (6) has also been used in human studies (30,31). The D_1 receptor antagonist, SCH-23390, (7) has been successfully labeled with carbon-11 (32-34).



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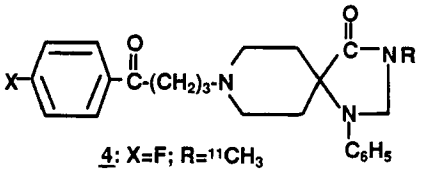
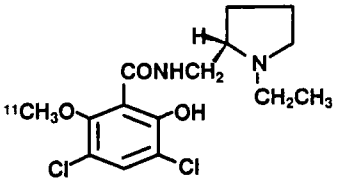


4: X=F; R=^{11}CH$_3$
5: X=^{18}F; R= CH$_3$



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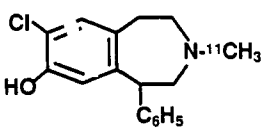

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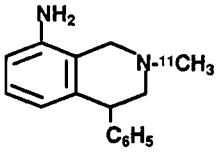

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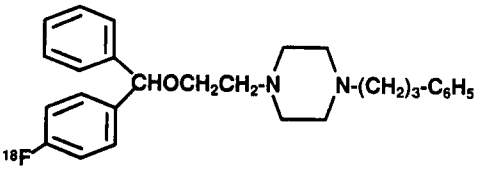

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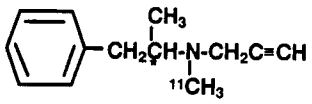

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[^{11}C]Nomifensine (8) and [^{18}F]GBR 13119 (9) have been used to probe the dopamine reuptake system (35,36). [^{11}C]L-Deprenyl (10) and [^{11}C]N,N-dimethylphenethylamine have been used for measuring monoamine oxidase activity (9,37). Radiotracers for the study of opiate (25,38,39), serotonin (25), muscarine-cholinergic (10) and benzodiazepine receptors (40) have also been developed.

Brain Protein Synthesis - A number of positron emitter labeled amino acids have been investigated as tracers for the quantitative measurement of the regional incorporation of amino acids into protein. For example, one strategy utilizes carboxyl-labeled amino acids such as [^{11}C-carboxyl]L-leucine whereby the products of amino acid metabolism (loss of [^{11}C]O_2) are not labeled, but proteins of interest are labeled (15,41). [^{11}C-S-methyl]L-Methionine and [1-^{11}C]L-methionine have also been examined as substrates for protein synthesis (42,43). [S-^{11}C-methyl]Methionine has been investigated most extensively, although studies are complicated by the fact that it can undergo both protein synthesis and transmethylation. The recent synthesis of carbon-11 and fluorine-18 labeled tyrosine (44,45) makes available additional radiotracers whose concentration in tissue may directly reflect labeled protein and free amino acid.

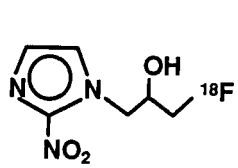
RADIOTRACERS FOR APPLICATION IN CARDIOLOGY

Radiotracers have been developed to probe the biochemical abnormalities which underly heart disease and to assess the viability of myocardial tissue so that appropriate treatments can be chosen. Myocardial blood flow, myocardial substrate metabolism, neuronal viability and receptor status have all been biochemical targets for radiotracer development.

Myocardial Blood Flow and Metabolism - The three major tracers for the measurement of myocardial blood flow are nitrogen-13 ammonia, oxygen-15 water and rubidium-82 (generator produced). A rather extensive body of knowledge on substrate utilization by the normal and ischemic myocardium has stimulated major efforts in the development of radiotracers to probe fatty acid and glucose metabolism. The first and most widely applied tracer for fatty acid metabolism was [1-^{11}C]palmitic acid (46). However, its use in the quantitative measurement of fatty acid metabolism is limited by recent evidence that multiple factors affect its clearance rate from myocardial tissue (47). Although β-methyl substituents were introduced into palmitic acid to make it non-metabolizable, (48), it still appears that partial metabolism of this tracer to [^{11}]CO_2 and partitioning into more than one lipid pool are limitations (49). [1-^{11}C]Acetate is under study as a marker for mitochondrial function (tricarboxylic acid cycle flux) (50).

Probes for Ischemic Tissue, Receptor Status and Neuronal Viability - ^{18}FDG has also been used to measure regional myocardial glucose metabolism, since it delineates ischemic but viable myocardial tissue via the shift from fatty acid metabolism (normal myocardium) to carbohydrate metabolism (ischemic tissue). Serial measurements of blood flow and glucose metabolism have shown predictive value for bypass surgery (51). [^{18}F]Fluoromisonidazole (11), a hypoxic cell sensitizer, is being developed to assess ischemic myocardial tissue (52). The formation of adenosine is a sensitive index for myocardial ischemia. [^{11}C]D,L-homocysteine thiolactone has recently been synthesized to probe the presence of adensoine via the formation of [^{11}C]S-adenosylhomocysteine (53).

Although the use of PET to assess receptor status and neuronal viability in heart has lagged behind the study of metabolism, a number of radiotracers have been developed including [^{11}C]quinuclidinyl benzilate [^{11}C]methiodide for muscarinic receptors (54) and [^{11}C]PK 11195 (12) for peripheral benzodiazepine receptors (55). A false neurotransmitter, [^{18}F]fluorometaraminol (13), has recently been developed to examine the neuronal viability of adrenergic nerves in the myocardium (56).



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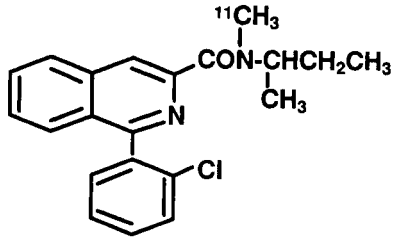

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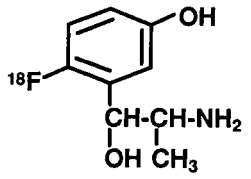

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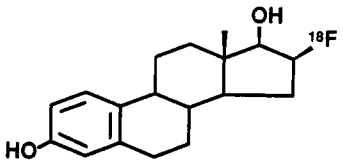
RADIOTRACERS FOR APPLICATION IN ONCOLOGY

The development of radiotracers for application in oncology has focussed on the following areas: the detection of malignant tissue and measurement of the degree of malignancy; the characterization of tumor tissue from a biochemical viewpoint; the differentiation of tumor tissue from normal tissue or from other pathologies; the detection of steroid hormone receptors, neurotransmitter receptors, tumor antigens or growth factors; the measurement of the delivery and efficacy of chemotherapeutic drugs.

Probes for Metabolism - A predominant biochemical feature of rapidly growing tumor cells is an ability to sustain high rates of glycolysis under anaerobic conditions. Glucose metabolism in human gliomas has been effectively probed with ^{18}FDG and used both to grade tumors (57) and to differentiate recurrent tumor from treatment-related necrosis (58). ^{18}FDG has also been used to study non-brain tumors (59). Oxygen-15 labeled tracers ([^{15}O]O_2 and H_2[^{15}O]) have been used to assess the relationship between the oxygen supply and demand of tumors (4). Enhanced protein synthesis in tumors is also a useful biochemical measure of tumor grade. Labeled methionine has been extensively utilized to delineate tumor tissue. [^{11}C]D and L-methionine have been used to show that methionine transport into tumor is non-selective (60). However, only [^{11}C]L-methionine showed continuous irreversible trapping in some tumors (61). The mechanism for the sequestratian of L-methionine by some tumors has not been fully characterized.

Receptor Status - Radiotracers for detecting the presence of receptors on tumors have been developed with a view to identifying patients who will respond to hormone targeted therapy. For example, 16α-[^{18}F]fluoroestradiol-17β (14) uptake in human breast cancer patients correlates with the density of estrogen receptors, as measured in biopsy samples, and reveals lymph node involvement (62). Radiotracers for the progesterone receptor are also under development (63). The presence of dopamine receptors which are expressed on some pituitary adenomas and which may be directly related to the cellular origins of the neoplastic cells has been detected by [N-^{11}C-methyl]spiroperidol, a dopamine D_2 receptor antagonist (64).



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Other Radiotracers for Tumors - The incorporation of labeled thymidine (tritium or carbon-14) into cells has long been the gold standard for measuring tissue proliferation and growth kinetics in cultured cells. The extension of this approach to in vivo studies using [^{11}C]thymidine was explored in the early 1970's (65) prior to modern PET instrumentation. Currently, efforts are focussed on the biochemistry and kinetics of [^{11}C]thymidine in tumors (66). Fluorine-18 labeled 5-fluorouridine and 5-fluoro-2-deoxyuridine have also been prepared and studied in small animals (8 and references therein). Polyamine biosynthesis, which accompanies rapid tissue growth, has been studied with [1-^{11}C]putrescine (67,68). Although PET studies of labeled putrescine have shown that this substance is taken up in high grade gliomas, the uptake is not specific for tumor tissue (69). Hypoxic cell sensitizers such as [^{18}F]fluoromisonidazole are also under investigation for the delineation of hypoxic regions of tumor tissue (52,70). PET has been used to probe the delivery of chemotherapeutic agents to tumors through the use of positron emitter labeled agents (71,72).

OUTLOOK FOR PET

It is safe to say that basic research in labeling biomolecules with positron emitters has shaped the PET field as we know it today. The growth of the field can be appreciated when one considers that in 1976 there were only four Cyclotron-PET facilities, all in the United States, whereas there are currently more than sixty centers worldwide. It is important to point out that PET is by no means a mature field. For example, new developments in rapid organic synthesis have a far-reaching impact, especially when they can be applied to radiotracers which are in demand for basic and clinical research. Basic research is still required to better understand and expand the physiological information that these tracer experiments can provide and accelerate the clinical availability of new tracers (73).

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73. This chapter was prepared at Brookhaven National Laboratory under contract DE-AC02-76CH00016 with the U. S. Department of Energy and supported by its Office of Health and Environmental Research.

Chapter 30. Polyamine Spider Toxins: Unique Pharmacological Tools

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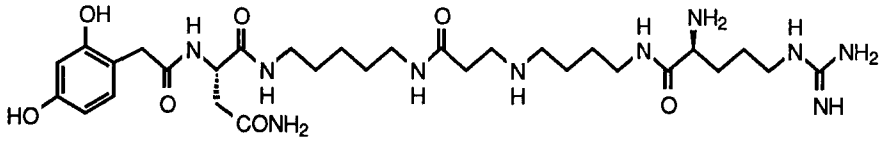
Introduction – The use of naturally derived venom constituents as research tools has allowed scientists to describe and understand the pharmacology and physiology of many important biological systems (1). Long-standing interest in spider venoms has primarily centered around the relatively large proteinaceous toxins (>3Kd) isolated from exotic spiders belonging to *Latrodectus, Loxosceles* and *Atrax.* genera (2). However, recent research from the laboratories of N. Kawai, P.N.R. Usherwood, and B.A. Tashmukhamedov has focused on the isolation and pharmacology of an exciting new class of low molecular weight (<1000 daltons) polyamine-derived constituents isolated from common "garden variety" spiders.

These polyamine toxins, obtained from the venom of a variety of orb-weaver spiders (family *Araneidae*; genera *Nephila, Argiope* and *Araneus*), are rapidly emerging as unique tools for understanding excitatory amino acid (EAA) transmission and related pharmacology/physiology (3). Moreover, the ability of these materials to potently affect excitatory amino acid neurotransmission underscores their potential both as tools and as novel lead structures for pharmaceutical and pesticide research (4,5). In this chapter we review the chemistry and pharmacology of *Araneidae* polyamine spider venom toxins. Also included is a brief discussion of the venom from the funnel-web spider (family *Agelenidae*).

Isolation and Structural Characterization of Polyamine Spider Toxins – Spiders produce very small quantities of venom containing complex mixtures of constituents. In addition, venom supply and content for a particular species may be subject to seasonal and regional variations (6). As a consequence, a major obstacle to spider toxin research is the availability of adequate and reliable amounts of whole venom. Several methods have been developed for the efficient removal of venom from the animal (7,8). The more common procedures include whole venom gland dissection/extraction and electrically stimulated milking of anesthetized spiders (9). Individual chemical entities are typically isolated from gland extracts or whole venom and purified by reverse-phase high performance liquid chromatography. A broad assortment of spectral (^{1}H, ^{1}H-COSY, ^{13}C NMR; UV; FAB-MS) and chemical (amino acid analysis, Edman degradation, hydrolyses and derivatizations) techniques have been employed for structure elucidation. Structural assignments have, in all cases thus far, been verified by independent chemical synthesis (*vide infra*).

The chemical characterizations of the insect neurotoxin, NSTX-3 (**1**), isolated from the Papua New Guinean spider (*Nephila maculata*) and a structurally related toxin, JSTX-3 (**2**), obtained from the Japanese Joro spider (*Nephila clavata*) were recently reported (10-12). Both toxin principles possess the 2,4-dihydroxyphenylacetyl-L-asparaginyl-cadaverine-β-alanine-putresine substructure but differ in that NSTX terminates with an L-arginine residue while JSTX ends with a propylamine moiety.

Several groups have independently isolated and proposed structures for the biologically active principles present in venom of spiders from the genus Argiope ("argiotoxins" (**3**-**5**)) (13-16). Sources of these argiotoxins, which closely resemble the nephilatoxins, include *A. aurentia* (13), *A. florida* (14), *A. lobata* (15) and *A.trifasciata* (14,16). The highly basic argiotoxins share the following common structural features: an internal polyamine flanked on one side by a hydrophilic N-terminal arginine and on the other by a 2,4-dihydroxy-phenylacetamide, or a 4-hydroxyindoleacetamide-containing terminal asparagine.



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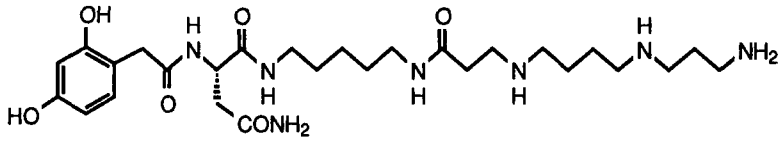

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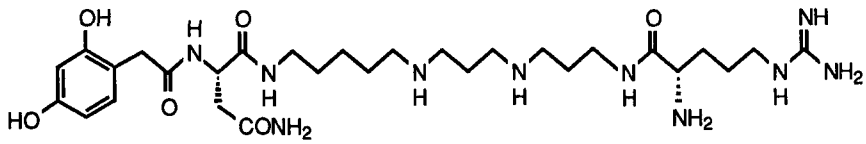

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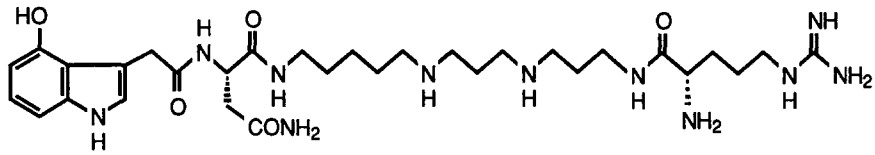

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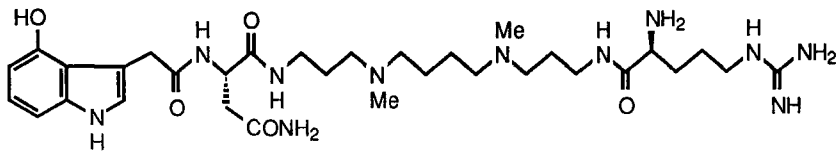

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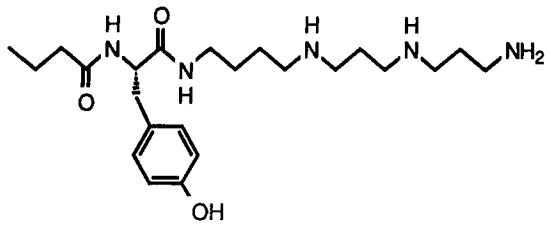
δ-Philanthotoxin (6), an insect neurotoxin structurally and pharmacologically related to the spider polyamines was found in the venom of the digger wasp *Philanthus triangulum* and subsequently assigned the structure 6 (17,18). The philanthotoxin molecule has a spermine nucleus appended to an N-n-butyryltyrosine residue by an amide bond.



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Synthesis of Polyamine Spider Toxins – The chemistry involved in the preparation of many of the naturally occurring polyamines has been summarized (19). Because polyamine spider venoms have highly polar hydroxyaryl and amino acid residues, synthetic strategy is dictated by the appropriate selection of chemically compatible basic amine, phenol and amino acid protecting groups. The polyamine backbones are routinely assembled in a stepwise fashion using amine alkylations, reductive aminations and acrylonitrile additions. Standard peptide coupling reactions are employed for the formation of amide bonds. This general strategy has been utilized for the preparation of NSTX-3 (1) (20), JSTX-3 (2) (21), argiotoxin-636 (3) (13,22-24), -659 (4) (13,23), -673 (5) (23), and δ-philanthotoxin (PTX-433) (6) (17,18). Synthesis is necessary to unambiguously verify structural assignments and to provide generous quantities of these and related toxins for continued biological investigations.

Polyamines Spider Venoms as Excitatory Amino Acid (EAA) Antagonists – Interest in the polyamine spider toxins is a result of the observation that these molecules affect those synapses at which an excitatory amino acid (glutamate or aspartate) is the neurotransmitter (*vide infra*). The invertebrate neuromuscular junction is a well recognized example of an EAA synapse at which functional antagonism will elicit paralysis. In addition, most excitatory synapses in mammalian brain use glutamate or aspartate as neurotransmitters, suggesting that EAA's are intimately involved in brain function (4,5). Moreover, increasingly strong and detailed evidence relating EAA function to important neurological disorders (e.g. stroke damage and Alzheimer's disease) now exists (25). Compounds that affect EAA function, particularly those that antagonize the action of such transmitters, are therefore of considerable agricultural (insect control) and therapeutic interest.

Excitatory amino acids produce synaptically-mediated depolarization of neurons by acting on specific receptors. The depolarizing effect produced by activation of excitatory synapses is referred to as an excitatory postsynaptic potential (EPSP) or excitatory postsynaptic current (EPSC). On the basis of extensive biochemical, pharmacological and electrophysiological data, three main classes of EAA receptor (named for their selective activation by known agonists) are recognized: N-methyl-D-aspartate receptors (NMDA) and the quisqualate and kainate receptors (both non-NMDA). The NMDA and non-NMDA receptors appear to have distinct functions (4,5).

Pharmacology of Polyamine Spider Toxins: Invertebrate Nervous Systems – Blockade of neuromuscular transmission in invertebrates by polyamine spider toxins reveals their glutamate antagonist activity (26-28). For example, JSTX-3 (2) blocks the excitatory postsynaptic potential (EPSP) elicited by iontophoretically applied glutamate at synapses in the lobster neuromuscular junction where an excitatory amino acid is thought to be the neurotransmitter. In this preparation, JSTX is reported to produce an irreversible, voltage-independent blockade at very low concentrations (28). ^{125}I-JSTX blocks neuromuscular transmission in the lobster (29) and binds exclusively to regions of the sacrolemma opposed to axon terminals (30). In studies published prior to the synthesis of 2, the JSTX used presumably consisted of a group of related toxins partially purified from extracts of whole venom glands by gel filtration and Sephadex chromatography (26). No data on the biological activities of any other components of *Nephila clavata* venom, save the single toxin ultimately identified as JSTX-3 (2), have been published. However, given the large number of components in this venom, it is unlikely that the effects attributed to the non-synthetic JSTX arose from a single toxin (31). The effects of JSTX have been less intensively studied in other invertebrate systems. In the giant synapse of the squid stellate

ganglion, venom gland extracts block EPSP's and glutamate-induced depolarization without affecting electrically evoked antidromic (presynaptic) responses (32). It is not clear whether the toxin binds to the normal attachment site of glutamate on the receptor molecule or to an external site on the associated ion channel, thereby preventing entry of ions (28). Therefore, it is uncertain from the available literature whether JSTX produces its antagonism of crustacean neuromuscular transmission *via* competitive or non-competitive mechanisms.

A venom-gland extract from *Argiope lobata* blocks neuromuscular transmission and the effects of applied glutamate in locust muscle (33). Similar activity was observed in venoms from other orb-weaving spiders and active fractions were isolated from *A. lobata* venom by gel chromatography (34). This toxin was further used for purification of glutamate receptors from crab muscle. The affinity-purified fraction was inserted into artificial membranes and shown to produce pharmacologically specific glutamate-induced ion conductances (35). One of the several active components of *Argiope lobata* venom was subsequently identified as argiopine **3** (15,34). This toxin was determined by electrophysiological methods to act functionally as a postsynaptic open-channel blocker ($K_D = 10^{-7}M$) in blowfly larval muscle (36).

Argiope aurantia venom contains two distinct classes of toxins that paralyze grasshoppers, cockroaches, flies and moths (37). Five toxins with molecular weights below 1,000 daltons including argiotoxins-636 (**3**), 659 (**4**) and 673 (**5**) and toxins above 5000 daltons have been isolated (13). In housefly paralysis assays, synthetic argiotoxin-659 has an ED_{50} of 0.9 pmol/mg, whereas synthetic argiotoxin-636 exhibits an ED_{50} of 3.3 pmol/mg. In *in vitro* assays, however, the potencies of the two toxins families are similar.

The effects of low molecular weight toxins from the venoms of *Argiope trifasciata*, *Argiope florida* and *Araneus gemma* on EAA-mediated synaptic transmission in the locust retractor unguis muscle have also been studied (38-40). These toxins block neurally evoked muscle twitch, the junctional potential elicited by glutamate iontophoresis, and the voltage-clamped EPSP in a slowly reversing fashion. Single channel studies of locust muscle suggest that the low molecular weight orb-weaver toxins (principally argiotoxins -636, -659 and -673) are very potent non-competitive antagonists of EAA receptor channel complexes. Their principal effect is a use-dependent blockade of open ion channels, although some action on closed channels is probable (41,3). The combination, in orb-weaver venom, of a high concentration of free glutamate, which activates the receptor-associated channels, with a potent open-channel non-competitive antagonist **3**, **4** or **5** provides the spider with a sophisticated and powerful mechanism for immobilizing insect prey (42).

The effects of δ-philanthotoxin (**6**) on a variety of insects have been extensively studied (17). This toxin causes reversible paralysis, apparently by blocking quisqualate-sensitive junctional and extra-junctional glutamate receptors on skeletal muscle. The specificity of **6** for a particular subtype of glutamate receptor has not yet been demonstrated.

Pharmacology of Polyamine Spider Toxins: Vertebrate Nervous Systems – Polyamine spider venoms also act on glutaminergic synapses in vertebrate systems. Partially purified JSTX blocks the responses of CA1 pyramidal neurons in rat hippocampus both to stimulation of the appropriate afferent neurons (Schaeffer collaterals) and to direct application of glutamate (43). 2,4-Dihydroxyphenylacetylasparagine, a common structural feature of JSTX (**2**) and argiotoxin-636 (**3**), inhibits binding of ^{3}H-L-glutamate to rat membranes with an IC_{50} of $10^{-5}M$ (44). JSTX also inhibits uptake of ^{3}H-glutamate into rat brain synaptosomes (45). The physiological actions of JSTX were assessed by recording EAA responses in voltage-clamped single rat hippocampal pyramidal neurons in culture (46). JSTX from partially purified gland extract completely blocked neuronal responses to quisqualic acid (QA) and kainic acid (KA) in a dose dependent (but use independent manner) at concentrations of 10^{-12} to $10^{-8}M$. Voltage dependent Na^+ and K^+ currents and glycine dependent Cl- currents were not affected by the toxin. Because JSTX was ineffective in blocking neuronal responses to L-aspartate, it appears that this toxin has little antagonist action at NMDA receptors.

In catfish lateral line organ, partially purified JSTX abolishes afferent impulses elicited by focal application of glutamate (47). In studies with the dogfish retina, purified JSTX

hyperpolarizes horizontal cells, blocks their response to light and antagonizes glutamate-induced depolarization of these cells in a slowly reversible manner (48). Interestingly, JSTX acts as an agonist at "on-bipolar" cells in a slowly reversible manner, opening the same ion channels as does glutamate. Thus, *Nephila* venom appears capable of exerting distinct and opposite effects at two classes of EAA receptor-channel complexes in vertebrates (48).

A variety of venoms have been screened for their ability to block synaptic transmission at glutaminergic synapses in the chick cochlear nucleus (49). Argiotoxin-659 (**4**), isolated from *Argiope aurantia*, produced a readily reversible use-independent blockade of transmission. Similarly, whole venom from *A. trifasciata* suppresses both spontaneous and sound-evoked electrical activity in guinea pig cochlear nerve (50). Synthetic argiotoxin-636 (**3**), -659 (**4**) and JSTX-3 (**2**) are functional antagonists of both NMDA and non-NMDA classes of glutamate receptor in rat brain. In addition, these materials greatly increase the binding of glycine, apparently by increasing the number (B_{max}) of strychnine-insensitive binding sites (51). Occupation of the strychnine-insensitive glycine binding site (found predominantly in rat forebrain) appears to be a prerequisite for activation of the NMDA receptor (52). It is not yet certain whether modulation of this site by polyamine spider toxins underlies their glutamate antagonist activity.

New Directions in Venom Research: Funnel Web Spider Toxins – A number of toxins from the funnel-web spider *Agelenopsis aperta* paralyze insects (53). These toxins contain low molecular weight acylpolyamine constituents (α – agatoxins), at least six 36-37 amino acid residue peptides (μ – agatoxins; reportedly sodium channel activators), and several larger polypeptides (ω – agatoxins; potent irreversible blockers of synaptic transmission, presumably by action at presynaptic calcium channels) (54). Other high molecular weight toxins found in the venom of the funnel web spider, *Hololena curta*, and the primitive hunting spider, *Plectreurys tristes*, are similar to the ω-agatoxins in inhibiting insect neuromuscular transmission (55,56). A (5Kd) toxin isolated from the venom of *Hololena curta* produces potent irreversible blockade of synaptic transmission by action on the postsynaptic neuron (50). Antidromic stimulation and responses to EAA agonists are also blocked by this toxin.

Two classes of toxins from *Agelenopsis aperta* venom that block transmission in the chick cochlear nucleus assay have also been studied (49,57). A multi-component fraction identified as "AG-1", containing polypeptides of 4-7 Kd irreversibly blocks synaptic transmission at very low doses without affecting antidromic stimulation or postsynaptic responses to applied EAA agonists. This fraction is likely to contain many if not all of the "μ" and "ω"-agatoxins (53). AG-1's action was shown to be use independent and is antagonized by extracellular calcium (58). In a dose-dependent manner, AG-1 potently blocks the binding of ^{125}I-ω-conotoxin (a calcium antagonist peptide from the venom of the marine snail, *Conus geographus*) to rat brain. However, AG-1 markedly increases binding of the dihydropyridine calcium antagonist ^{3}H-nitrendipine to brain membranes, apparently by increasing the number of binding sites (59).

The second fraction from *Agelenopsis aperta* contains polyamine toxins of <1 Kd, whose structures have not been reported. This low molecular weight fraction, AG-2, suppresses synaptic transmission in the chick cochlear nucleus in a fully-reversible, dose-dependent and use-independent manner (57). In sharp contrast to AG-1, AG-2 substantially increases binding of ^{125}I-ω-conotoxin but decreases the binding of ^{3}H-nitrendipine to rat brain (59). AG-2, administered intravenously or intracerebroventricullarly, protects rats against otherwise-lethal doses of the convulsants, kainic acid, picrotoxin or bicuculline. At doses effective in preventing lethal seizures, AG-2 produces only mild sedation in test animals (60).

Conclusion – To date, venoms from only a handful of the estimated one hundred thousand spider species have been studied in any detail. The results of these studies already suggest that considerable structural and functional diversity exists within the polyamine toxin class and that these compounds differ significantly from previously-known classes of EAA and calcium antagonists. For these reasons, it seems likely that spider venoms will continue, for some time, to provide novel molecules useful for pharmaceutical and pesticide research.

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Section VII. Trends and Perspectives

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Somerville, New Jersey 08876

Chapter 31. To Market, To Market - 1988

Helen H. Ong and Richard C. Allen
Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ 08876

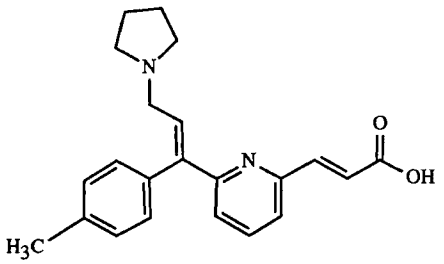
The new chemical entities (NCEs) for human therapeutic use introduced into the world marketplace for the first time during 1988 totaled 52 in number, reflecting a 20% decline from 1987 (1) and a return to the level of 1986 (2). As in the past six years, Japan was the leading country in which the most NCEs were introduced, with 15, followed by France, with 8. Sharing third place are Italy, the United States, the United Kingdom and Sweden, each introduced four NCEs. Again, nearly 50% of the new launches in 1988 originated in two countries: 12 in Japan and 10 in the United States, followed by France, Italy, and West Germany.

As a continuation of recent trends, cardiovascular and antiinfective products comprised more than half of the new NCE's launched in 1988. Several new classes of agents were introduced; these include the first proton pump inhibitor, omeprazole, as an antiulcer agent; the first triazole antifungal agent, fluconazole; recombinant human erythropoietin for use in anemia; and the first partial $beta_1$-agonist, xamoterol, for the treatment of congestive heart failure.

During 1988, 20 new chemical entities were approved in the United States (3) and 29 reached the marketplace, including α-1 anti-trypsin, apraclonidine hydrochloride, octreotide and pergolide mesylate which were also first worldwide launches. It is worth noting that, as a departure from previous trends, no systemic antiinfectives and only one cardiovascular agent were introduced in 1988 in the United States.

Acrivastine (antihistamine) (4,5)

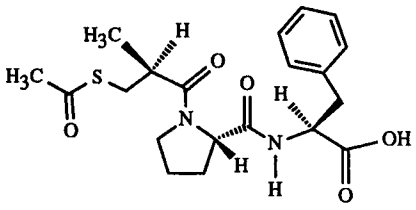
Country of Origin:	**USA**
Originator:	**Burroughs Wellcome**
First Introduction:	**United Kingdom**
Introduced by:	**Burroughs Wellcome**
Trade Name:	**Semprex**
CAS Registry No.:	**87848-99-5**



Acrivastine is an orally-active H_1 receptor antagonist reportedly useful in the treatment of allergic rhinitis. The main advantages of acrivastine in comparison to older antihistamines are its low sedative potential and the absence of anticholinergic side-effects.

Alacepril (antihypertensive) (6-8)

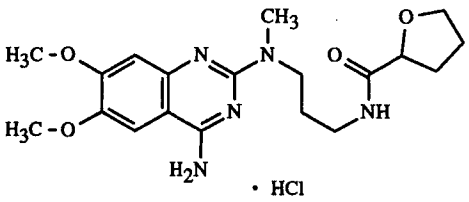
Country of Origin:	**Japan**
Originator:	**Dainippon**
First Introduction:	**Japan**
Introduced by:	**Dainippon**
Trade Name:	**Cetapril**
CAS Registry No.:	**74258-86-9**



Alacepril is an angiotensin-converting enzyme (ACE) inhibitor useful in the treatment of essential and renal hypertension. Compared with captopril, alacepril is a weaker ACE inhibitor *in vitro*, yet clinically it has a longer duration of action and appears to be more active due to an added component of norepinephrine antagonism.

Alfuzosin Hydrochloride (antihypertensive) (9,10)

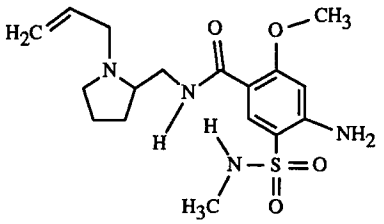
Country of Origin:	**France**
Originator:	**Synthelabo**
First Introduction:	**France**
Introduced by:	**Synthelabo**
Trade Name:	**Xatral**
CAS Registry No.:	**81403-80-7**



Alfuzosin hydrochloride is a post-synaptic α-antagonist useful in the symptomatic treatment of benign prostatic hypertrophy and acute adenoma by lowering urethral contractions induced by alpha$_1$ stimulation. Alfuzosin is also being developed as an antihypertensive; its efficacy in this indication is comparable to prazosin with no significant change in heart rate.

Alpiropride (antimigraine) (11,12)

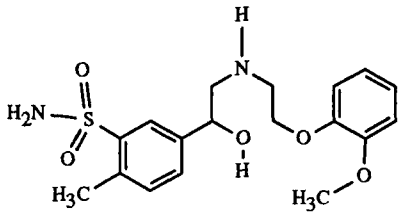
Country of Origin:	**France**
Originator:	**Delagrange**
First Introduction:	**Portugal**
Introduced by:	**Delagrange**
Trade Name:	**Rivestel**
CAS Registry No.:	**81982-32-3**



Alpiropride is a sulpiride-like dopamine antagonist reportedly useful in the treatment of migraine. Among the various sulpiride analogs, alpiropride has shown the largest separation between affinities for D_2 and D_4 binding sites in the brain.

Amosulalol (antihypertensive) (13-15)

Country of Origin:	**Japan**
Originator:	**Yamanouchi**
First Introduction:	**Japan**
Introduced by:	**Yamanouchi**
Trade Name:	**Lowgan**
CAS Registry No.:	**85320-68-9**



Amosulalol is a potent antihypertensive with combined alpha and beta blocking properties. Indicated for the treatment of essential hypertension, it appears to possess a minimum of side-effects. Its beta-blocking property reduces the tachycardia associated with alpha-blockers, while its alpha-blocking activity reduces the peripheral vasoconstriction associated with beta-blockade.

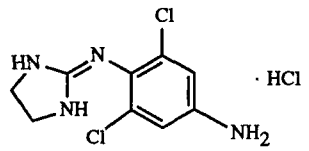
α-1 Antitrypsin (protease inhibitor) (16, 17)

Country of Origin:	**USA**	Introduced by:	**Cutter (Bayer)**
Originator:	**Cutter (Bayer)**	Trade Name:	**Prolastin**
First Introduction:	**USA**		

α-1 Antitrypsin (AAT) is a protease inhibitor useful in retarding the progression of emphysema in patients with a deficiency of α-1 antitrypsin. Based on recent clinical data, it appears unlikely that AAT would have any beneficial effect on emphysema developed as a result of environmental factors or cigarette smoking.

Apraclonidine Hydrochloride (antiglaucoma) (18-20)

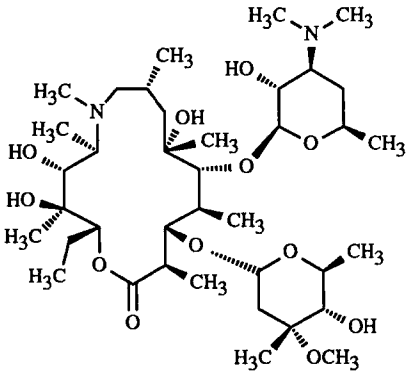
Country of Origin:	**W.Germany**
Originator:	**Boehringer Ingelheim**
First Introduction:	**USA**
Introduced by:	**Alcon**
Trade Name:	**Lopidine**
CAS Registry No.:	**87913-86-8**



Alpraclonidine hydrochloride is a selective α_2-adrenergic agonist useful in the postsurgical control of intraocular pressure with minimal systemic side-effects. Its efficacy in chronic therapy of open angle glaucoma is currently under investigation.

Azithromycin (antibiotic) (21,22)

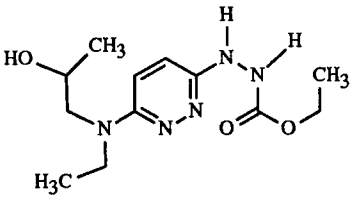
Country of Origin:	**Yugoslavia**
Originator:	**Pliva**
First Introduction:	**Yugoslavia**
Introduced by:	**Pliva**
Trade Name:	**Sunamed**
CAS Registry No.:	**83905-01-5**



Azithromycin is a long-acting macrolide antibiotic structurally related to erythromycin A (EA), having a methyl-substituted nitrogen at position 9a in the aglycone ring. Azithromycin is reported to be highly effective in the treatment of respiratory and urinary infections; it has the advantages of being acid-stable and requiring a shorter course of treatment than EA.

Cadralazine (antihypertensive) (23-25)

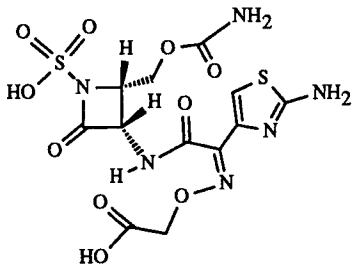
Country of Origin:	**Italy**
Originator:	**ISF(Smith Kline)**
First Introduction:	**Italy**
Introduced by:	**ISF; Ciba Geigy**
Trade Name:	**Cadraten; Cadrilan**
CAS Registry No.:	**64241-34-5**



Cadralazine is a vasodilatory antihypertensive reportedly better-tolerated and longer-acting than hydralazine. It is useful as a second-line treatment in the management of hypertension.

Carumonam (antibiotic) (26-28)

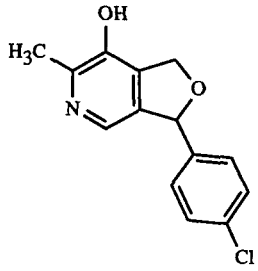
Country of Origin:	**Japan**
Originator:	**Takeda**
First Introduction:	**Japan**
Introduced by:	**Takeda**
Trade Name:	**Amasulin**
CAS Registry No.:	**87638-04-8**



Carumonam is an injectable antibiotic, the second monobactam ever developed (the first being Squibb's aztreonam). It is highly active against Gram negative bacteria, particularly *pseudomonas*, and is reportedly effective in the treatment of pneumonia, cystitis, peritonitis and secondary infections.

Cicletanine (antihypertensive) (29, 30)

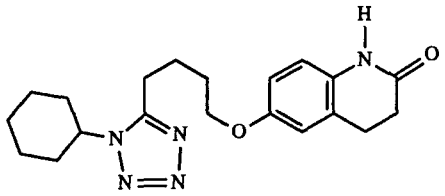
Country of Origin:	**United Kingdom**
Originator:	**Ipsen-Beaufour**
First Introduction:	**France**
Introduced by:	**Ipsen-Beaufour**
Trade Name:	**Tenstaten**
CAS Registry No.:	**82747-56-6**



Cicletanine is an antihypertensive diuretic indicated for the treatment of essential hypertension. Its unique mechanism of action is thought to involve the decrease of peripheral resistance and increase of venous compliance without modification of arterial diameter and venous tone. Chemically cicletanine belongs to a series of furopyridines not associated with any currently known pharmacological classes.

Cilostazol (antithrombotic) (31, 32)

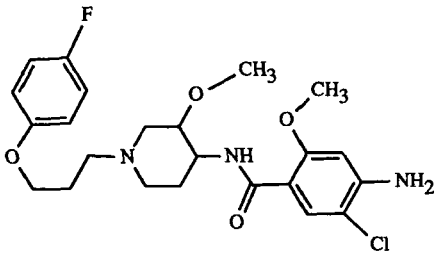
Country of Origin:	**Japan**
Originator:	**Otsuka**
First Introduction:	**Japan**
Introduced by:	**Otsuka**
Trade Name:	**Retal**
CAS Registry No.:	**73963-72-1**



Cilostazol is a platelet aggregation inhibitor with cerebral vasodilating activity, indicated for use in stroke and myocardial infarction. In patients with cerebral thrombosis, transient ischemia and cerebral arteriosclerosis, cilostazol significantly inhibits ADP-, collagen- and epinephrine-induced platelet aggregation. Side effects include headache and tachycardia.

Cisapride (gastroprokinetic) (33-35)

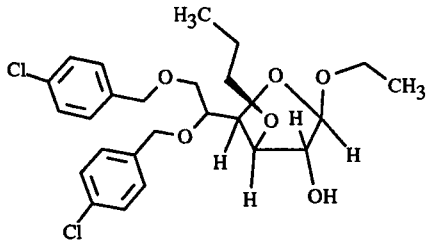
Country of Origin:	**Belgium**
Originator:	**Janssen**
First Introduction:	**Sweden**
Introduced by:	**Janssen**
Trade Name:	**Prepulsid**
CAS Registry No.:	**81098-60-4**



Cisapride is a gastroprokinetic useful in the treatment of reflux esophagitis, constipation, and a variety of gastro-intestinal motility disorders. Its novel mechanism of action is thought to involve the enhancement of acetylcholine release in the mysenteric plexus of the gut. It is reportedly devoid of CNS and cardiac side-effects.

Clobenoside (vasoprotective) (36, 37)

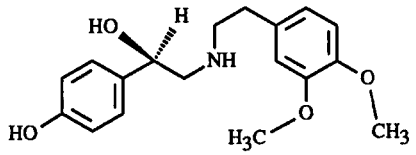
Country of Origin:	**Switzerland**
Originator:	**Ciba Geigy**
First Introduction:	**Switzerland**
Introduced by:	**Ciba Geigy**
Trade Name:	**Arvigol**
CAS Registry No.:	**29899-95-4**



Clobenoside is a vasoprotective agent with antiinflammatory activity. It is indicated for the topical treatment of chronic venous insufficiency and post-thrombotic syndrome. Clobenoside is also reportedly effective in treating traumatic edema through its inhibitory effects on histamine and/or kinins.

Denopamine (cardiostimulant) (38, 39)

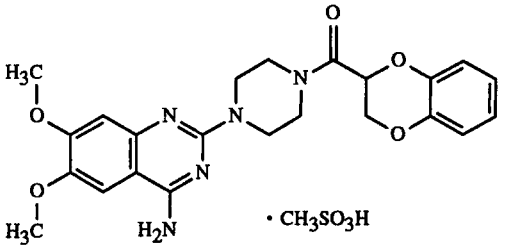
Country of Origin:	**Japan**
Originator:	**Tanabe Seiyaku**
First Introduction:	**Japan**
Introduced by:	**Tanabe Seiyaku**
Trade Name:	**Kalgut**
CAS Registry No.:	**71771-90-9**



Denopamine is a potent orally-active cardiotonic with selective effects on the $beta_1$ adrenergic receptors. Unlike isoproterenol or dobutamine, denopamine's positive inotropic activity is not mediated through the release of catecholamines; its lack of effect on heart rate and myocardial oxygen consumption thus offers significant advantages in the management of congestive heart failure.

Doxazosin Mesylate (antihypertensive) (40-42)

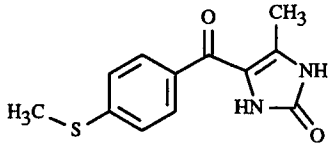
Country of Origin:	**United Kingdom**
Originator:	**Pfizer**
First Introduction:	**Denmark**
Introduced by:	**Pfizer**
Trade Name:	**Carduran**
CAS Registry No.:	**74191-85-8**



Doxazosin mesylate is a selective $alpha_1$ blocker indicated for the treatment of hypertension, reportedly of special benefit as a first-line agent in the most hypertonic patient population. Another advantage of doxazosin is its favorable effect on blood lipids; it significantly increases the HDL/total cholesterol ratio and decreases the total cholesterol as well as triglycerides levels.

Enoximone (cardiostimulant) (43, 44)

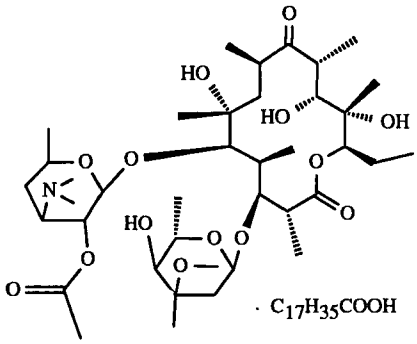
Country of Origin:	**USA**
Originator:	**Merrell Dow**
First Introduction:	**France**
Introduced by:	**Merrell Dow**
Trade Name:	**Perfan IV**
CAS Registry No.:	**77671-31-9**



Enoximone is a phosphodiesterase inhibitor indicated for selective use as a cardiostimulant in heart transplant patient maintenance. It is currently being investigated for other indications including acute congestive heart failure.

Erythromycin Acistrate (antibiotic) (45, 46)

Country of Origin:	**Finland**
Originator:	**Orion**
First Introduction:	**Finland**
Introduced by:	**Orion**
Trade Name:	**Erasis**
CAS Registry No.:	**96128-89-1**



Erythromycin acistrate is a prodrug of erythromycin with lowered liver toxicity, improved pharmacokinetics, and greatly reduced gastro-intestinal side-effects. It is indicated for respiratory tract infections, skin infections and chlamydial urethritis.

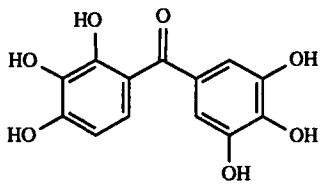
Erythropoietin (hematopoietic) (47, 48)

Country of Origin:	**USA**	Introduced by:	**Johnson & Johnson**
Originator:	**Amgen**	Trade Name:	**Eprex**
First Introduction:	**Switzerland**		

Recombinant erythropoietin (EPO) is currently indicated for use only in anemia associated with renal transplant or end-stage renal disease. Widened indications for use in other forms of anemia, accompanied by price reductions as a result of keen competition, have been projected for EPO in the future.

Exifone (nootropic) (49, 50)

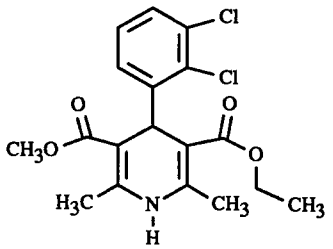
Country of Origin:	**France**
Originator:	**Pharmascience**
First Introduction:	**France**
Introduced by:	**Pharmascience**
Trade Name:	**Adlone**
CAS Registry No.:	**52479-85-3**



Exifone is a nootropic indicated for the treatment of cognitive dysfunctions in geriatric patients. Its reported clinical efficacy is ascribed to pharmacological activities including activation of neuronal oxygen and glucose metabolism, free radical scavenging, and indirect effects on serotonergic and dopaminergic pathways.

Felodipine (antihypertensive) (51, 52)

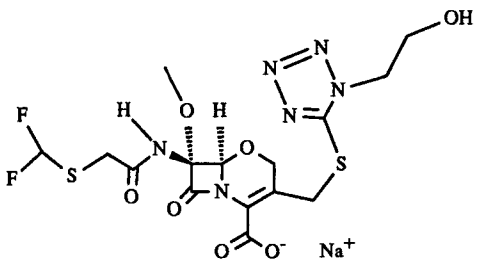
Country of Origin:	**Sweden**
Originator:	**Astra**
First Introduction:	**Sweden/Denmark**
Introduced by:	**Astra**
Trade Name:	**Plendil**
CAS Registry No.:	**72509-76-3**



Felodipine is a vasodilatory calcium antagonist with a high degree of vascular selectivity. It is currently indicated for use only in hypertension, either as monotherapy or in conjunction with diuretics or beta blockers.

Flomoxef Sodium (antibiotic) (53, 54)

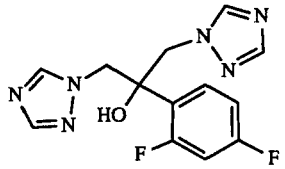
Country of Origin:	**Japan**
Originator:	**Shionogi**
First Introduction:	**Japan**
Introduced by:	**Shionogi**
Trade Name:	**Flumarin**
CAS Registry No.:	**99665-00-6**



Flomoxef sodium is a β-lactamase resistant oxacephalosporin. Its spectrum of activity is similar to that of cefazolin and moxalactam, but with better efficacy against *Staphylococcus aureus* and *Bacteroides fragilis*. Flomoxef is reported effective in the treatment of post-operative, urinary tract and abdominal cavity infections.

Fluconazole (antifungal) (55, 56)

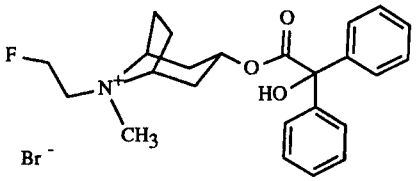
Country of Origin:	**United Kingdom**
Originator:	**Pfizer**
First Introduction:	**United Kingdom**
Introduced by:	**Pfizer**
Trade Name:	**Diflucan**
CAS Registry No.:	**86386-73-4**



Fluconazole is the first member of a new generation of stable and orally active antifungals, the triazoles. It is highly effective in the treatment of dermal and vaginal fungal infections; new indications currently under investigation include severe systemic mycoses such as disseminated candidiasis and cryptococcal meningitis in immunocompromised patients.

Flutropium Bromide (antitussive) (57)

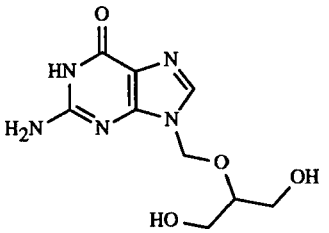
Country of Origin:	**W.Germany**
Originator:	**Boehringer Ingelheim**
First Introduction:	**Japan**
Introduced by:	**SS Pharmaceutical**
Trade Name:	**Flubron**
CAS Registry No.:	**63516-07-4**



Flutropium bromide is an antitussive useful in the treatment of allergic rhinitis and in the symptomatic relief of bronchial asthma and chronic bronchitis. Recent studies suggest that flutropium bromide may have potential as a long-acting alternative to β-sympathomimetics or the xanthines in the prophylaxis of obstructive airways disease.

Ganciclovir (antiviral) (58-60)

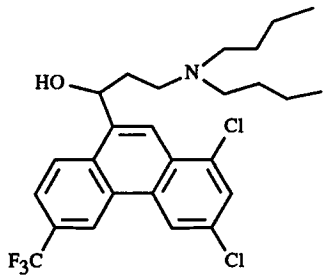
Country of Origin:	**United Kingdom**
Originator:	**Enscor**
First Introduction:	**United Kingdom**
Introduced by:	**Syntex**
Trade Name:	**Cymevene**
CAS Registry No.:	**82410-32-0**



Ganciclovir is a parenterally-active antiviral agent indicated for sight- or life-threatening cytomegalovirus (CMV) infections in immunocompromised patients. Its suppressive effects on bone marrow and renal tubular secretion/absorption are reported to present potential limitations on adjunct therapies involving zidovudine, vincristine, adriamycin and amphotericin B. Recently, the emergence of CMV strains resistant to ganciclovir therapy has been reported.

Halofantrine (antimalarial) (61, 62)

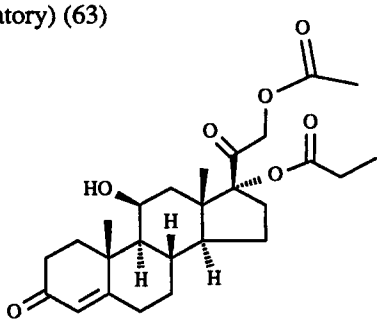
Country of Origin:	**USA**
Originator:	**Smith Kline & French**
First Introduction:	**Ivory Coast**
Introduced by:	**Smith Kline & French**
Trade Name:	**Halfan**
CAS Registry No.:	**69756-53-2**



Halofantrine is an orally-active blood schizonticide reportedly highly effective in the treatment of *falciparum* malaria and other types of parasitemia. Cure rate is claimed to be over 95%.

Hydrocortisone Aceponate (topical antiinflammatory) (63)

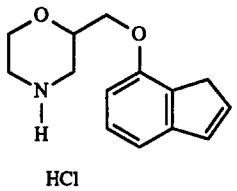
Country of Origin:	**W.Germany**
Originator:	**Beiersdorf**
First Introduction:	**W.Germany**
Introduced by:	**Beiersdorf**
Trade Name:	**Retef**
CAS Registry No.:	**74050-20-7**



Hydrocortisone aceponate is the 17-α-propionate derivative of hydrocortisone-21-acetate. Its is reportedly significantly superior to the latter in the treatment of dermatoses of varying severity.

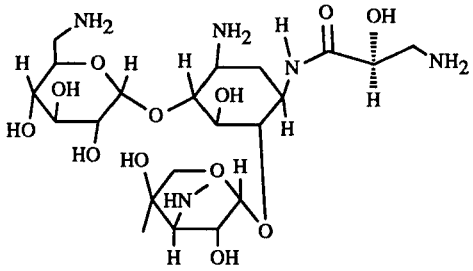
Indeloxazine Hydrochloride (nootropic) (64-66)

Country of Origin:	**Japan**
Originator:	**Yamanouchi**
First Introduction:	**Japan**
Introduced by:	**Yamanouchi**
Trade Name:	**Elen**
CAS Registry No.:	**65043-22-3**



Indeloxazine hydrochloride is a nootropic indicated for the treatment of senile dementia. In animal models indeloxazine reportedly enhances brain energy metabolism and monoamine content; its claimed protective effect on ischemia-induced amnesia is supported by the prolonged step-through latency in passive avoidance tests.

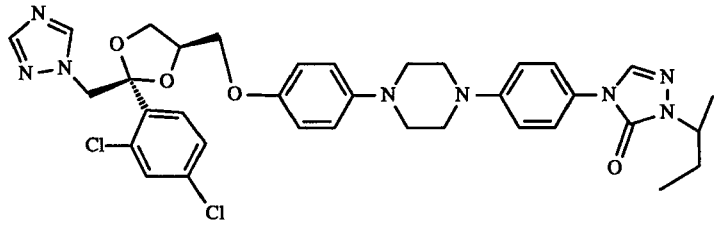
Isepamicin (antibiotic) (67, 68)



Country of Origin:	**USA**
Originator:	**Schering Plough**
First Introduction:	**Japan**
Introduced by:	**Schering; Toyo Jozo**
Trade Name:	**Isepacin; Exacin**
CAS Registry No.:	**58152-03-7**

Isepamicin is a aminoglycoside antibiotic indicated for use in the treatment of urinary and respiratory tract infections. Although it is less potent than most other aminoglycosides, it is also less nephrotoxic. The broad-spectrum activity of isepamicin generally parallels that of amikacin *in vitro*.

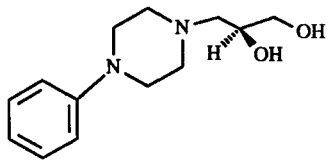
Itraconazole (antifungal) (69, 70)



Country of Origin:	**Belgium**	Introduced by:	**Johnson & Johnson**
Originator:	**Janssen**	Trade Name:	**Sporanox**
First Introduction:	**Mexico**	CAS Registry No.:	**84625-61-6**

Itraconazole is an orally-active triazole antifungal indicated for use in the treatment of dermal, vaginal and systemic mycoses. In immunocompromised and AIDS patients, itraconazole has been shown to significantly reduce the incidence of relapses of cryptococcal meningitis.

Levodropropizine (antitussive) (71, 72)

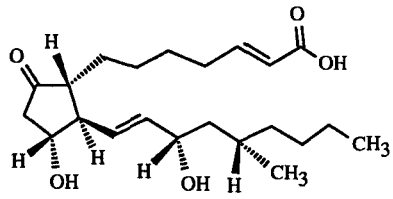


Country of Origin:	**Italy**
Originator:	**Dompé**
First Introduction:	**Italy**
Introduced by:	**Dompé**
Trade Name:	**Levotuss**
CAS Registry No.:	**99291-24-4**

Levodropropizine is an antitussive with antiinflammatory activity, the L-isomer of dropropizine. Compared with the racemate, levodropropizine is reported to have less sedative potential and better therapeutic index, while maintaining the the same efficacy levels as an antitussive and antiinflammatory.

Limaprost (antithrombotic) (73, 74)

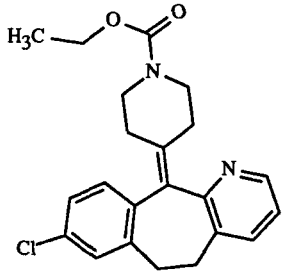
Country of Origin:	**Japan**
Originator:	**Ono**
First Introduction:	**Japan**
Introduced by:	**Ono; Dainippon**
Trade Name:	**Opalmon; Prorenal**
CAS Registry No.:	**88852-12-4**



Limaprost is an orally-active antithrombotic and vasodilator structurally related to alprostadil. Oral administration of limaprost causes a dose-dependent inhibition of platelet aggregation, an increase in platelet cAMP and a mild suppression of platelet adhesiveness. In patients with pulmonary emphysema, tuberculosis, and chronic lung disease, treatment with limaprost reportedly produces significant cardiac improvements.

Loratadine (antihistamine) (75, 76)

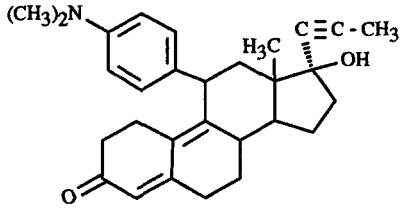
Country of Origin:	**USA**
Originator:	**Schering Plough**
First Introduction:	**Belgium**
Introduced by:	**Schering Plough**
Trade Name:	**Claritin**
CAS Registry No.:	**79794-75-5**



Loratadine is a non-sedating antihistamine indicated for use in allergic rhinitis and chronic urticaria. Its major advantage over other non-sedating antihistamines such as astemizole and terfenadine is its very fast onset of action. Loratadine is claimed not to cross the blood-brain barrier.

Mifepristone (abortifacient) (77)

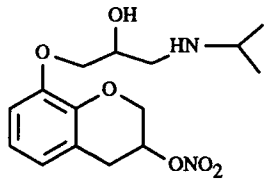
Country of Origin:	**France**
Originator:	**Roussel-Uclaf**
First Introduction:	**France**
Introduced by:	**Roussel-Uclaf**
Trade Name:	**Mifegyne**
CAS Registry No.:	**84371-65-3**



Mifepristone is an orally-active progesterone and glucocorticoid receptor antagonist indicated for use as a post-coital contraceptive. In addition to being an abortifacient, mifepristone is reported to be effective in the treatment of ocular hypertension; its potential therapeutic effect in hormone-dependent tumors is currently under investigation.

Nipradilol (antihypertensive) (78, 79)

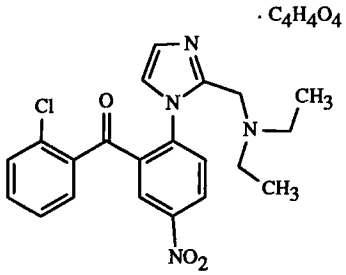
Country of Origin:	**Japan**
Originator:	**Kowa**
First Introduction:	**Belgium**
Introduced by:	**Kowa**
Trade Name:	**Hypadil**
CAS Registry No.:	**81486-22-8**



Nipradilol is a beta-blocker with vasodilatory activity. As an antihypertensive nipradilol decreases systemic/pulmonary arterial blood pressure, heart rate, cardiac output and left ventricular volume without causing tachycardia. Nipradilol is also reported to have antiangina activity in dogs.

Nizofenzone Fumarate (nootropic) (80, 81)

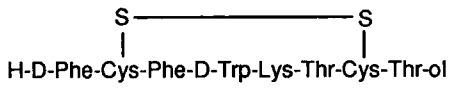
Country of Origin:	**Japan**
Originator:	**Yoshitomi**
First Introduction:	**Japan**
Introduced by:	**Yoshitomi**
Trade Name:	**Ekonal**
CAS Registry No.:	**54533-85-6**



Nizofenzone fumarate is a potent antianoxic agent reported of benefit to geriatric and stroke patients. In animal models nizofenzone is reported to have protective effects against anoxia, global ischemia or other conditions resulting from cerebral infarction. It is also claimed to be a sedative and anticonvulsant.

Octreotide (antisecretory) (82-84)

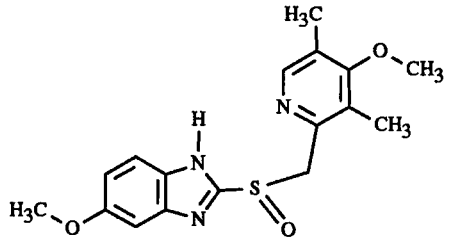
Country of Origin:	**Switzerland**
Originator:	**Sandoz**
First Introduction:	**New Zealand**
Introduced by:	**Sandoz**
Trade Name:	**Sandostatin**
CAS Registry No.:	**83150-76-9**



Octreotide is a long-acting somatostatin analog indicated for symptomatic control in acromegaly and gastroenteropancreatic tumors. Other potential uses under investigation include diabetes, psoriasis and Alzheimer's disease.

Omeprazole (antiulcer) (85-87)

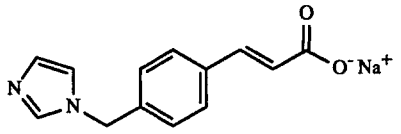
Country of Origin:	**Sweden**
Originator:	**Astra**
First Introduction:	**Sweden**
Introduced by:	**Astra**
Trade Name:	**Losec**
CAS Registry No.:	**73590-58-6**



Omeprazole is a potent gastric antisecretory agent with selective inhibitory effect on the H^+,K^+-ATPase proton pump. It is highly effective in the treatment of duodenal ulcer and Zollinger-Ellison syndrome, and is reportedly superior to ranitidine in the management of reflux esophagitis.

Ozagrel Sodium (antithrombotic) (88, 89)

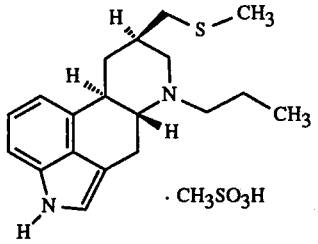
Country of Origin:	**Japan**
Originator:	**Ono**
First Introduction:	**Japan**
Introduced by:	**Kissei; Ono**
Trade Name:	**Xanbon; Cataclot**
CAS Registry No.:	**82571-53-7**



Ozagrel sodium is the first thromboxane (TX2) synthetase inhibitor to be developed as an antithrombotic agent. In patients with angina pectoris, ozagrel sodium reportedly decreases the frequency of anginal attacks and increases exercise tolerance. Other indications for ozagrel sodium currently under study include bronchial asthma and pulmonary thromboembolism.

Pergolide Mesylate (antiparkinsonian) (90, 91)

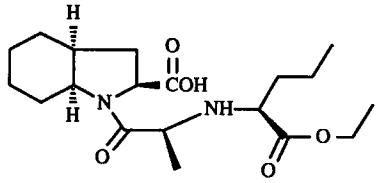
Country of Origin:	**USA**
Originator:	**Lilly**
First Introduction:	**USA**
Introduced by:	**Lilly**
Trade Name:	**Permax**
CAS Registry No.:	**66104-23-2**



Pergolide mesylate is a potent, long-acting dopamine agonist useful in the treatment of Parkinson's disease and hyperprolactinemia. Compared with lergotrile, pergolide mesylate has shown less toxicity and lower propensity for inducing psychosis.

Perindopril (antihypertensive) (92, 93)

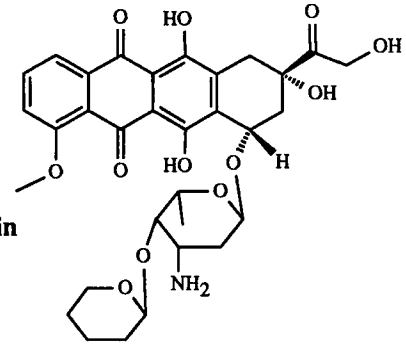
Country of Origin:	**France**
Originator:	**Servier**
First Introduction:	**France**
Introduced by:	**Servier**
Trade Name:	**Coversyl**
CAS Registry No.:	**82834-16-O**



Perindopril is a potent, orally-active angiotensin-converting enzyme (ACE) inhibitor useful in the management of hypertension. Against rat ACE, perindopril appears to be more potent than enalapril and enalaprilat, and is approximately equipotent to ramipril (HOE 498). Its long duration of action suggests the possibility of once-daily dosing.

Pirarubicin (antineoplastic) (94-96)

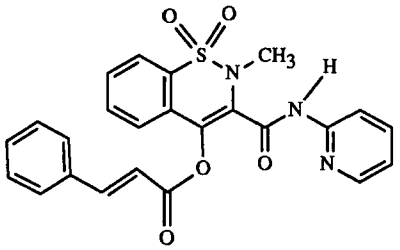
Country of Origin:	**Japan**
Originator:	**Meiji Seika**
First Introduction:	**Japan**
Introduced by:	**Sanraku; Meiji Seika**
Trade Name:	**Pinorubicin; Therarubucin**
CAS Registry No.:	**72496-41-4**



Pirarubicin is a new anthracycline anticancer agent structurally related to doxorubicin. In acute leukemia, non-Hodgkin's lymphoma, breast and ovarian carcinoma, pirarubicin is reportedly superior to doxorubicin and better tolerated. Its major side-effect appears to be myelosuppression, mainly in the form of leucopenia, which is potentially dose-limiting.

Piroxicam Cinnamate (antiinflammatory) (97)

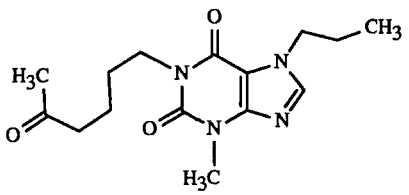
Country of Origin:	**Italy**
Originator:	**SPA**
First Introduction:	**Italy**
Introduced by:	**SPA**
Trade Name:	**Sinartrol**
CAS Registry No.:	**87234-24-0**



Piroxicam cinnamate is a long-acting antiinflmmatory useful in the once-daily therapy of rheumatoid- and osteoarthritis.

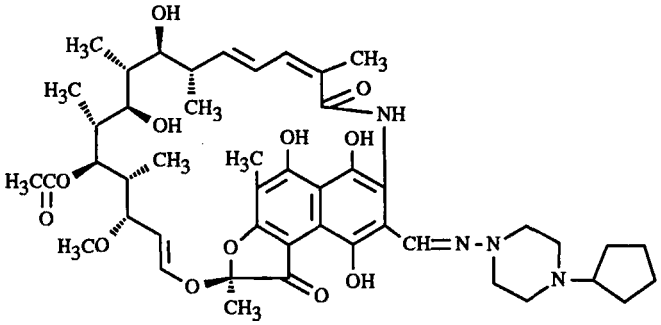
Propentofylline (nootropic) (98, 99)

Country of Origin:	**W.Germany**
Originator:	**Hoechst AG**
First Introduction:	**Japan**
Introduced by:	**Hoechst AG**
Trade Name:	**Hextol**
CAS Registry No.:	**55242-55-2**



Propentofylline is a nootropic indicated for cerebrovascular disorders. Other indications currently under investigation include senile dementia, stroke and hypoxia.

Rifapentine (antibiotic) (100-102)

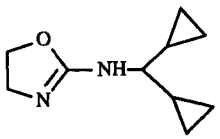


Country of Origin:	**China**	Introduced by:	**Merrell Dow**
Originator:	**Shanghai No. 5 Factory**	Trade Name:	**Rifampin**
First Introduction:	**China**	CAS Registry No.:	**61379-65-5**

Rifapentine is a broad spectrum antibiotic highly active against Gram-positive bacteria and *Neisseria gonorrhoea*. It is reported to be 10 times more active than the structurally related rifampicin against *Mycobacterium tuberculosis*. Unlike rifampicin the bioavailability of rifapentine is significantly increased when administered after a meal.

Rilmenidine (antihypertensive) (103, 104)

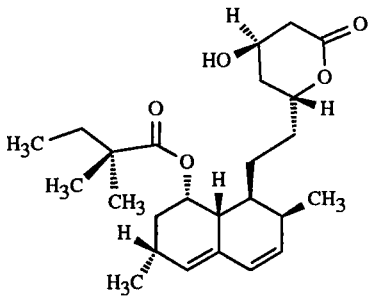
Country of Origin:	**France**
Originator:	**Servier**
First Introduction:	**France**
Introduced by:	**Servier**
Trade Name:	**Hyperium**
CAS Registry No.:	**54187-04-1**



Rilmenidine is a new antihypertensive agent with a selective alpha$_2$-agonist/antagonist profile, reportedly useful in the management of mild to moderate hypertension. Its first-line indication is possible because of the clear dissociation of its antihypertensive activity from other neuropharmacological effects

Simvastatin (hypocholesterolemic) (105-107)

Country of Origin:	**USA**
Originator:	**Merck**
First Introduction:	**Sweden**
Introduced by:	**Merck**
Trade Name:	**Zocord**
CAS Registry No.:	**79902-63-9**



Simvastatin is an once-daily hypolipemic, an analog of lovastatin indicated for the treatment of atherosclerosis. In patients with Type IIA or IIB hypercholesterolemia, simvastatin reportedly produces significant reductions in total serum cholesterol, LDL, triglycerides and apolipoprotein-B, while HDL and apolipoprotein-A levels are increased.

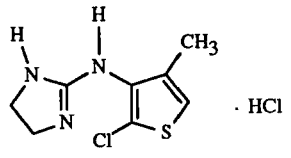
Teicoplanin (antibiotic) (108, 109)

Country of Origin:	**Italy**	Introduced by:	**Merrell Dow**
Originator:	**Merrell Dow**	Trade Name:	**Targocid**
First Introduction:	**Italy/France**	CAS Registry No.:	**61036-62-2; 61036-63-3; 61036-64-4**

Teicoplanin is a new antibiotic, the second glycopeptide to be developed in over 30 years. Compared with vancomycin, the only such agent currently available, teicoplanin is equiefficacious, and has milder side-effects and a longer half-life, allowing once-daily dosing and bolus injection. Teicoplanin is claimed to have an overall cure rate of 92% in infections involving skin, joint and bone, endocarditis and septicemia.

Tiamenidine Hydrochloride (antihypertensive) (110, 111)

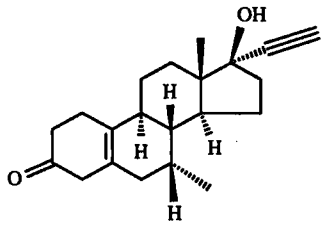
Country of Origin:	**W.Germany**
Originator:	**Hoechst AG**
First Introduction:	**W.Germany**
Introduced by:	**Delande**
Trade Name:	**Sundralen**
CAS Registry No.:	**51274-83-0**



Tiamenidine hydrochloride is a centrally-acting antihypertensive structurally related to clonidine. In patients with mild to moderate hypertension, tiamenidine exerts similar activity to clonidine, but with fewer sympathomimetic side-effects.

Tibolone (anabolic) (112, 113)

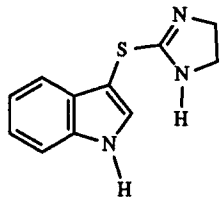
Country of Origin:	**The Netherklands**
Originator:	**Akzo (Organon)**
First Introduction:	**The Netherlands**
Introduced by:	**Akzo**
Trade Name:	**Livial**
CAS Registry No.:	**5630-53-5**



Tibolone is a synthetic steroid with weak progestational and estrogenic properties, reportedly useful in controlling symptoms resulting from natural or surgical menopause. It has thus far shown no significant antithrombotic effect in post-menopausal patients.

Tinazoline (nasal decongestant) (114)

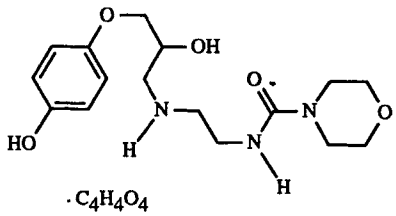
Country of Origin:	**India**
Originator:	**Ciba Geigy**
First Introduction:	**India**
Introduced by:	**Ciba Geigy**
Trade Name:	**Varsyl**
CAS Registry No.:	**62882-99-9**



Tinazoline is a potent, long-acting vasoconstrictor useful as a nasal decongestant.

Xamoterol Fumarate (cardiotonic) (115-118)

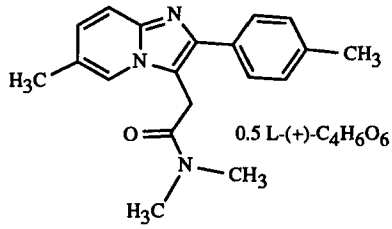
Country of Origin:	**United Kingdom**
Originator:	**ICI**
First Introduction:	**United Kingdom**
Introduced by:	**ICI**
Trade Name:	**Corwin**
CAS Registry No.:	**73210-73-8**



Xamoterol fumarate is a cardioselective partial beta-agonist, the first of its class to be developed for use in heart failure. The main advantage of xamoterol fumarate as a cardiotonic lies in the fact that its beta-blocking effect exerted during high sympathetic drive will protect the heart from overstimulation. It is claimed to be a viable but safer alternative to digoxin.

Zolpidem Hemitartrate (hypnotic) (119, 120)

Country of Origin:	**France**
Originator:	**Synthelabo**
First Introduction:	**France**
Introduced by:	**Synthelabo**
Trade Name:	**Stilnox**
CAS Registry No.:	**82626-48-0**



Zolpidem hemitartrate is a non-benzodiazepine hypnotic with specific agonist activity at type 1 benzodiazepine receptors, and is indicated for use in insomnia and other sleep disorders. Structurally zolpidem belongs to a chemically distinct class, thus lacking the side-effects and abuse potential of classical benzodiazepines. It is currently being studied as a pre-operative sedative.

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APD	calcium regulator	1987	23, 326
APSAC	thrombolytic	1987	23, 326
acetohydroxamic acid	hypoammonuric	1983	19, 313
acipimox	hypolipidemic	1985	21, 323
acrivastine	antihistamine	1988	24, 295
adamantanium bromide	antiseptic	1984	20, 315
adrafinil	psychostimulant	1986	22, 315
afloqualone	muscle relaxant	1983	19, 313
alacepril	antihypertensive	1988	24, 296
alclometasone dipropionate	topical antiinflammatory	1985	21, 323
alfentanil HCl	analgesic	1983	19, 314
alfuzosin hydrochloride	antihypertensive	1988	24, 296
alminoprofen	analgesic	1983	19, 314
alpiropride	antimigraine	1988	24, 296
alteplase	thrombolytic	1987	23, 326
amfenac sodium	antiinflammatory	1986	22, 315
amisulpride	antipsychotic	1986	22, 316
amlexanox	antiasthmatic	1987	23, 327
amosulalol	antihypertensive	1988	24, 297
amrinone	cardiotonic	1983	19, 314
amsacrine	antineoplastic	1987	23, 327
alpha-1 antitrypsin	protease inhibitor	1988	24, 297
apraclonidine hydrochloride	antiglaucoma	1988	24, 297
arotinolol HCl	antihypertensive	1986	22, 316
artemisinin	antimalarial	1987	23, 327
aspoxicillin	antibiotic	1987	23, 328
astemizole	antihistamine	1983	19, 314
astromycin sulfate	antibiotic	1985	21, 324
auranofin	chrysotherapeutic	1983	19, 314
azelastine HCl	antihistamine	1986	22, 316
azithromycin	antibiotic	1988	24, 298
azosemide	diuretic	1986	22, 316
aztreonam	antibiotic	1984	20, 315
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
benexate HCl	antiulcer	1987	23, 328
betaxolol HCl	antihypertensive	1983	19, 315
bevantolol HCl	antihypertensive	1987	23, 328
bifemelane HCl	nootropic	1987	23, 329
bifonazole	hypnotic	1983	19, 315
binifibrate	hypolipidemic	1986	22, 317
bisoprolol fumarate	antihypertensive	1986	22, 317
bopindolol	antihypertensive	1985	21, 324
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317
bucillamine	immunomodulator	1987	23, 329
bucladesine sodium	cardiostimulant	1984	20, 316
budralazine	antihypertensive	1983	19, 315
bunazosin HCl	antihypertensive	1985	21, 324
buserelin acetate	hormone	1984	20, 316

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL.,PAGE
buspirone HCl	anxiolytic	1985	21, 324
butoconazole nitrate	topical antifungal	1986	22, 318
butoctamide succinate	hypnotic	1984	20, 316
butyl flufenamate	topical antiinflammatory	1983	19, 316
cadexomer iodine	wound healing agent	1983	19, 316
cadralazine	antihypertensive	1988	24, 298
camostat mesylate	protease inhibitor	1985	21, 325
carboplatin	antineoplastic	1986	22, 318
carumonam	antibiotic	1988	24, 298
cefbuperazone sodium	antibiotic	1985	21, 325
cefixime	antibiotic	1987	23, 329
cefmenoxime HCl	antibiotic	1983	19, 316
cefminox sodium	antibiotic	1987	23, 330
cefonicid sodium	antibiotic	1984	20, 316
ceforanide	antibiotic	1984	20, 317
cefotetan disodium	antibiotic	1984	20, 317
cefpimizole	antibiotic	1987	23, 330
cefpiramide sodium	antibiotic	1985	21, 325
ceftazidime	antibiotic	1983	19, 316
cefteram pivoxil	antibiotic	1987	23, 330
cefuroxime axetil	antibiotic	1987	23, 331
cefuzonam sodium	antibiotic	1987	23, 331
celiprolol HCl	antihypertensive	1983	19, 317
cetirizine HCl	antihistamine	1987	23, 331
chenodiol	anticholelithogenic	1983	19, 317
cibenzoline	antiarrhythmic	1985	21, 325
cicletanine	antihypertensive	1988	24, 299
cilostazol	antithrombotic	1988	24, 299
cimetropium bromide	antispasmodic	1985	21, 326
ciprofibrate	hypolipidemic	1985	21, 326
ciprofloxacin	antibacterial	1986	22, 318
cisapride	gastroprokinetic	1988	24, 299
clobenoside	vasoprotective	1988	24, 300
cloconazole HCl	topical antifungal	1986	22, 318
clodronate disodium	calcium regulator	1986	22, 319
cyclosporine	immunosuppressant	1983	19, 317
dapiprazole HCl	antiglaucoma	1987	23, 332
defibrotide	antithrombotic	1986	22, 319
deflazacort	antiinflammatory	1986	22, 319
denopamine	cardiostimulant	1988	24, 300
diacerein	antirheumatic	1985	21, 326
divistyramine	hypocholesterolemic	1984	20, 317
doxazosin mesylate	antihypertensive	1988	24, 300
doxefazepam	hypnotic	1985	21, 326
doxifluridine	antineoplastic	1987	23, 332
doxofylline	bronchodilator	1985	21, 327
dronabinol	antinauseant	1986	22, 319
emorfazone	analgesic	1984	20, 317
enalapril maleate	antihypertensive	1984	20, 317
enalaprilat	antihypertensive	1987	23, 332
encainide HCl	antiarrhythmic	1987	23, 333
enocitabine	antineoplastic	1983	19, 318
enoxacin	antibacterial	1986	22, 320
enoxaparin	antithrombotic	1987	23, 333

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL.,PAGE
enoximone	cardiostimulant	1988	24, 301
enprostil	antiulcer	1985	21, 327
eperisone HCl	muscle relaxant	1983	19, 318
epidermal growth factor	wound healing agent	1987	23, 333
epirubicin HCl	antineoplastic	1984	20, 318
epoprostenol sodium	platelet aggreg. inhib.	1983	19, 318
eptazocine HBr	analgesic	1987	23, 334
erythromycin acistrate	antibiotic	1988	24, 301
erythropoietin	hematopoetic	1988	24, 301
esmolol HCl	antiarrhythmic	1987	23, 334
etizolam	anxiolytic	1984	20, 318
etodolac	antiinflammatory	1985	21, 327
exifone	nootropic	1988	24, 302
famotidine	antiulcer	1985	21, 327
felbinac	topical antiinflammatory	1986	22, 320
felodipine	antihypertensive	1988	24, 302
fenbuprol	choleretic	1983	19, 318
fenticonazole nitrate	antifungal	1987	23, 334
fisalamine	intestinal antiinflam.	1984	20, 318
flomoxef sodium	antibiotic	1988	24, 302
fluconazole	antifungal	1988	24, 303
flumazenil	benzodiazepine antag.	1987	23, 335
flunoxaprofen	antiinflammatory	1987	23, 335
fluoxetine HCl	antidepressant	1986	22, 320
flupirtine maleate	analgesic	1985	21, 328
flutamide	antineoplastic	1983	19, 318
flutazolam	anxiolytic	1984	20, 318
flutoprazepam	anxiolytic	1986	22, 320
flutropium bromide	antitussive	1988	24, 303
fluvoxamine maleate	antidepressant	1983	19, 319
formoterol fumarate	bronchodilator	1986	22, 321
fosfosal	analgesic	1984	20, 319
gallopamil HCl	antianginal	1983	19, 319
gemeprost	abortifacient	1983	19, 319
ganciclovir	antiviral	1988	24, 303
gestodene	progestogen	1987	23, 335
gestrinone	antiprogestogen	1986	22, 321
goserelin	hormone	1986	22, 321
goserelin	hormone	1987	23, 336
guanadrel sulfate	antihypertensive	1983	19, 319
halofantrine	antimalarial	1988	24, 304
halometasone	topical antiinflammatory	1983	19, 320
hydrocortisone aceponate	topical antiinflammatory	1988	24, 304
hydrocortisone butyrate propionate	topical antiinflammatory	1983	19, 320
ibopamine HCl	cardiostimulant	1984	20, 319
idebenone	nootropic	1986	22, 321
imipenem/cilastatin	antibiotic	1985	21, 328
indalpine	antidepressant	1983	19, 320
indeloxazine hydrochloride	nootropic	1988	24, 304

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL.,PAGE
indobufen	antithrombotic	1984	20, 319
isepamicin	antibiotic	1988	24, 305
isofezolac	antiinflammatory	1984	20, 319
isoxicam *	antiinflammatory	1983	19, 320
itraconazole	antifungal	1988	24, 305
ivermectin	antiparasitic	1987	23, 336
ketanserin	antihypertensive	1985	21, 328
lenampicillin HCl	antibiotic	1987	23, 336
lentinan	immunostimulant	1986	22, 322
leuprolide acetate	hormone	1984	20, 319
levacecarnine HCl	nootropic	1986	22, 322
levobunolol HCl	antiglaucoma	1985	21, 328
levodropropizine	antitussive	1988	24, 305
lidamidine HCl	antiperistaltic	1984	20, 320
limaprost	antithrombotic	1988	24, 306
lisinopril	antihypertensive	1987	23, 337
lobenzarit sodium	antiinflammatory	1986	22, 322
lonidamine	antineoplastic	1987	23, 337
loprazolam mesylate	hypnotic	1983	19, 321
loratadine	antihistamine	1988	24, 306
lovastatin	hypocholesterolemic	1987	23, 337
loxoprofen sodium	antiinflammatory	1986	22, 322
mabuterol HCl	bronchodilator	1986	22, 323
malotilate	hepatoprotective	1985	21, 329
medifoxamine fumarate	antidepressant	1986	22, 323
mefloquine HCl	antimalarial	1985	21, 329
meglutol	hypolipidemic	1983	19, 321
melinamide	hypocholesterolemic	1984	20, 320
mepixanox	analeptic	1984	20, 320
meptazinol HCl	analgesic	1983	19, 321
metaclazepam	anxiolytic	1987	23, 338
metapramine	antidepressant	1984	20, 320
mexazolam	anxiolytic	1984	20, 321
mifepristone	abortifacient	1988	24, 306
miokamycin	antibiotic	1985	21, 329
misoprostol	antiulcer	1985	21, 329
mitoxantrone HCl	antineoplastic	1984	20, 321
mizoribine	immunosuppressant	1984	20, 321
mometasone furoate	topical antiinflammatory	1987	23, 338
mupirocin	topical antibiotic	1985	21, 330
muromonab-CD3	immunosuppressant	1986	22, 323
muzolimine	diuretic	1983	19, 321
nabumetone	antiinflammatory	1985	21, 330
nafamostat mesylate	protease inhibitor	1986	22, 323
naftifine HCl	antifungal	1984	20, 321
naltrexone HCl	narcotic antagonist	1984	20, 322
nedocromil sodium	antiallergic	1986	22, 324
nicorandil	coronary vasodilator	1984	20, 322
nilutamide	antineoplastic	1987	23, 338
nimesulide	antiinflammatory	1985	21, 330
nimodipine	cerebral vasodilator	1985	21, 330
nipradilol	antihypertensive	1988	24, 307
nitrefazole *	alcohol deterrent	1983	19, 322
nitrendipine	antihypertensive	1985	21, 331

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nizatidine	antiulcer	1987	23, 339
nizofenzone fumarate	nootropic	1988	24, 307
nomegestrol acetate	progestogen	1986	22, 324
norfloxacin	antibacterial	1983	19, 322
norgestimate	progestogen	1986	22, 324
octreotide	antisecretory	1988	24, 307
ofloxacin	antibacterial	1985	21, 331
omeprazole	antiulcer	1988	24, 308
ornoprostil	antiulcer	1987	23, 339
osalazine sodium	intestinal antiinflam.	1986	22, 324
oxaprozin	antiinflammatory	1983	19, 322
oxiconazole nitrate	antifungal	1983	19, 322
oxiracetam	nootropic	1987	23, 339
oxitropium bromide	bronchodilator	1983	19, 323
ozagrel sodium	antithrombotic	1988	24, 308
pefloxacin mesylate	antibacterial	1985	21, 331
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
picotamide	antithrombotic	1987	23, 340
piketoprofen	topical antiinflammatory	1984	20, 322
pimaprofen	topical antiinflammatory	1984	20, 322
pinacidil	antihypertensive	1987	23, 340
pirarubicin	antineoplastic	1988	24, 309
piroxicam cinnamate	antiinflammatory	1988	24, 309
plaunotol	antiulcer	1987	23, 340
prednicarbate	topical antiinflammatory	1986	22, 325
progabide	anticonvulsant	1985	21, 331
promegestone	progestogen	1983	19, 323
propacetamol HCl	analgesic	1986	22, 325
propentofylline	cerebral vasodilator	1988	24, 310
propofol	anesthetic	1986	22, 325
quazepam	hypnotic	1985	21, 332
quinfamide	amebicide	1984	20, 322
ranimustine	antineoplastic	1987	23, 341
repirinast	antiallergic	1987	23, 341
rifapentine	antibacterial	1988	24, 310
rifaximin	antibiotic	1985	21, 332
rifaximin	antibiotic	1987	23, 341
rilmenidine	antihypertensive	1988	24, 310
rimantadine HCl	antiviral	1987	23, 342
rokitamycin	antibiotic	1986	22, 325
ronafibrate	hypolipidemic	1986	22, 326
rosaprostol	antiulcer	1985	21, 332
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antibiotic	1987	23, 342
schizophyllan	immunostimulant	1986	22, 326
setastine HCl	antihistamine	1987	23, 342
simvastatin	hypocholesterolemic	1988	24, 311
sodium cellulose PO4	hypocalciuric	1983	19, 323
sofalcone	antiulcer	1984	20, 323
somatropin	hormone	1987	23, 343
spizofurone	antiulcer	1987	23, 343
sufentanil	analgesic	1983	19, 323
sulbactam sodium	B-lactamase inhibitor	1986	22, 326

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sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344
teicoplanin	antibacterial	1988	24, 311
temocillin disodium	antibiotic	1984	20, 323
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terconazole	antifungal	1983	19, 324
tertatolol HCl	antihypertensive	1987	23, 344
thymopentin	immunomodulator	1985	21, 333
tiamenidine hydrochloride	antihypertensive	1988	24, 311
tianeptine sodium	antidepressant	1983	19, 324
tibolone	anabolic	1988	24, 312
timiperone	neuroleptic	1984	20, 323
tinazoline	nasal decongestant	1988	24, 312
tioconazole	antifungal	1983	19, 324
tiquizium bromide	antispasmodic	1984	20, 324
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
toloxatone	antidepressant	1984	20, 324
trientine HCl	chelator	1986	22, 327
trimazosin HCl	antihypertensive	1985	21, 333
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
xamoterol fumarate	cardiotonic	1988	24, 312
zidovudine	antiviral	1987	23, 345
zolpidem hemitartrate	hypnotic	1988	24, 313
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

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gemeprost	ABORTIFACIENT	1983	19, 319
mifepristone		1988	24, 306
nitrefazole *	ALCOHOL DETERRENT	1983	19, 322
quinfamide	AMEBICIDE	1984	20, 322
tibolone	ANABOLIC	1988	24, 312
mepixanox	ANALEPTIC	1984	20, 320
alfentanil HCl	ANALGESIC	1983	19, 314
alminoprofen		1983	19, 314
emorfazone		1984	20, 317
eptazocine HBr		1987	23, 334
flupirtine maleate		1985	21, 328
fosfosal		1984	20, 319
meptazinol HCl		1983	19, 321
propacetamol HCl		1986	22, 325
sufentanil		1983	19, 323
suprofen *		1983	19, 324
propofol	ANESTHETIC	1986	22, 325
nedocromil sodium	ANTIALLERGIC	1986	22, 324
repirinast		1987	23, 341
gallopamil HCl	ANTIANGINAL	1983	19, 319
cibenzoline	ANTIARRHYTHMIC	1985	21, 325
encainide HCl		1987	23, 333
esmolol HCl		1987	23, 334
amlexanox	ANTIASTHMATIC	1987	23, 327
ciprofloxacin	ANTIBACTERIAL	1986	22, 318
enoxacin		1986	22, 320
norfloxacin		1983	19, 322
ofloxacin		1985	21, 331
pefloxacin mesylate		1985	21, 331
aspoxicillin	ANTIBIOTIC	1987	23, 328
astromycin sulfate		1985	21, 324
azithromycin		1988	24, 298
aztreonam		1984	20, 315
carumonam		1988	24, 298
cefbuperazone sodium		1985	21, 325
cefixime		1987	23, 329
cefmenoxime HCl		1983	19, 316
cefminox sodium		1987	23, 330
cefonicid sodium		1984	20, 316
ceforanide		1984	20, 317
cefotetan disodium		1984	20, 317

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cefpimizole		1987	23, 330
cefpiramide sodium		1985	21, 325
ceftazidime		1983	19, 316
cefteram pivoxil		1987	23, 330
cefuroxime axetil		1987	23, 331
cefuzonam sodium		1987	23, 331
erythromycin acistrate		1988	24, 301
flomoxef sodium		1988	24, 302
imipenem/cilastatin		1985	21, 328
isepamicin		1988	24, 305
lenampicillin HCl		1987	23, 336
miokamycin		1985	21, 329
rifapentine		1988	24, 310
rifaximin		1985	21, 332
rokitamycin		1986	22, 325
roxithromycin		1987	23, 342
sultamycillin tosylate		1987	23, 343
teicoplanin		1988	24, 311
temocillin disodium		1984	20, 323
mupirocin	ANTIBIOTIC, TOPICAL	1985	21, 330
chenodiol	ANTICHOLELITHOGENIC	1983	19, 317
progabide	ANTICONVULSANT	1985	21, 331
fluoxetine HCl	ANTIDEPRESSANT	1986	22, 320
fluvoxamine maleate		1983	19, 319
indalpine		1983	19, 320
medifoxamine fumarate		1986	22, 323
metapramine		1984	20, 320
tianeptine sodium		1983	19, 324
toloxatone		1984	20, 324
fenticonazole nitrate	ANTIFUNGAL	1987	23, 334
fluconazole		1988	24, 303
itraconazole		1988	24, 305
naftifine HCl		1984	20, 321
oxiconazole nitrate		1983	19, 322
terconazole		1983	19, 324
tioconazole		1983	19, 324
butoconazole nitrate	ANTIFUNGAL, TOPICAL	1986	22, 318
cloconazole HCl		1986	22, 318
sulconizole nitrate		1985	21, 332
apraclonidine HCl	ANTIGLAUCOMA	1988	24, 297
befunolol HCl		1983	19, 315
dapiprazole HCl		1987	23, 332
levobunolol HCl		1985	21, 328
acrivastine	ANTIHISTAMINE	1988	24, 295
astemizole		1983	19, 314

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azelastine HCl		1986	22, 316
cetirizine HCl		1987	23, 331
loratadine		1988	24, 306
setastine HCl		1987	23, 342
alacepril	ANTIHYPERTENSIVE	1988	24, 296
alfuzosin HCl		1988	24, 296
amosulalol		1988	24, 297
arotinolol HCl		1986	22, 316
betaxolol HCl		1983	19, 315
bevantolol HCl		1987	23, 328
bisoprolol fumarate		1986	22, 317
bopindolol		1985	21, 324
budralazine		1983	19, 315
bunazosin HCl		1985	21, 324
cadralazine		1988	24, 298
celiprolol HCl		1983	19, 317
cicletanine		1988	24, 299
doxazosin mesylate		1988	24, 300
enalapril maleate		1984	20, 317
enalaprilat		1987	23, 332
felodipine		1988	24, 302
guanadrel sulfate		1983	19, 319
ketanserin		1985	21, 328
lisinopril		1987	23, 337
nipradilol		1988	24, 307
nitrendipine		1985	21, 331
perindopril		1988	24, 309
pinacidil		1987	23, 340
rilmenidine		1988	24, 310
terazosin HCl		1984	20, 323
tertatolol HCl		1987	23, 344
tiamenidine HCl		1988	24, 311
trimazosin HCl		1985	21, 333
AF-2259	ANTIINFLAMMATORY	1987	23, 325
amfenac sodium		1986	22, 315
deflazacort		1986	22, 319
etodolac		1985	21, 327
flunoxaprofen		1987	23, 335
isofezolac		1984	20, 319
isoxicam *		1983	19, 320
lobenzarit sodium		1986	22, 322
loxoprofen sodium		1986	22, 322
nabumetone		1985	21, 330
nimesulide		1985	21, 330
oxaprozin		1983	19, 322
piroxicam cinnamate		1988	24, 309
tenoxicam		1987	23, 344
fisalamine	ANTIINFLAMMATORY,	1984	20, 318
osalazine sodium	INTESTINAL	1986	22, 324

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL.,PAGE
alclometasone dipropionate	ANTIINFLAMMATORY,TOPICAL	1985	21, 323
butyl flufenamate		1983	19, 316
felbinac		1986	22, 320
halometasone		1983	19, 320
hydrocortisone aceponate		1988	24, 304
hydrocortisone butyrate propionate		1983	19, 320
mometasone furoate		1987	23, 338
piketoprofen		1984	20, 322
pimaprofen		1984	20, 322
prednicarbate		1986	22, 325
artemisinin	ANTIMALARIAL	1987	23, 327
halofantrine		1988	24, 304
mefloquine HCl		1985	21, 329
alpiropride	ANTIMIGRAINE	1988	24, 296
dronabinol	ANTINAUSEANT	1986	22, 319
amsacrine	ANTINEOPLASTIC	1987	23, 327
carboplatin		1986	22, 318
doxifluridine		1987	23, 332
enocitabine		1983	19, 318
epirubicin HCl		1984	20, 318
flutamide		1983	19, 318
lonidamine		1987	23, 337
mitoxantrone HCl		1984	20, 321
nilutamide		1987	23, 338
ranimustine		1987	23, 341
pirarubicin		1988	24, 309
ivermectin	ANTIPARASITIC	1987	23, 336
pergolide mesylate	ANTIPARKINSONIAN	1988	24, 308
lidamidine HCl	ANTIPERISTALTIC	1984	20, 320
gestrinone	ANTIPROGESTOGEN	1986	22, 321
amisulpride	ANTIPSYCHOTIC	1986	22, 316
zuclopenthixol acetate		1987	23, 345
timiperone		1984	20, 323
diacerein	ANTIRHEUMATIC	1985	21, 326
octreotide	ANTISECRETORY	1988	24, 307
adamantanium bromide	ANTISEPTIC	1984	20, 315
cimetropium bromide	ANTISPASMODIC	1985	21, 326
tiquizium bromide		1984	20, 324

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL.,PAGE
tiropramide HCl		1983	19, 324
cilostazol	ANTITHROMBOTIC	1988	24, 299
defibrotide		1986	22, 319
enoxaparin		1987	23, 333
epoprostenol sodium		1983	19, 318
indobufen		1984	20, 319
limaprost		1988	24, 306
ozagrel sodium		1988	24, 308
picotamide		1987	23, 340
flutropium bromide	ANTITUSSIVE	1988	24, 303
levodropropizine		1988	24, 305
benexate HCl	ANTIULCER	1987	23, 328
enprostil		1985	21, 327
famotidine		1985	21, 327
misoprostol		1985	21, 329
nizatidine		1987	23, 339
omeprazole		1988	24, 308
ornoprostil		1987	23, 339
plaunotol		1987	23, 340
rosaprostol		1985	21, 332
roxatidine acetate HCl		1986	22, 326
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