ANNUAL REPORTS IN MEDICINAL CHEMISTRY Volume 23

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

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

HOECHST-ROUSSEL PHARMACEUTICALS, INC.
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Volume 23

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PREFACE

Volume 23 of *Annual Reports in Medicinal Chemistry* is similar in format to previous volumes of this series. Section II (formerly "Pharmacodynamic Agents") has been renamed "Cardiopulmonary and Vascular Agents" to better reflect its content. The 33 chapters of this volume are distributed among this section and the standard sections: CNS Agents; Chemotherapeutic Agents; Endocrinology, Immunology, and Metabolic Disorders; Topics in Biology; Topics in Chemistry and Drug Design; and Special Topics. The scope of the volume index has been expanded to include selected subject citations as well as compound names and code numbers, and a Cumulative NCE Introductions Index has been added.

Traditional annual updates include chapters on antihypertensives, analgesics, new NCE introductions, β-lactam antibiotics, and quinolone antibacterials. Complementing the latter two chapters is a contribution on novel approaches to the discovery of antibacterial agents. Current directions in AIDS research are covered in a chapter on antiviral agents, as well as in one on the disease itself. Companion chapters (18 and 19) treat immunomodulatory and arachidonic acid cascade-based approaches to the treatment of rheumatoid arthritis. Modulators of this cascade, among other agents, are also covered in a chapter on pulmonary and antiallergy agents. Use of chemically modified antibodies in cancer chemotherapy is reviewed (Chapter 16), as is the metabolic basis of anticancer drug resistance (Chapter 27). Metabolic enzymes and their implications in the drug design process are discussed in Chapter 32. Application of enzymes in organic media as synthetic tools is also reviewed.

A number of chapters touch upon the increasingly recognized role of serotonin in both CNS and peripheral functions, including Chapter 6 (serotonin receptors), Chapter 1 (antipsychotics), Chapter 3 (anxiolytics), Chapter 20 (obesity), and Chapter 21 (GI motility). The ubiquitous neuromodulator adenosine is also the subject of a review. Complementary chapters treat both the central (cognitive disorders) and peripheral applications of cholinergic agents. The importance of peptides in therapy and as models for non-peptidergic drugs is underscored in chapters on vasopressin antagonists, plasminogen activators, and LHRH, and in companion chapters (28 and 29) dealing with NMR protein structure determination and search strategies for determining bioactive conformers of peptides. An overview of our current understanding of important drug receptors and aspects of signal transduction is provided in three sequential chapters (23–25) on G-protein receptor structure, G-proteins, and protein kinase C. A look beyond receptors as drug targets is the subject of a provocative chapter on antisense oligonucleotides as inhibitors of gene expression.

It has been a distinct pleasure to work with the Section Editors on this volume. Although many of them were new to *Annual Reports* this year, they responded in a heroic fashion to join with the "oldtimers" and the contributors to assemble a fine collection of reports. Special thanks go to Ms. Susan K. Campbell, Marc N. Agnew, Christian Raabe, and the many secretaries to the authors and Section Editors without whose efforts there would

be no volume. Lastly, I wish to thank two former Editors-in-Chief of *Annual Reports*, Hans-Jürgen Hess and Denis M. Bailey, who prepared me to carry on this tradition of almost two and one-half decades.

Richard C. Allen
Somerville, New Jersey
May 1988

Section I - CNS Agents

Editor: Joel G. Berger, Schering-Plough Corporation
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Chapter 1. Antipsychotic Agents

James S. New and Katherine S. Takaki
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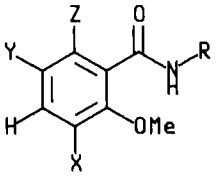
Introduction - Emphasis on the development of antipsychotic agents with fewer side effects continues to stimulate research on new animal models which may refine the drug discovery process in schizophrenia (1-4). This is also reflected in the extensive studies on the mechanism of action of antipsychotic agents which have appeared this year. The emerging technologies of functional and anatomical brain imaging are finding more frequent application in the study of brain structural abnormalities which may underlie the etiology of schizophrenia (5). General discussions of current biological studies as well as treatment strategies in schizophrenia have been thoroughly reviewed (6,7).

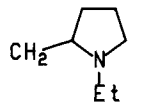
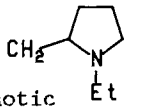
Substituted Benzamides - The substituted benzamides form a distinct class of agents characterized by high selectivity for dopamine (DA) D_2 receptors. Sulpiride (1) in high doses shows clinical efficacy against the positive symptoms of schizophrenia and in low doses has been suggested to possess activating and possibly antidepressant properties (8). A recent double-blind comparison of normal and low dose sulpiride showed a statistical improvement for the low dose regime on the negative symptoms of schizophrenia (9). In healthy volunteers, stimulation of prolactin (PRL) release by 1 has been seen in doses as low as 1 mg (10). Levosulpiride is an effective antipsychotic at lower doses than the racemate and its pharmacological and clinical data have been recently summarized (11). Binding studies utilizing [^{3}H]levosulpiride and [^{3}H]spiperone have demonstrated an identical binding site for the two ligands (12).

A concise review of the major pharmacological and clinical effects of remoxipride (2) shows that this agent displays superior bioavailability to 1 and selectively blocks DA D_2 receptors with specific affinity for limbic areas (13). In open clinical studies, 2 exerted an antipsychotic effect similar to conventional agents with a low propensity to induce extrapyramidal, autonomic, or cardiovascular side effects. Based on a comparison of median therapeutic doses, an equivalency of haloperidol to 2 of 1:7 has been suggested (14). Treatment of rats with 2 dose-dependently increased dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels, but no significant change in DA turnover was observed at doses which were active in the inhibition of apomorphine-induced hyperactivity (15). Recent clinical trials with 2 continue to suggest antipsychotic efficacy for this agent (16,17). In a small study, 2 was shown to be useful in the treatment of acute mania (17). A more recent evaluation in manic patients showed only a slight, transient effect of drug treatment on rating scales, but confirmed previous reports of a low incidence of side effects and a lack of elevation of basal PRL levels (18).

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Raclopride (3) has shown a behavioral profile in rats similar to haloperidol but with a greater separation between doses suppressing locomotor activity and those inducing catalepsy (19). Most of the relevant pharmacology of 3 has been outlined (20). A dose-dependent tolerance to the effect of chronic raclopride treatment on DA turnover has been observed, although the degree of tolerance was marginal at lower but behaviorally active doses (21). In an open trial in schizophrenic patients, 3 significantly decreased mean rating scores (22). The high selectivity and affinity of raclopride for central D_2 receptors, as well as its rapid association of specific binding, have made ^{11}C-raclopride a useful agent for positron emission tomography (PET) studies of schizophrenia (23). A significant blockade of D_2 but not D_1 receptors by clinical antipsychotic treatment has been demonstrated by PET analysis using ^{11}C-3 and the specific D_1 antagonist ^{11}C-SCH 23390 (24). Although a PET study conducted with ^{11}C-N-methylspiperone reported a two- to three-fold elevation of D_2 receptors in drug-naive and drug-treated patients (25), this has not been confirmed by a ^{11}C-raclopride study of drug-naive patients (26).

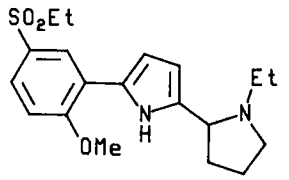


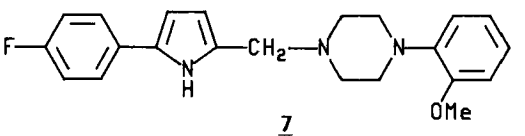
	R	X	Y	Z
1		H	SO_2NH_2	H
2	"	Br	H	OMe
3	"	Cl	Cl	OH
4	$CH_2CH_2NEt_2$	H	SO_2Me	H
6		H	SO_2Et	H

The benzamide tiapride (4) has failed to exhibit antipsychotic efficacy in clinical trials (27), but a new study concerned with the treatment of permanent tardive dyskinesia appears to confirm its usefulness in treating extrapyramidal motor disturbances (28). In the rat, repeated administration of a therapeutic dose of 4 did not modify the kinetic characteristics of DA receptors in the anterior pituitary (AP) or change basal PRL levels (29). Long-term dosing with 4 does appear to cause a slight elevation of DA agonist-labeled receptors in limbic and striatal areas and induces some tolerance to DA turnover (30).

A structural modification which appears to enhance the activity of some benzamides is the use of pyrrole as a bioisosteric replacement for the amide group. For example, 5, a conformationally restricted analog of sultopride (6), was shown to be three times more potent than 6 in vivo (31). From this work, 7 has emerged as the prototype of a new class of sodium-independent D_2 antagonists with low potential to induce catalepsy.

5

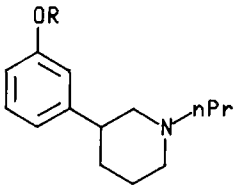




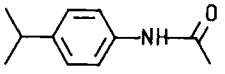
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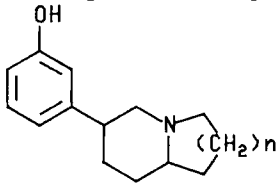
DA-Autoreceptor Agonists - The hope of reducing dopaminergic neurotransmission via stimulation of dopamine autoreceptors has continued to fuel the search for selective DA autoreceptor agonists as potential antipsychotic agents. On-going synthetic work has been aimed at improved derivatives of 3-(3-hydroxyphenyl)-N-n-propylpiperidine (3-PPP) (8). The low oral bioavailability of (-)-8 has been enhanced by derivatization of the

compound into aryl-carbamate prodrugs containing electron-donating substituents on the aromatic ring (32). In particular, **9** escaped first pass metabolism and generated a plasma level of (-)-3-PPP after 4h which was 90 times that achieved by the oral administration of (-)-**8** itself. Conformationally restricted indolizidine and quinolizidine derivatives (**10** and **11**) of 3-PPP have been shown to conform to the dopamine model proposed by Wikstrom, et. al.(33,34). The racemic, equatorial derivative **11** (n=1) appeared to show autoreceptor activity similar to racemic 3-PPP but the separate enantiomers displayed no presynaptic selectivity. The (-)-enantiomer of the related compound, HW-165 (**12**), exhibits pharmacological activity similar to (-)-3-PPP and appears to exert a preferential effect in limbic tissue without inducing catalepsy (35). A systematic investigation of N-substitution of **12**, as well as its 9-hydroxy isomer, showed a general trend toward increasing DA receptor affinity with increasing alkyl size (36). This data has also been subjected to detailed QSAR and eudismic analyses of presynaptic dopamine activity and D_2 and σ receptor affinities (37).

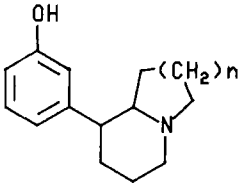


8 R = H

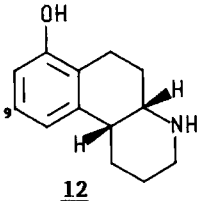
9 R = 



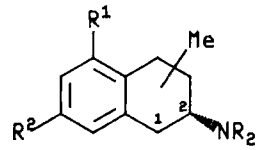
10 (n=1,2)



11 (n=1,2)



12

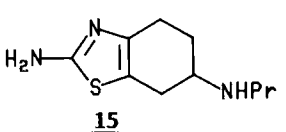


13 R_1=OH R_2=H

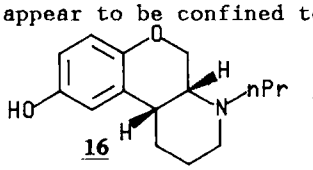
14 R_1=H R_2=OH

Systematic investigations of C_1 and C_3-methylated-5-hydroxy-2-(dialkylamino)tetralins (**13**) as central DA agonists and antagonists have been reported (38-40). Data derived from crystallographic, NMR, and force field calculations has been used to identify two structural parameters which define conformation and are predictive of agonist activity (38,40). Autoreceptor agonist activity requires the S configuration at C_2 and is favored in the C_1-methyl series by decreasing the size of the N-alkyl groups (39). An analogous study of C_1 and C_3-methylated-7-hydroxy-2-(dialkylamino)tetralins (**14**) has been published, but all of the compounds reported were less active than 7-OH-DPAT itself (41). Substitution of an aminothiazole for the phenolic subunit of the aminotetralins has produced **15** which is active in the γ-butyrolactone (GBL) model of DA autoreceptor activity and does not induce stereotyped behavior (42).

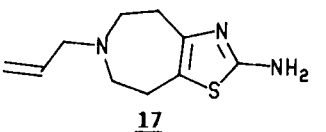
CGS 15855A (**16**) is an autoreceptor agonist which produces a dose dependent decrease in 3-methoxytyramine levels in both the mouse and the rat (43,44). Weak behavioral stereotypies have been observed with **16** but only at doses 20 times the ED_{50} for activity in the GBL model (45). Behavioral effects appear to be confined to the (+) enantiomer.



15



16

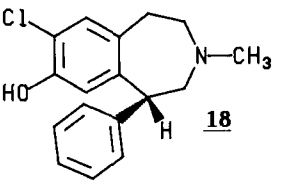


17

The α_1-agonist B-HT 920 (17) appears to function as a DA autoreceptor agonist in normasensitive receptors and as a post-synaptic agonist in receptors rendered supersensitive by DA depletion or denervation (46-48). A pilot study of B-HT 920 in patients with chronic, paranoid-hallucinatory psychosis showed no overall superiority of 17 over placebo, although isolated cases appeared to show improvement (49). No significant effects on blood pressure or heart rate were noted and the compound did not appear to cause extrapyramidal symptoms (EPS).

Mechanistic Studies and D_1 Antagonists - The degree of selectivity dopaminergic antagonists display for limbic versus striatal structures in the brain has become a reliable predictor for potential antipsychotic agents with lower EPS liability. Acute i.v. clozapine increases the number of spontaneously active DA neurons in the ventral tegmental area (VT or A10) but not in the substantia nigra pars compacta (SNC or A9), in contrast to haloperidol which increases the firing rate in both subpopulations of DA neurons. The regioselective effects observed with clozapine are not thought to be due to its antimuscarinic activity (50). Parallel studies demonstrated that repeated treatment with classical antipsychotic drugs reduced basal DA release in the striatum and nucleus accumbens (NA), whereas repeated treatment with atypical antipsychotics decrease DA release only in the accumbens (51). The depolarization-induced inactivation of both A9 and A10 dopaminergic neurons by haloperidol during chronic administration is dependent on the continuous presence of the drug, since spontaneous activity in these neurons is normalized after cessation of drug treatment. Extracellular single-unit recording experiments suggest that the DA autoreceptors in the VT area are of the D_2 subtype, while the autoreceptors in the NA, which receives projections from VT, are composed of both D_1 and D_2 subtypes (52).

SCH 23390 (18), a selective D_1 antagonist, potently reverses d-amphetamine-induced suppression of A9 and A10 DA neuronal firing in the chloral hydrate anesthetized rat. This may be a consequence of a nonselective D_2 antagonist effect or the 5-HT_2 receptor antagonist properties of the molecule (53). SCH 23390 produced only a partial reversal of apomorphine-induced suppression of A9 and A10 DA cells; without any drug pretreatment, 18 did not significantly alter the basal activity of either of these cell subpopulations in the anesthetized rat (54). This observation differs from the potent activating effects of the drug recorded from A9 DA units in unanesthetized rats, suggesting some aspect of the unanesthetized preparation may potentiate the effects of SCH 23390, or that chloral hydrate interferes with the effects of this drug (54,55).



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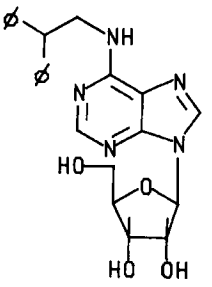
The postsynaptic expression of D_2 agonist effects has been demonstrated to involve D_1 receptor activation (56,57). The synergistic effects resulting from the functional interaction of these two receptor subtypes was observed in the firing rates of basal ganglia neurons and on the expression of stereotyped behavior in rats. Additional evidence for a functional D_1-D_2 receptor interaction was provided by the ability of the D_1 selective agonist SKF 38393 to restore the diminished inhibitory effect of the D_2 agonist quinpirole in rat NA preparation depleted of catecholamines by pretreatment with α-methyl-p-tyrosine (58). Intracerebral dialysis methods conclude that DA release in the striatum is autoregulated by independent D_1 and D_2 receptor-linked mechanisms, but that the level of DA metabolism is controlled by the D_2 receptor which can be modulated by the D_1 receptor (59).

The chronic blockade of D_1 receptors in the rat striatum, NA and substantia nigra (SN) by SCH 23390 induced an up-regulation of the D_1 receptors without changing the functional activity of D_2 receptors. Similarly, chronic blockade of D_2 receptors by (-)sulpiride caused up-regulation of D_2 but not D_1 receptors in the SN, NA striatum and pituitary. Neither compound produced an up-regulation with either D_1 or D_2 receptors in the limbic-cortical areas (60). These results contrast with those from a recent study involving long-term treatment with haloperidol, which suggest an up-regulation of both D_1 and D_2 subpopulations of DA receptors occurs in the striatum and NA of the rat (61). Specific inhibition of [^{3}H]SCH 23390 binding by several divalent metal cations and N-ethylmaleimide suggest a thiol group is involved in ligand binding to the D_1 receptor protein (62). A model for the D_2 receptor, which addresses only the molecular subunits involved in ligand-specific binding and not associated regulator or effector units, was proposed from radiation inactivation studies. The target size or functional unit required for DA agonist binding was found to be smaller (81,000 daltons) than that required for the D_2 antagonist spiroperidol (137,000 daltons) (63); the opposite was found for D_1 binding sites (64).

The stereoselective and highly specific labelling of the D_1 receptor by SCH 23390 and related analogues has been used to map the topography and estimate the target size of the D_1 receptor (65,66). The binding of SCH 23390 in rat and mouse brain tissue indicates the density of D_1 sites is higher in striatum than in limbic structures, which in turn was greater than that found in frontal cortex (67). Unilateral 6-OHDA lesions did not affect SCH 23390 binding; however, a significant decrease in binding was observed 3 weeks after unilateral kainic acid lesions. Further studies demonstrated decrements in the activity of DA-sensitive adenyl cyclase and SCH 23390 binding in rat nigra 15 days after the injection of 6-OHDA into the medial forebrain bundle (68). In contrast, the 6-OHDA-induced degeneration of the NS DA pathway enhanced these D_1 responsive parameters. The changes observed in both membrane preparations resulted from changes in the total number of binding sites with no modifications in the apparent affinity of SCH 23390.

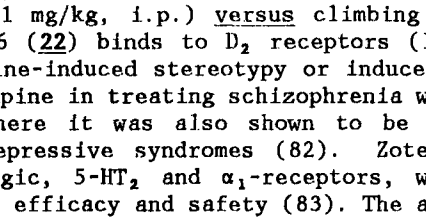
Circling behavior in rats unilaterally lesioned with 6-OHDA, and administered either a D_1 or D_2 agonist, could be antagonized in a mutually exclusive fashion by the D_1 or D_2 antagonists SCH 23390 or spiperone, respectively (69); in this model, therefore, the D_1 and D_2 receptors appear functionally independent. Quantitative autoradiographic studies employing [^{3}H]SCH 23390 in ibotenic acid or 6-OHDA lesioned rat brain indicate that D_1 receptors are located postsynaptically on striatal neurons in the caudate-putamen, but have a presynaptic location on nerve terminals in the SN which originate in the caudate-putamen (70). Activation of the EEG in the rabbit has been shown to be a common response to the stimulation of either D_1 or D_2 receptors (71). Induction of EEG activation by L-DOPA was stereospecifically blocked by SCH 23390 but not by haloperidol, which was effective in blocking only the behavioral effects (72). Rats trained to intravenously self-administer cocaine on a fixed schedule, when subjected to a series of systemic injections of SCH 23390, produced a dose-dependent increase in cocaine intake (73). Since an analogous result is not observed with spiperone, it is suggested that selective D_1 receptor activation is a contributing component in psychostimulant reward. The hypermotility evoked in mice from administration of morphine could be blocked by pretreatment with SCH 23390, inferring a role for D_1 receptors in the mediation of this hypermotility response (74).

Atypical Agents - The characterization of an antipsychotic agent as atypical is usually based on its pharmacological profile which predicts the compound will have less propensity to induce the typical neuroleptic side effects of EPS or tardive dyskinesia. Clozapine still reigns as the archetype antipsychotic which embodies this ideal. The antipsychotic effects of CI-936 (19) are suggested to arise from its potent affinity at the A1 and A2 adenosine receptors, since the locomotor inhibition properties of this compound are reversed in a dose-dependent manner by the adenosine receptor antagonist, theophylline. CI-936 inhibits dark-stimulated motor activity at doses below those that produce ataxia and it is active in the Sidman conditioned avoidance (75). Numerous analogues of CI-936 have recently been reported and an excellent discussion of adenosine's CNS effects has appeared (76-78).

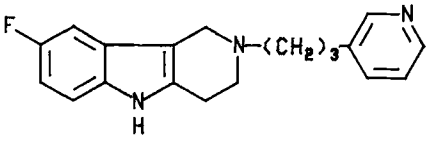


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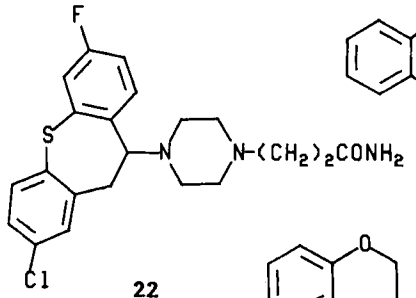
Compound 20 is active in the Sidman avoidance and self-stimulation test in rats with ED_{50} values comparable to those of thioridazine (79). It inhibits locomotor activity, does not bind to D_1 or D_2 receptors, and is predicted to have low EPS potential since it did not produce any dystonic signs in the haloperidol - sensitized cebus monkey model. The β-carboline derivative Wy-47,384 (21) binds only weakly to D_2 sites and blocks avoidance responding in rats (80). The atypical aspect of its profile with regard to low side effect liability is evidenced by the differential potency it displays in antagonizing apomorphine-induced stereotyped behavior (ED_{50}= 11 mg/kg, i.p.) versus climbing behavior (ED_{50}=4 mg/kg, i.p.). VUFB-15496 (22) binds to D_2 receptors (IC_{50}=49.7 nM) but neither blocks apomorphine-induced stereotypy or induces catalepsy (81). The efficacy of fluperlapine in treating schizophrenia was observed in an open crossover study where it was also shown to be effective in the treatment of apathic depressive syndromes (82). Zotepine (23), which blocks mainly dopaminergic, 5-HT_2 and α_1-receptors, was compared with thiothixene for clinical efficacy and safety (83). The antipsychotic properties of this drug were affirmed and its use led to marked improvement in motor retardation, suspiciousness, and posturing symptoms, indicating zotepine may possess activating properties as well. Cipazoxapine's (24)



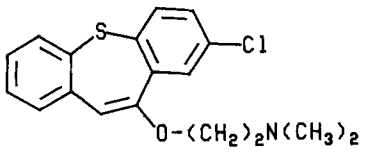
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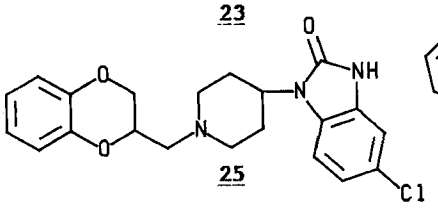
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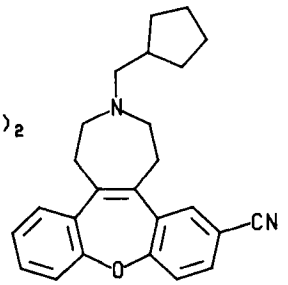
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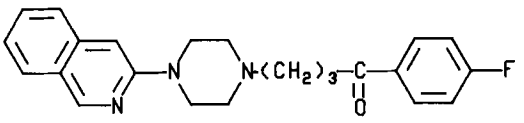


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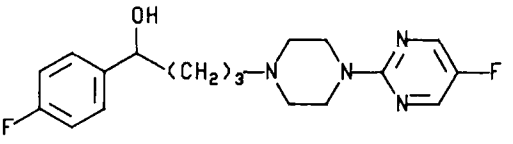


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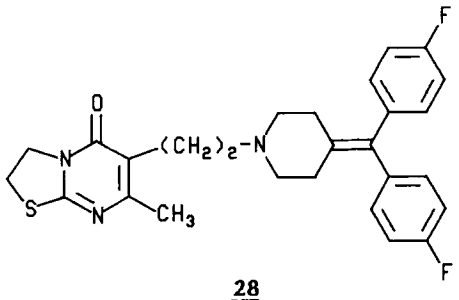
reduced side effects may be derived from its selective affinity for D_2 receptors in the hippocampus versus the striatum (84). In a preliminary trial with eight schizophrenic patients, cipazoxapine demonstrated antipsychotic effects without EPS. HR 723 (25) is equipotent to haloperidol in suppressing apomorphine-induced climbing in mice and potently binds to spiroperidol labelled sites. The compound exhibits a large difference in the desired antiapomorphine activity and its cataleptogenic potency in rats (85). Cinuperone (26), which inhibits [^{3}H]spiperone binding to D_2 receptors and (+)[^{3}H]SKF 10,047 binding to σ receptors, is speculated to derive some of its antipsychotic-like properties from its interaction with σ receptors (86). Unlike cinuperone, the potential antipsychotic BMY 14802 (27) binds only weakly to D_2 receptors, but demonstrates a potent and stereospecific interaction at the σ receptor (87,88). It inhibits both the conditioned avoidance response and apomorphine-induced stereotypy, and effects a depolarization block of spontaneously active A10 dopaminergic cells but not A9 cells after chronic administration. Ritanserin (28) and setoperone (29), potent 5-HT_2 antagonists, continue to be investigated for anxiolytic and antipsychotic indications (89). Ritanserin offered significant improvement in the negative symptoms of schizophrenia with a concurrent decrease in extrapyramidal symptomatology (90). The D_2 receptor blocking activity of setoperone was observed in its elevation of plasma prolactin levels (91). An anecdotal report of setoperone in schizophrenic patients indicates efficacy in the treatment of delusional symptoms. Setoperone on chronic administration



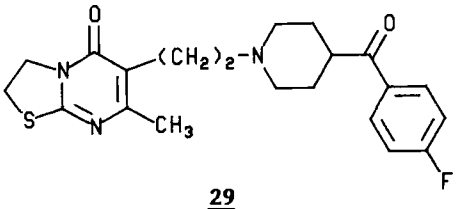
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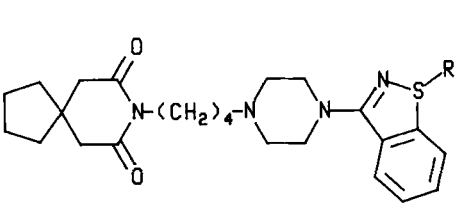


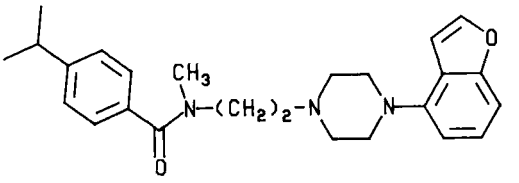
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leads to a desensitization of 5-HT_2 receptors, possibly by uncoupling the receptor site and its transducer system (92). An autoradiographic analysis of typical and atypical antipsychotics binding to D_2 and 5-HT_2 receptors indicates the atypical agents were more effective at competing for 5-HT_2 sites in the frontal cortex than for D_2 sites in the basal ganglia. Relative to the typical agents, the atypicals also had a greater selectivity for D_2 sites in the NA or olfactory tubercle, than in the caudate-putamen (93). Tiospirone (30) in a single-blind cross-over trial demonstrated antipsychotic efficacy with no reports of EPS (94,95). Derivatization of tiospirone as a S-oxide, as in BMY 20366 (31), attenuates the molecules affinity for DA D_2 receptors without disrupting its potency in the blockade of apomorphine stereotypy or the conditioned avoidance response (96).

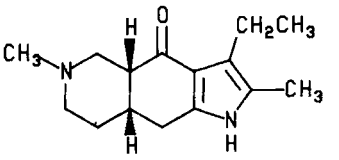




30; R=unsubstituted
31; R=O

32

Befiperide (**32**) which also lacks a direct interaction with DA receptors, demonstrates antipsychotic-like effects in animal behavior models (97). The preclinical pharmacological profile of piquindone (**33**), predictive of antipsychotic activity with low EPS liability, has been corroborated in a double-blind, placebo-controlled clinical study (98).



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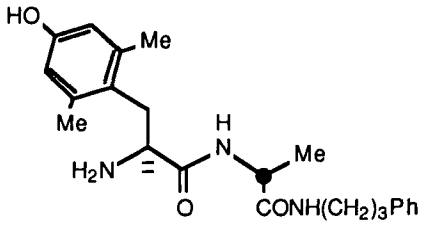
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Chapter 2. Analgesics

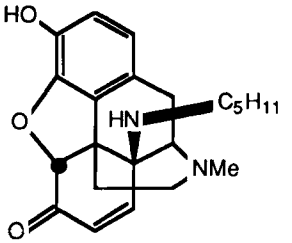
Richard E. Chipkin and Federico C.A. Gaeta
Schering Corporation, Research Division, Bloomfield, NJ 07003

Introduction - The last five years have seen a substantial increase in the amount of information available regarding the neurochemistry of central and spinal systems regulating the perception of pain (1). Understanding of the mechanism of action of opioids beyond the receptor has increased (2) and the pivotal role of G-proteins and inhibition of cAMP formation by opioids is being investigated (3). The isolation and partial purification of the opioid receptors has been reported (4,5), and the startling discovery that mammals seem capable of synthesizing morphine and codeine (6,7) endogenously suggests that these alkaloids may have some undiscovered role in the control of nociception. In spite of these advances, a unified theory of sensory processing has not emerged, and the search for an ideal pain reliever continues. However, recently acquired knowledge of how different nociceptive stimuli (heat, cold, pressure, etc.) recruit different neuronal systems could result in new therapies which may be specifically directed toward the kind of pain being treated, rather than being a general "analgesic". A number of general review articles on pain and analgesia have been published in the last two years (7-13), and migraine pain has been reviewed in volume 22 of this series (14).

Full Opioid Agonists - SC-39566 (**1**) was reported to be an orally active opiate in rodents and primates, producing less respiratory depression than morphine at analgesically relevant doses (15). However, its preclinical profile does not appear radically different from standard opiates, and its clinical benefits are unknown. Another modified enkephalin opioid agonist is BW433C (Tyr-D-Arg-Gly-(4-NO_2)Phe-Pro-NH_2). BW433C is antinociceptive in animals (16) and in clinical experimental (cold-induced) pain (17) after intravenous administration. BW433C is thought to produce analgesia by a peripheral mechanism based on its antagonism by quaternary opioid antagonists, and its failure to induce analgesia when given directly into the cerebral ventricles (18). Its pain-relieving effects in man occurred at doses that did not alter central indices such as miosis, respiratory depression or self-reported mood changes (17). However, like previous enkephalin analogs tested in man, BW433C caused feelings of warmth, heavy legs with pain in the calf muscles, and increased bowel sounds (19). BW433C has not been reported to be orally active and has been noted to produce constipation after subcutaneous administration (20). Although SC-39566 is the most advanced peptide-related compound, other agents reported include D-Arg substituted tetrapeptides (21), opioid peptides derived from hemoglobin (TyrProTrpThr) (22), and metorphamide (TyrGlyGlyPheMetArgArgVal-NH_2) (23). Additionally, work has progressed on non-peptide full opioid agonists. The most advanced of these is the aminomorphinone RX77989 (**2**), which is being developed because of its short duration of action (24).



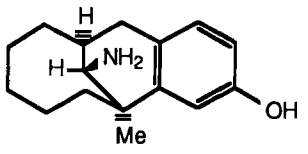
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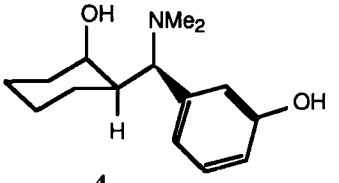
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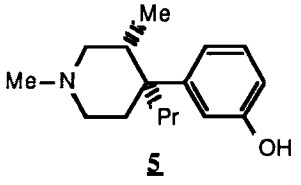
Partial Opioid Agonists and Agonist/Antagonists - Dezocine (3) has been shown to alleviate post-operative pain at 10-15mg po (25,26). However, it has positive subjective effects in humans similar to morphine (27), suggesting that its abuse liability may be higher than with other partial agonists. Ciramadol (4) is a partial agonist which shows clinical activity by all routes of administration (28-30). Like other drugs of this class it seems to produce fewer gastrointestinal (GI), cardiovascular (CV) or respiratory (R) side-effects. Picenadol (5) is of interest since the *d*-enantiomer is an agonist, and the *l*-isomer an antagonist. This drug is clinically active and may have less sedative and psychotomimetic effects than previous agonist-antagonists (31,32). Tonazocine (6) is orally active against post-operative pain and produces less sedation than standards (33). A newer agent, still under pre-clinical evaluation, is zenazocine (WIN42964, 7) (34).



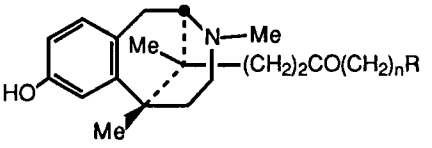
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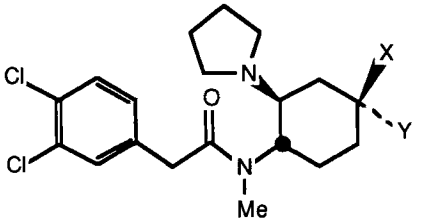


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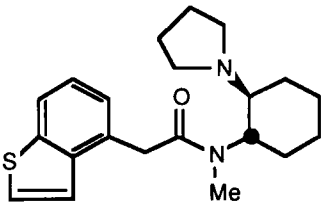
6 , n=4, R=Me
7 , n=2, R=i-Pr

Kappa Agonists - Preclinical studies using U-50488 (8) show that this drug produces strong analgesia, but has a low propensity to cause GI or R side effects and has a different tolerance and dependence profile from morphine (35). Although clinical utility of the κ agonists has not yet been demonstrated, these agents could have unique side effect profiles limiting their clinical utility. Primary among them are an ability to cause diuresis due to a blockade of vasopressin release (36), and the risk of psychotomimetic effects (37,38). Despite this, work on κ agonists has progressed, and spiradoline (U-62066, 9) is in clinical trials (39). Another κ agonist under investigation is PD 117302 (10) , which showed good analgesic activity, but did not suppress morphine withdrawal in the rhesus monkey (40,41). None of these compounds have undergone rigorous clinical investigation, and thus the concept of κ agonist-induced analgesia as an alternative to morphine still remains untested.



8 X=Y=H

9 X-Y=$OCH_2CH_2CH_2$

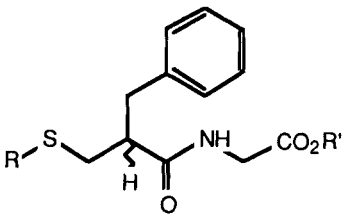


10

Delta Receptor Agonists - The finding that the specific δ agonist [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) produced analgesia in standard *in vivo* tests (42) implied that agents of this type could be useful thereapeutically. Additional studies have shown that genetically mutant mice deficient in μ but not δ receptors still display analgesia when given morphine or DPDPE, thus confirming δ-mediated analgesia (43). Intrathecal administration of D-Ala^2-

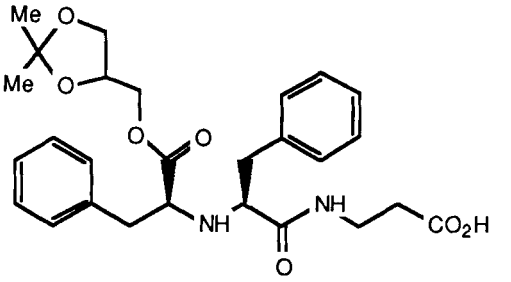
Leu^5-enkephalin (δ selective) to morphine (μ selective) tolerant patients produced clinically relevant pain relief (44). This raises the possibility that problems of tolerance may be avoided by use of agents that have different pharmacological specificity.

Enkephalinase Inhibitors - The strategy of inhibiting the enzymes which terminate the action of endogenously released opioids has received considerable attention recently (45,46). Acetorphan (**11**), a prodrug analog of thiorphan (**12**) (47), the first reported enkephalinase inhibitor, was tested intravenously in humans, producing clinically relevant analgesia in post-myelography headache pain (48), but not in shock-induced experimental pain (49). These studies were limited by lack of an orally active agent which would have made large scale clinical tests possible. The finding that the enantiomers of thiorphan were equally able to produce analgesia was quite surprising considering that they have substantially different *in vitro* activity vs. the enzyme (50). However, this work has not been reproduced, and, as expected, the ability of these drugs to produce analgesia was correlated with their effects on enkephalinase *in vitro* (51).



11 R=Ac, R'=Bzl

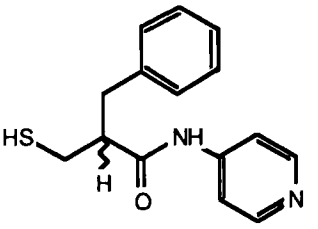
12 R=R'=H



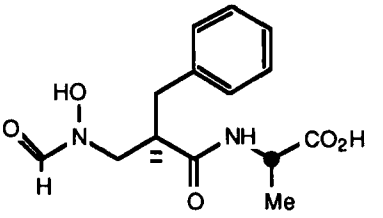
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SCH 34826 (**13**) has been reported to be an orally active enkephalinase inhibitor (52). This drug selectively blocks the enzyme and produces naloxone-reversible analgesia. It is a member of a wider series of compounds which can inhibit enkephalinase *in vitro*, but which are not orally active (46). It has been reported that the thiorphan-related compound RU 42827 (**14**) is an orally active analgesic (53). In contrast to thiorphan, RU 42827 does not bear a carboxylic acid moiety at the C-terminus of the pseudodipeptide, consistent with the endopeptidase properties of enkephalinase. These drugs are of particular interest because they should exhibit few CV or R side effects. Acetorphan and thiorphan have been reported to inhibit castor oil-induced diarrhea (54); however, the meaning of this finding with regard to potential side-effects of these drugs is unclear. One side-effect that does not seem to occur with enkephalinase inhibitors is tolerance or dependence (47). Thus, based on pre-clinical studies, enkephalinase inhibitors produce opiate-like analgesia without any of the opiate side-effects. If this profile can be clinically verified, then enkephalinase inhibitor analgesics will represent the first novel approach in the field in quite some time. Of further interest is a series of mixed enzyme inhibitors, exemplified by kelatorphan (**15**) and phelorphan (**16**), which block degradation of enkephalin not only by enkephalinase, but also by aminopeptidase and by dipeptidylaminopeptidase (DAP-III) (55,56). Their potential advantages over selective enkephalinase inhibitors remains to be determined.

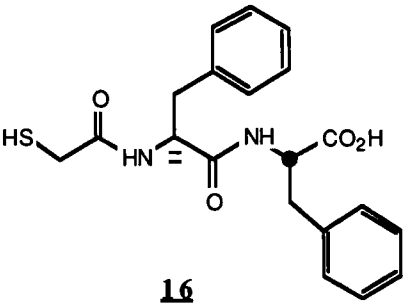
Serotonergic Agents - Multiple categories of serotonin receptors have been identified (see chapter 6, this volume), although the function of selective agonists and antagonists at each of these sites is controversial (57). Nonetheless, the concept of using serotonergic drugs as analgesics continues to be of interest. For example, fluoxetine (**17**), a selective serotonin uptake inhibitor with little effect on noradrenergic or dopaminergic systems (58), produces analgesia in mice after intrathecal administration (59). In spite of this, there are no clinically useful analgesics which operate solely via this mechanism. However, trazodone (**18**), used clinically as an antidepressant acting via serotonergic systems was inactive when tested in man for analgesic efficacy (60).



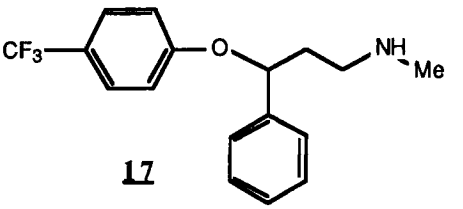
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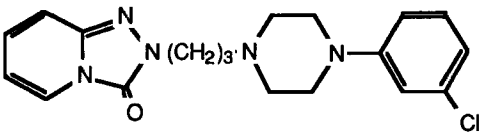
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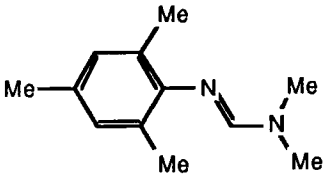


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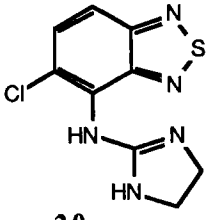


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Noradrenergic Agents - The most promising work in this area appears to be on α-2 agonists (61). U-47476A (19) was found to be active in standard animal models of analgesia (writhing, tail flick, and hot-plate) and was blocked by yohimbine, an α-2 antagonist (62). U-47476A did not affect blood pressure and had only moderate effects on locomotor activity, suggesting the separability of analgesic and CV effects (62). Other α-2 agonists reported on included tizanidine (20), which has been demonstrated to be active in the mouse writhing and the mouse and rat tail-flick tests (63,64). Tizanidine analgesia has also been reported to be reversed by the α-2 antagonist yohimbine and tolerance has been demonstrated after repeated dosing (65).



19

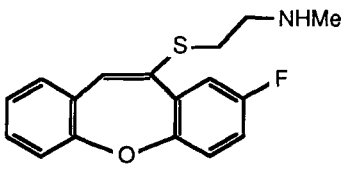


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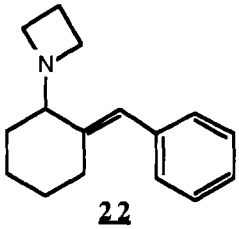
Although most of the work has been done with α agonists, some preliminary data exist on other adrenergic receptor subtype agonists that might have analgesic effects. Thus, the beta receptor agonists (-)-isoproterenol and (±)-isoxsuprine were able to suppress writhing behavior in mice (66).

Antidepressants as Analgesics - Antidepressants are effective in the treatment of various types of pain, particularly chronic pain conditions (67,68); these effects are achievable even in absence of an effect on depression. The effect seems best with agents (e.g. amitriptyline) which inhibit the reuptake of both norepinephrine and serotonin. The precise mechanism of action of these drugs remains controversial. Among the possibilities are: effects on adrenergic systems either in the brain or in the spinal cord (69); effects on an endogenous opioid system (70) which is unlikely to be enkephalinergic, since enkephalinase inhibitors do not potentiate the antinociceptive effects of antidepresssants in animals (71), but which may be β-endorphinergic since antidepressant treatment increases brain levels of this peptide (72); effects on GABA systems, since chronic antidepressant treatment increases the number of $GABA_B$ binding sites in the brain (73) and GABAergic systems have been purported to be involved in analgesia (74).

Two specific attempts have been made to capitalize on the idea of an analgesic/antidepressant. Fluradoline (21) had antidepressant properties in preclinical tests (75), and has been reported to relieve clinical post-operative pain (76). Likewise, tazadolene (22) is active in models of both analgesia and depression in animals (77); no clinical data are currently available. Thus, the concept of an analgesic/antidepressant for the relief of both pain and mood changes associated with pain may be viable.



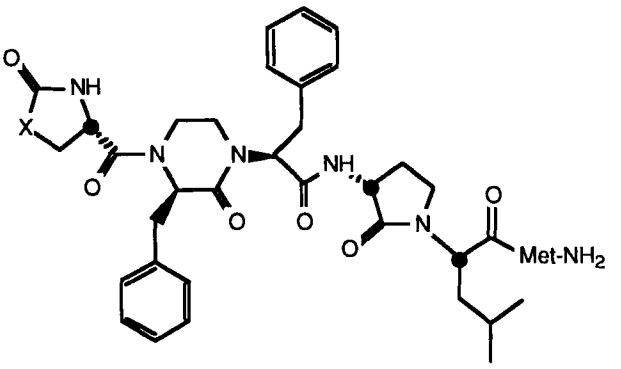
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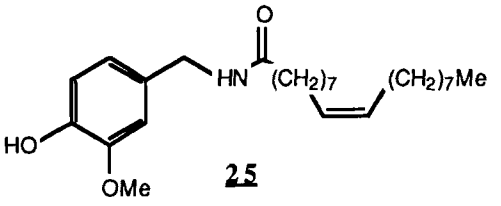
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Peptides - A variety of neuropeptides have been implicated in either the transmission or inhibition of pain and have been the subject of analgesic studies. Each of these will be discussed briefly. Substance P (SP) has been purported to be the transmitter at the spinal level for nociceptive impulses. Hence, SP antagonists should be analgesics. However, present SP antagonists have been disappointing because of their neurotoxic effects (78). In contrast, the piperazinone-SP derivatives U-66,739 (23) and U-67,202 (24) may have promise since they have little action on locomotor activity (79). Also, since SP is known to come from a family of endogenous tachykinins (80) it is possible that modifing these related peptides may lead to more specific effects. An alternative to SP antagonists are drugs which selectively deplete SP or some as yet unidentified transmitter in the CNS. This work has centered around capsaicin, which is able to deplete SP from C-fibers in the spinal cord and cause analgesia (81). One promising lead is olvanil, NE19550 (25). This drug has been reported to be orally active in rats and mice, and not to have the thermoregulatory and direct nociceptive problems associated with capsaicin itself (82). Bradykinin (BK) is known to produce pain when administered topically (83), and the recent discovery of selective BK antagonists (84,85) has led to their preclinical testing as analgesics. Mixed results have been reported (86,87). Part of the problem may be the lack of suitably potent, bioavailable peptides with a sufficiently long duration of action to permit testing. Calcitonin (CAL) has been reported to be analgesic in mice (88), rats (89) , and man (90). The mechanism of action is not clear, but may be related to calcium mobilization in the brain (91). Somatostatin (SOM) levels are reportedly decreased in the spinal cord of chronic pain patients (92) and in an anecdotal clinical report, intrathecal SOM caused pain relief (93). Arg-Vasopressin has been reported to be analgesic in preclinical tests (94) and in an anecdotal clinical report (93). Delta Sleep-Inducing Peptide (DSIP) has been shown to work both in mice (95) and in an open clinical trial on migraine headache (96). Finally, a

novel tetrapeptide (AsnAlaGlyAla) has been reported to produce naloxone-reversible anagesic effects in mice (97).

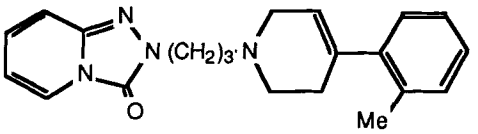


23 X=NH
24 X=CH_2

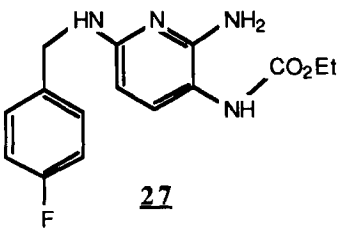


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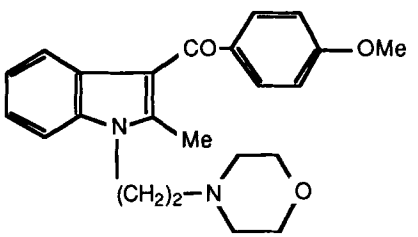
Miscellaneous Non-Opioid Analgesics - A variety of non-opioid analgesic drugs have been recently reported. Their mechanism of action is unknown. SCH 30497 (26), a structure somewhat related to trazodone (18), was analgesically active in the mouse, rat and squirrel monkey, and had attributes similar to both narcotic and non-narcotic analgesics (98). Flupirtine (27) has been reported to be a non-narcotic-like analgesic based on preclinical tests (99), and to work orally in post-operative clinical pain (100). WIN48098 (28) has been reported to be antinociceptive in mice and rats after oral administration (101). Phase I clinical trials have been uneventful (102). Although this drug may be operating via inhibition of cyclooxygenase, WIN48098 appears to have a distinct central action which differentiates it from typical NSAID's.



26



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28

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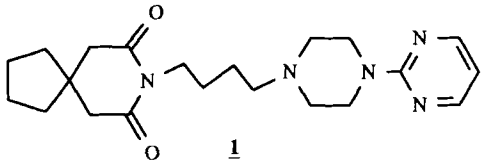
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Chapter 3. Antianxiety Agents and Anticonvulsants

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ANTIANXIETY AGENTS

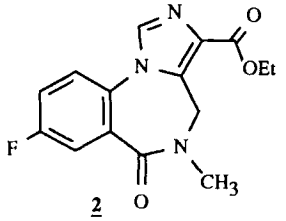
Introduction - The search for the ideal nonsedating anxiolytic agent continues to maintain a prominant position in CNS research (1). Agents acting *via* the benzodiazepine (BZ) receptor now receive ample competition from their serotonergic receptor counterparts such as buspirone (**1**), which is the prototype (2). Reviews of pharmacological (3) and clinical (4) studies continue to contrast the slightly superior efficacy and undesirable side effects (5) of the BZs to the improved therapeutic profile and reduced sedative properties of buspirone (6). Both classes of compounds may be acting similarly in the amygdala as seen with punished responding behavior in rodents (7). Mechanistic receptor probing with new specific affectors is now a central issue.



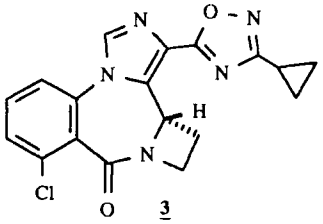
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BZ Receptor Mediated Anxiolysis - Refinement of the understanding of the structure and function of the GABA/BZ receptor complex with its associated chloride ion channel continues (8, 9). New models include a three-domain "static" model that accommodates the cyclopyrrolones (10) and a generalized stereochemically-controlled binding model (11). Considerable excitement surrounds the recent preparation of a functional synthetic receptor/ion channel complex via the coexpression of α- and β-subunit RNAs (prepared from cloned cDNAs from bovine cortical receptor) in *Xenopus* oocytes (12). The role of newly found astrocytic BZ receptors is under investigation (13). BZ receptor pharmacology (14) and animal conflict procedures (15) have been reviewed. Recent animal model studies include yohimbine-induced seizures in mice (16), stress-induced ultrasonic vocalization in rats (17-19), stimulation of saline intake in rats (20), the staircase test in mice (21), the "plus-maze" in mice (22), the corridor-type runway in rats (23), the "two-compartment (light/dark) activity box" in mice (24), and a short stress-induced electrocorticogram arousal assay in rats (25). The place-conditioning paradigm in rats may detect abuse potential (26). *Ex vivo* [^{3}H]flunitrazepam binding has been used to determine the onset and duration of action of BZs (27).

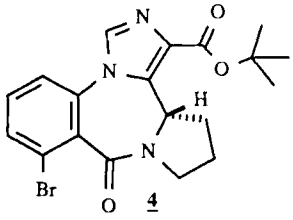
BZ Receptor Related Compounds - The entrance of the BZ antagonist flumazenil (Ro 15-1788, **2**) into the Swiss market (28) highlights the therapeutic significance of BZ receptor manipulation. This and other BZs which possess the complete affector range including full agonist Ro 19-1880 (**3**), partial agonist Ro 16-6028 (**4**), and inverse agonist



2



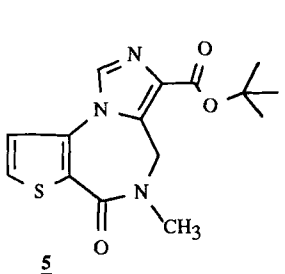
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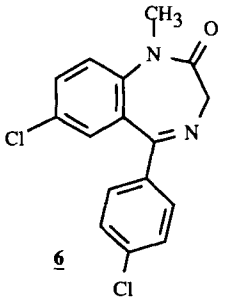
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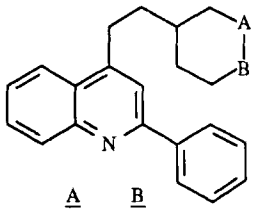
Ro 19-4603 (5) were reviewed (29). A novel binding site for Ro 5-4864 (6), associated with the chloride channel of central BZ receptors in rat brain, has been proposed from [^{35}S]TBPS binding studies; pipequaline (PK 8165, 7) and PK 9084 (8) also modulate this site (30). Clinically, the partial agonist character of pipequaline was confirmed with anxiolytic activity in stressed, anxious patients and antagonism of some diazepam (DZ) effects in EEG (31). The cyclopyrrolone suriclone (RP 31264, 9) compared favorably



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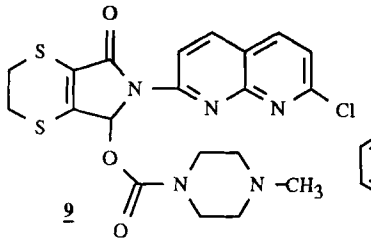


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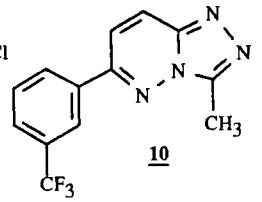


	A	B
7	CH_2	NH
8	NH	CH_2

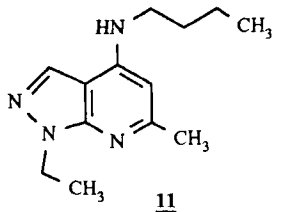
with DZ and lorazepam in a battery of animal motor function and EEG assays (32). Tolerance studies on CL 218,872 (10) and tracazolate (11), with mice in the holeboard test, reveal that their unique affector qualities do not preclude the same tolerance to depressant effects and lack thereof to stimulant effects as seen with DZ (33). Antagonist/partial inverse agonist CGS 8216 (12) was well tolerated in healthy volunteers with no apparent anxiogenic effects (34). Thienyl analog S-135 (14) is equipotent to 12 in [^{3}H]DZ binding but is >10x as potent orally in inverse agonist activity (proconvulsant with pentylenetetrazole (PTZ) in mice), while not causing convulsions alone. Conversely, regioisomer 15 is a potent agonist orally (PTZ in mice) with similar receptor binding (35). The profile of new partial agonist CGS 20625 (16) looks promising with potent oral activty in conflict responding and antagonism of DZ-induced rotorod deficit, and little or no effect in rotorod or in either ethanol or hexobarbital potentiation (36). Quazepam (Sch 16134, 17) and antagonist AHR-11797 (18) showed similar but less pronounced effects on suppressed behavior of squirrel monkeys when compared with lorazepam and flumazenil, respectively (37). The profile of Gö 4962 (19) indicates agonist character, somewhat weaker than DZ, residing primarily in the (-)-isomer (38).



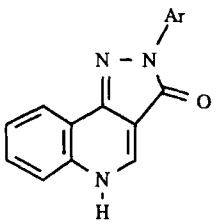
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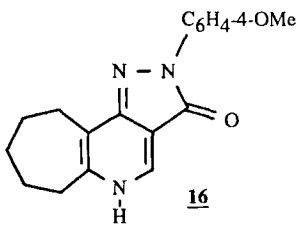
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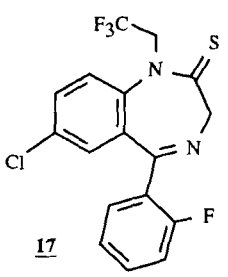


	Ar
12	C_6H_5
13	4-ClC_6H_4
14	3-(5-Me-C_4H_2S)
15	2-(5-Me-C_4H_2S)

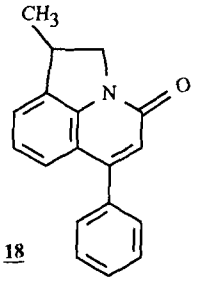


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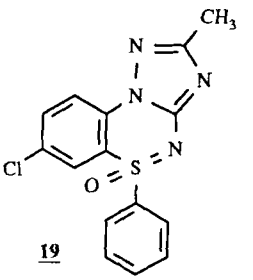
β-Carbolines continue to play a prominent role in BZ receptor manipulation. The anxiogenic character of inverse agonist, β-CCM (20) was confirmed in a light/dark choice model in mice, and flumazenil antagonized this effect (39). β-CMC (22) has shown selective antagonism of sedative effects of BZs in mice (40). ZK 91296 (23) and ZK 93423 (24) have clear BZ-like agonist effects on schedule-controlled behavior in squirrel monkeys (41) and enhance GABA binding in rat cerebral cortex membranes (42).



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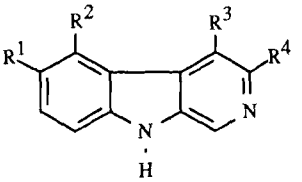


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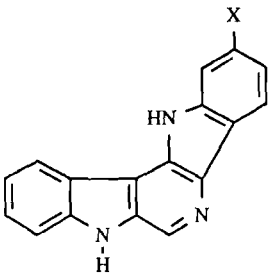


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Antagonist properties similar to CGS 9896 (13) are also shown by 23 *in vitro* (43). The *in vitro* and *in vivo* profile of a host of β-carbolines and other receptor ligands were correlated. Full agonist 24 appears more potent (ip) than DZ in many assays and new partial agonist ZK 95962 (25) potently antagonized PTZ cue but was inactive in lick-suppression (44). Also, 24 prevents stress-induced enhancement of DA turnover in rat prefrontal cortex as seen with BZs (45). 6-Benzylamino-β-CCM (26) resembles flumazenil in mouse PTZ assays. Remarkably, 27 and 28 also possess potent BZ receptor affinity, with 28 being a partial inverse agonist (46). New indolo-fused analogs 29 and 30 are partial inverse agonists in proconvulsant and DZ-antagonism PTZ assays (mice, ip), while 31 is a pure antagonist (47). The role of GABA (48) and the behavioral effects of GABA agonists (49) on BZ receptor anxiolysis were reviewed. The antianxiety potential of a "brain delivery system" for GABA was reported (50).



	R^1	R^2	R^3	R^4
20	H	H	H	COOMe
21	H	H	H	CONHMe
22	H	H	H	NHCOOMe
23	H	OBn	CH_2OMe	COOEt
24	OBn	H	CH_2OMe	COOEt
25	H	O-i-Pr	CH_2OMe	COOEt
26	NHBn	H	H	COOMe
27	NHBn	H	H	CH_2OH
28	NHBn	H	H	H

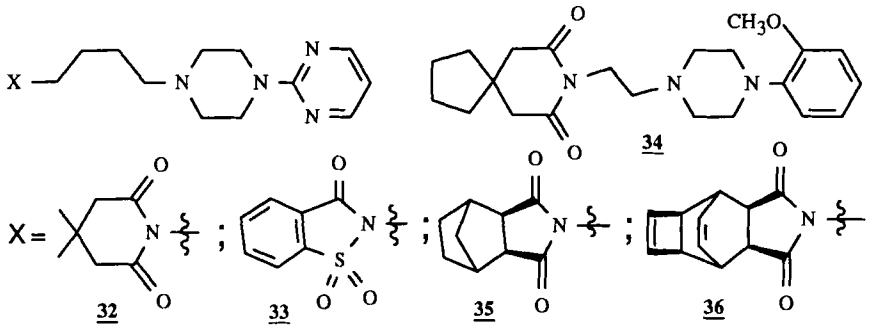


	X
29	H
30	OMe
31	Cl

5-HT Receptor Mediated Anxiolysis - The mechanism of action of the putative 5-HT_{1A} receptor associated antianxiety agents remains unresolved. In *in vitro* studies in rat hippocampal (5-HT_{1A}-rich CA1 region) slices, buspirone exhibited effects suggesting postsynaptic partial agonism at 5-HT_{1A} receptors (51). Subsequent detailed analysis of field potential effects in these slices using subtype specific agents, indicated that 5-HT and the 5-HT_{1A}-selective agents act via independent receptor subpopulations. Combined with radioligand binding data, considerable doubt has been cast as to whether the physiological effects of these agents in the hippocampus are truly 5-HT_{1A} dependent (52). Rat behavioral studies in the elevated plus-maze, failed to provide consistent, dose related effects supportive of a major role for any specific 5-HT subtype (53). Reported animal model studies include 8-OH-DPAT discrimination in rats (54), buspirone discrimination in the pigeon (55), inhibition of isolation-induced intraspecies aggression in mice (56), and stimulation of saline intake in rats (20), which also detects BZ receptor agents.

5-HT Receptor Related Compounds - Clinical evidence supporting the relative safety of buspirone (1) versus BZs is accumulating. The combination of buspirone and DZ produced no further decrement in DZ-induced psychomotor deficit in healthy subjects

(57). Buspirone fails to show an interaction with ethanol or BZ-hypnotics supporting the potential for BZ combination therapy (58). In addition, memory deficit was absent in placebo-controlled study with DZ (59), sexual function normalized in anxious patients with dysfunction (60), BZ withdrawal could not be alleviated (61), and no interaction was observed with cimetidine (62). The lack of significance of D_2 receptor blockade is supported by unaffected serum prolactin levels at anxiolytic doses (63). Gepirone (BMY



13805, 32) appeared promising in an uncontrolled pilot study for anxiety (64). The biological profile of buspirone, gepirone and ipsapirone (TVXQ 7821, 33) were reviewed (65, 66). Buspirone (but not gepirone) attenuated learned helplessness in rats (67). Gepirone, which lacks the dopaminergic effects of buspirone, appears equipotent to buspirone in inhibiting mouse aggressive behavior but is a weaker postsynaptic 5-HT agonist, acting primarily via reduction of 5-HT neurotransmission (68). Studies with ipsapirone confirm the negative coupling of hippocampal 5-HT_{1A} receptors with adenylate cyclase (69). BMY 7378 (34), with its high affinity but low intrinsic activity in rat and guinea pig hippocampal membranes, should be a promising tool for elucidating 5-HT_{1A} receptor function (70). It is a partial agonist in rat dorsal raphe nucleus (71). The biological profile of SM-3997 (35) includes potent inhibition of 5-HT neurotransmission with minimal dopaminergic effects (72). Similarly, WY-47,846 (36) has weak D_2 effects with potent binding (*in vitro* and *ex vivo*) and dorsal raphe firing inhibition (73).

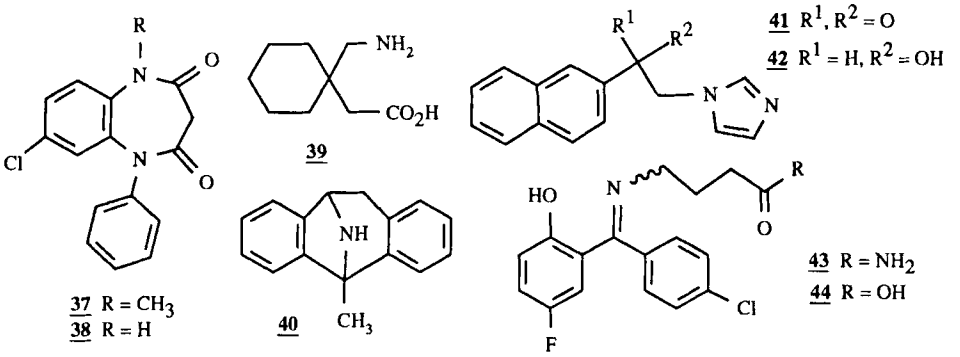
ANTICONVULSANTS

Introduction - Estimates suggest that epilepsy affects between 0.5 and 2% of the world's population (74). For the available anticonvulsant (AC) agents, a surprising lack of efficacy for many seizure types and/or tolerance development leads to polytherapy and/or increased dosage regimens, often with the emergence of undesirable side effects or toxicity (75, 76). Improved ACs are clearly required and, with poor prospects for non-pharmacologic therapeutic advances, the development of new drugs offers the most reasonable hope for patients with uncontrolled seizures (75). Since both the primary pathology of epilepsy and the precise mechanisms by which the available ACs act are not well understood, the development of new agents remains a challenging problem.

With respect to both epilepsy and excitatory amino acids, abstracts of various meetings (77, 78) and a symposium (79) appeared, and several books (75, 80-84) were published. Numerous reviews describing the mechanisms of AC drug action with respect to generalized tonic-clonic and partial epilepsies (85) and absence epilepsy (86), the neurophysiological and neurochemical fundamentals of the mechanism of antiepileptic drug action (87), excitatory amino acids and mammalian CNS function (88), a focus on the N-methyl-D-asparate (NMDA) receptor (89) and protection against hypoxic/ischemic brain damage with excitatory amino acid antagonists (90) also appeared. A model of the NMDA receptor channel complex was proposed (91).

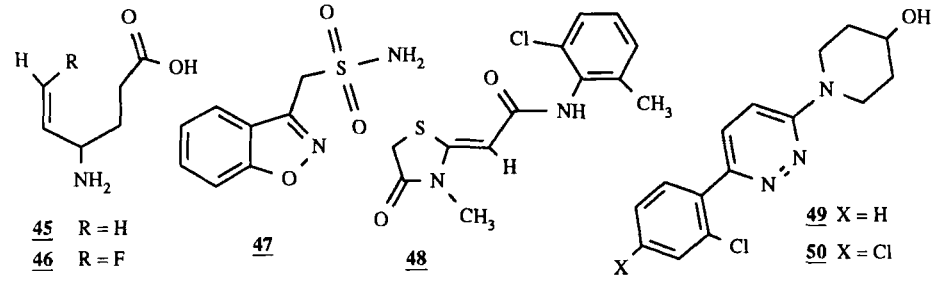
Highlights - Compounds Under Clinical Investigation - The majority of the compounds under clinical evaluation (clobazam, denzimol, eterobarb, felbamate, flunarizine, flupirtine, gabapentin, lamotrigine, milacemide, MK-801, nafimidone, Org 6370,

oxcarbazepine, progabide, stiripentol, vigabatrin and zonisamide) have been reviewed in depth as a series of collected monographs (75). Tolerance develops in a signficant proportion of patients treated with clobazam (37); however, animal studies, along with some clinical evidence, suggests that the active N-desmethyl-metabolite 38 produces signficantly less tolerance (92). As add-on therapy, gabapentin (39) produced a dose-related antiepileptic effect in therapy-resistant patients with partial and generalized seizures, with no evidence of significant interaction with other ACs (93). Clinical trials of the non-competitive NMDA receptor antagonist MK-801 (40) in epileptic patients were suspended, presumably because oral administration did not achieve adequate plasma levels (94). However, 40 protected neonatal rats from hypoxic-ischemic brain damage (95) and the development of 40 as a neuroprotective agent for the prevention of hypoxic/ischemic brain injury of fetal or other origins is under consideration (94, 95).



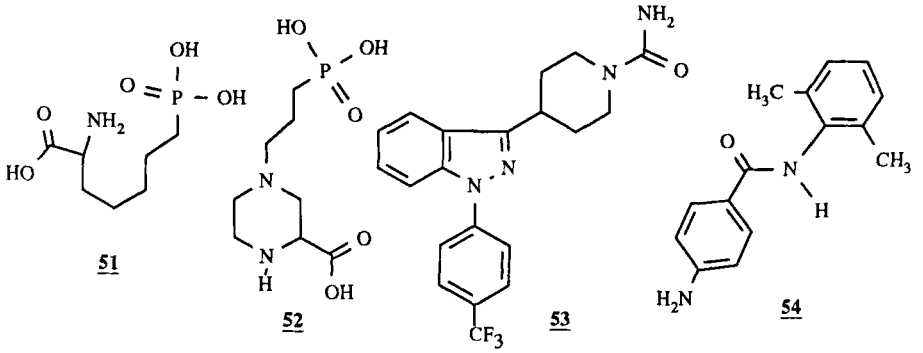
Nafimidone (41) is metabolized to the alcohol 42, which was suggested to be the pharmacologically more important species (96). Progabide (43), designed to deliver GABA across the blood brain barrier and recently marketed in France (97), is metabolized to the acid SL 75102 (44) at a much faster rate than any other changes (98). Both 43 and 44 display intrinsic GABA-mimetic activity, with 44 being about 50 times more potent than 43 in displacing [^{3}H]GABA from human cerebellum binding sites (98). In a long term multicenter trial, add-on 43 produced more than a 50% reduction in seizure frequency in 71% of drug-resistant epileptic patients, with only mild, transient side effects (99). However, 43 may interact with phenytoin, carbamazepine and phenobarbital, and adjustments in the dosage of these ACs may be required with add-on 43 (100). Vigabatrin (45), a selective enzyme-activated irreversible inhibitor of GABA-transaminase, is an efficacious, well tolerated AC in therapy-resistant patients with complex partial seizures (101, 102); it is also effective in reducing dyskinetic symptoms associated with tardive dyskinesia in drug-free schizophrenics (103). The occurrence of microvacuoles in brain tissue of rats and dogs treated with 45 has caused concern; however, these microvacuoles have not been observed in primates nor in autopsy samples from the brain of a patient (104) who received high doses of 45. A series of fluorinated analogues of 45 were prepared and the mono-fluorinated analogue 46 was as potent as 45 in elevating brain GABA levels, despite a lesser inhibition of GABA-transaminase *in vitro* (105). The general pharmacology (106, 107) and neuropharmacology (108) of zonisamide (47) were reviewed. In a trial with therapy-resistant epileptics, 47 was more effective against simple and complex partial seizures than generalized seizures (109). A review to clarify the incidence of fatal hepatatoxicity associated with the marketed AC valproic acid was published. The primary risk group was found to be children 0 to 2 years old, who were receiving valproate as polytherapy (110). In children with drug-resistant epilepsy, a medium chain triglyceride diet was partially successful in controlling seizures (111).

Preclinical Highlights - Several promising preclinical ACs (13, 23, 24, ralitoline (48), CM 40907 (49), SR 41378 (50) and 2-amino-7-phosphonoheptanoic acid (51)) were also reviewed in depth (75). CPP (52), a rigid analogue of the competitive NMDA antagonist 51, was proposed as the ligand of choice for *in vitro* evaluation of NMDA receptors due to its specificity and high affinity (112). In audiogenic DBA/2 mice, 52 was 15-fold more



45 R = H
46 R = F
47
48
49 X = H
50 X = Cl

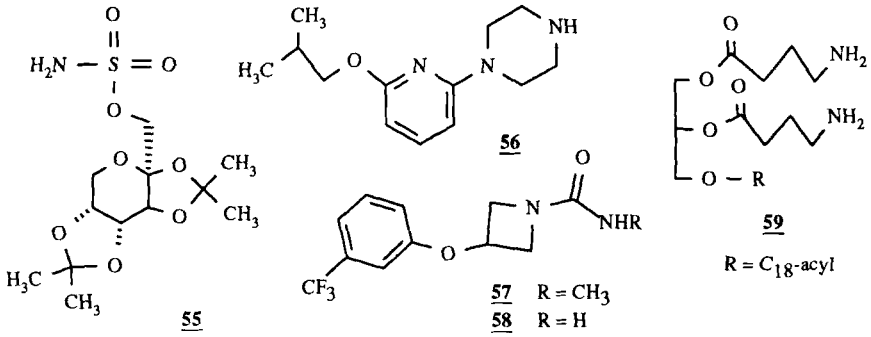
potent than **51** in preventing sound-induced seizures and was considered to be the most potent NMDA-receptor antagonist so far identified in this model (113). Because phencyclidine (PCP) is an NMDA antagonist, concern was expressed as to whether or not other NMDA antagonists such as **40** (non-competitive) and **51** (competitive) might exhibit undesirable PCP-like effects (74, 94, 114). Both PCP and **51** were effective ACs in the mouse maximal electroshock model (MES); however, in the locomotor activity model, PCP greatly stimulated locomotion whereas **51** was without effect. The results suggest that not all of PCP's effects are mediated by NMDA receptor antagonism (114).



51
52
53
54

HP 654 (**53**) was characterized as a non-toxic, long-acting AC with a preclinical profile suggesting utility for grand mal and absence seizures (115). LY201116 (**54**) was also selected from a series of compounds for development against generalized tonic-clonic and partial seizures (116). The sugar sulfamate topiramate (McN-4853, **55**) exhibited phenytoin-like activity in preclinical evaluation and was selected for clinical development (117). The 2,6-disubstituted pyridine **56** is active in MES, has a profile resembling phenytoin and was selected for more complete evaluation (118). Although clinical investigation of fluzinamide (**57**) was halted (119), the active metabolite AHR-11748 (**58**) may be under consideration for development. In genetically epileptic Mongolian gerbils, **58** is active against tonic-clonic seizures and has a carbamazepine-like profile (120), and is also active in the amygdaloid kindling model (119). A series of C-18 glyceryl lipid esters of GABA (**59**), with a varying degree of unsaturation in the C-18 chain, were prepared as GABA prodrugs (121).

Mechanisms of Anticonvulsant Action - Although much of the available literature pertains to either the facilitation of inhibitory GABAergic systems or the inhibition of excitatory amino acid neurotransmission, AC activity can be mediated by many mechanisms. Calcium-dependent mechanisms in neurons are affected by AC drugs and a number of studies imply that calcium plays an important role in epileptogenesis (122). Recent evidence suggests that diazepam and phenytoin can inhibit voltage-dependent calcium channels, which may contribute to their AC effects (123). Seizures induced in mice by the calcium channel agonist, BAY k 8644 were antagonized by several calcium channel blocking agents but not by standard ACs (122). Nimodipine suppressed PTZ-induced seizures when administered intra-arterially to anesthesized cats (124) and orally to rabbits (125). The reduction of seizure severity in audiogenic rats by metoprine, a



55 56 57 R = CH_3 58 R = H 59 R = C_{18}-acyl

histamine-N-methyltransferase inhibitor, led to the suggestion that histamine may be an endogenous AC (126). Essential trace metals copper (127) and zinc (128) may prevent the accumulation of convulsant concentrations of superoxide or related oxyradical products (Cu), or reduce postsynaptic NMDA receptor mediated excitation (Zn). Deficiencies of these metals may lead to convulsions or neuronal death. In addition to being an inhibitory neurotransmitter, glycine may facilitate excitatory neurotransmission by allosteric activation of the NMDA receptor (129). In certain seizure models, stimulation of central α_1- or α_2-adrenoceptors produces AC effects. Both St 587, a selective α_1-agonist, and clonidine, a selective α_2-agonist, afforded significant AC activity in kindled rats and epileptic gerbils, but not in MES-induced seizures (130). The ability of α_2-antagonists to block the α_2-agonist reversal of seizures induced by **6** suggests a modulatory role for α_2-adrenoceptors on central GABAergic function (131). Structure-activity relationships for the convulsant and AC barbiturates suggested that barbiturate-induced convulsant and AC activities are exerted at similar but conformationally distinct sites (132).

Seizure Models - The various seizure models have been reviewed (75). The kindling model (focal epilepsy) is well established and several variations are employed, including seizures induced by low frequency stimulation (133) and chemically-induced kindling (134) with the BZ receptor partial inverse agonist FG 7142 (**21**). Evidence was presented suggesting that the substantia nigra is involved in the development of amygdaloid kindled seizures in rats (135). *In vitro* application of tetanic electrical stimuli to the stratum radiatum pathway in the hippocampus suggests that the kindling stimulus produces a reduction of GABAergic inhibition, due to postsynaptic activation of NMDA receptors (136). The μ-opioid receptor, rather than the σ-receptor, is implicated in the limitation of amygdaloid kindled seizures (137). Pilocarpine administered to lithium-pretreated rats induces a status epilepticus, which models human generalized epilepsy (138). Results with seven BZ receptor ligands of the β-carboline class, including the partial inverse agonist **21**, the partial agonist **23** and the full agonist **24**, implicated the involvement of the GABA/BZ receptor complex in the control of seizures in a model of petit mal epilepsy, utilizing a genetically predisposed strain of Wistar rats (139).

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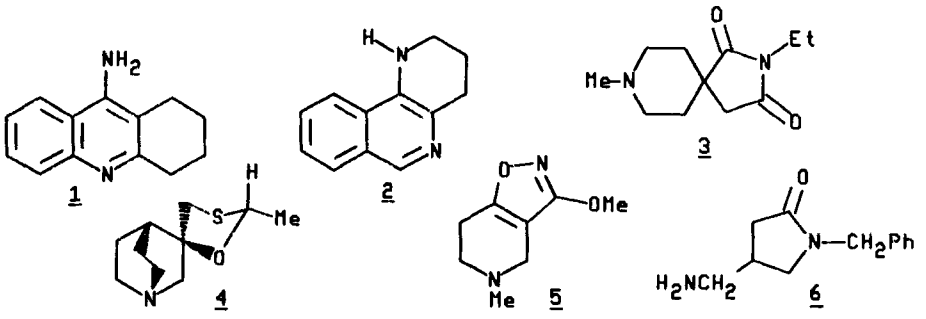
Chapter 4. Agents for the Treatment of Cognitive Disorders

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Bristol-Myers Co., Wallingford, CT 06492

Introduction - Cognitive dysfunction is the prominent symptomology associated with aging, stroke, and some dementias such as Alzheimer's Disease (AD). Memory mechanisms have many subclassifications that are mediated by a variety of neural processes (1,2). In a manner similar to the use of dopaminergics for the treatment of Parkinson's disease, one approach to the treatment of cognitive disorders targets a specific endogenous chemical system, whether a neurotransmitter, modulator, trophic factor, or hormone (3). Another approach attempts to alter global brain function. This chapter will review the progress in these areas since the last review in this series in 1986 (4).

Cholinergics - The cholinergic system has long been implicated in normal memory functioning. There is central cholinergic decline with age and severe loss of cholinergic neurons in AD (5,6). Whether cholinergic receptors are altered in AD is still debated; perhaps only a subset of AD patients have a decline in muscarinic and/or nicotinic binding (7). Attempts to enhance the central cholinergic system have been reviewed (8).

Treatment with cholinesterase inhibitors is assumed to be limited by the availability of released acetylcholine. Animal studies with the cholinesterase inhibitor physostigmine, showed mixed results depending on the species, behavioral task, and length of treatment (9-12), but clinical results have been generally disappointing (13,14). A large, multi-center clinical project was undertaken after a report of dramatic memory improvement in AD patients treated with lecithin and tacrine (1), a cholinesterase inhibitor with a longer half-life (15). It was temporarily halted after 8/40 patients treated with 1 showed liver abnormalities (16). In the guinea pig hippocampal slice preparation, 1 had a strong excitatory action that could not be attributed to anticholinesterase activity and may be due to blockade of potassium-associated conductance (17). An equally potent cholinesterase inhibitor, 2, also showed activity against scopolamine-induced amnesia (18).

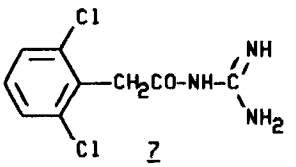


Muscarinic (M) agonists have shown mixed effects clinically. Oxotremorine resulted in depressive reactions, anxiety, and confusion, while RS-86 (3) showed clinically obvious improvement in only 2/12 AD patients

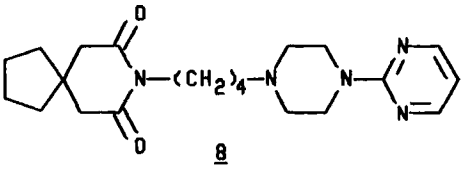
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(19,20). AF102B (4) was more selective for M_1 than M_2 receptors, and it was claimed to reverse cognitive deficits without peripheral side effects in cholinotoxin-treated mice (21). Arecoline reportedly improved scopolamine-disrupted retention in mice (22). Bioisoteres of norarecoline and arecoline, such as 5, have been reported to be active in anticonvulsant and antinociception assays, but not in assays more suggestive of cognition enhancement (23). Another M_1 agonist, WEB 1881 FU (6) showed evidence of efficacy in hypoxia tests with healthy volunteers, but not in scopolamine-induced amnesia (24). Meclofenoxate, a tertiary amine analog of acetylcholine, increased acetylcholine release and reversed electroconvulsive shock-induced (ECS) amnesia (25).

Monoamines - The influence of monoamines on learning and memory, and their dysfunction in dementias have been reviewed (8,26). In postmortem AD tissue, decreased concentrations of dopamine, norepinephrine (NE), serotonin (5-HT), and their metabolites have been reported (26). Low doses of a monoamine oxidase inhibitor, l-deprenyl, which should enhance central monoamines, resulted in statistical improvement in recall memory (27). Treatment with clonidine, an α_2-adrenergic agonist, improved memory in schizophrenics, but its use with AD patients led to unacceptable hypotension (28,29). Guanfacine 7, another α_2 agonist, gave favorable preliminary results in aged monkeys without extreme hypotension and sedation (29).



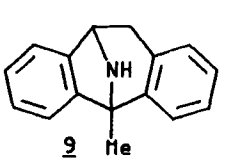
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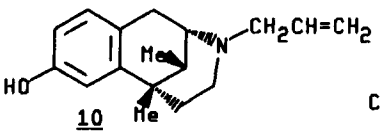
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The influence of 5-HT on learning and memory may be inhibitory, since serotonin antagonists (pirenperone, ketanserin, mianserin, methysergide and metergoline) enhanced the memory of previously learned behavior (30). 5-HT antagonists may also be useful in the reduction of damage and high mortality rate in rabbits after ischemia (31). Fluoxetine, a 5-HT reuptake blocker, enhanced the memory of avoidance tasks and reduced amnesia induced by scopolamine or anisomycin (32). The amelioration by minaprine of cycloheximide-induced amnesia was antagonized by the 5-HT releaser p-chloroamphetamine (33). Minaprine also reduced scopolamine-induced amnesia, improved memory after ischemia, and hastened the normalization and stabilization of theta waves in the hippocampus (34,35). Compared to diazepam or placebo in anxious patients, buspirone (8) tended to enhance immediate recall after repeated daily doses (36).

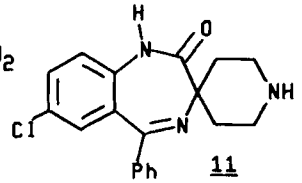
Amino Acids - Glutamate neurotoxicity has been implicated in cerebrovascular accidents, epilepsy, Huntington's disease, and AD (37,38). In AD, the distribution of diminished glutamate binding, particularly the N-methyl-D-aspartate (NMDA) receptor subtype, has been shown to correspond to the location of histopathology (38). NMDA applied to the cortex produced retrograde degeneration of the cholinergic neurons in the rat nucleus basalis (39). NMDA antagonists such as 2-amino-7-phosphonoheptanoic acid prevented cell death induced by glutamate, but did not protect against hypoxic or ischemic damage (40,41). 3,4-diaminopyridine, which promotes calcium influx into nerve terminals, reduces the potassium stimulated release of glutamate and dopamine during anoxia, and also improves the radial maze performance of senescent rats (42,43).



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The chemically diverse, noncompetitive NMDA agents have the advantage of lipophilicity (40). MK-801 (9), which blocks the NMDA receptor-associated ion channel, protected neurons against death caused by local injection of NMDA or quinolinic acid, but not kainate (39). In animals, it protects against focal or global ischemic damage whether administered before or after the occlusion, perhaps by blocking lethal cation entry or diminishing hyperexcitability (40,44). The noncompetitive NMDA antagonists, phencyclidine and its thienyl analog, TCP, demonstrated neuroprotection against focal ischemia (40). Apparently binding to the same site, dextrorphan and dextramethorphan lessened hypoxic damage without stereoselectivity and diminished glutamate neurotoxicity in cortical cell culture (45,46). N-allyl-N-normetazocine (SKF 10,047, 10) similarly blocked NMDA-induced neuronal toxicity (40). Long term potentiation (LTP), which apparently involves activation of the NMDA receptor, is believed to be associated with learning and memory formation (47,48). MK-801, phencyclidine, ketamine, and also NMDA competitive antagonists, blocked LPT in the rat hippocampal slice preparation (49,50). However, anti-convulsant doses of MK-801 did not affect spatial memory and long term potentiation, in vivo (51,52).

With agents acting at the GABA/benzodiazepine receptor complex, the extent of memory impairment seen may vary (53). Methyl β-carboline-3-carboxylate, a benzodiazepine inverse agonist, enhanced learning and memory in rat and chick (54). Some new benzodiazepines, such as 11, that do not bind to typical benzodiazepine receptors and are devoid of anxiolytic action, appeared to improve performance in learning and memory tasks in normal rats and those with hippocampal lesions (55).

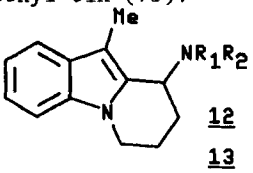
Peptides - Neuropeptidergic changes have been demonstrated in certain neurologic diseases (56). Vasopressin (VP), has been suggested to influence learning and memory, through heightened attention (57). In AD, VP concentrations are reduced in some brain regions and elevated in others (56). Reports of the cognition-enhancing ability of VP and analogs have been inconsistent (58,59). When memory enhancement has been shown in rodents, generally shorter fragments (e.g. Lys^{8}VP, Arg^{8}VP and their desglycinamide analogs) were more active than the parent molecule, and the dosing regimen was thought to be critical (60). However, in one study, not only did Arg^{8}VP fail to ameliorate an electroshock-induced deficit, it negatively affected the performance of the sham-treated controls (61). The clinical data with VP and ACTH have been hard to evaluate due to the differences in patient populations and drug regimens (62).

There may be a global somatostatin defect in AD that occurs beyond the genomic level since somatostatin-linked deficits are found in cortex and hippocampus, hypothalamohypophyseal levels, and pancreatic endocrine function (63). The entire sequence may be necessary for memory enhancement (64).

There may be a lack of specific trophic factors in AD (65). The overlapping anatomy of the nerve growth factor (NGF) system and AD lesions in the adult brain is a powerful observation to support such

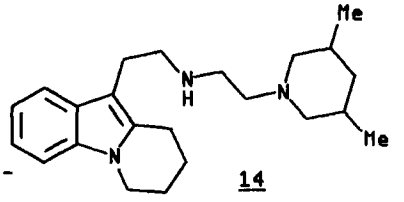
speculation (66,67). Continuous infusion of 7S NGF over a four-week period in aged rats partly reversed cholinergic cell atrophy and improved retention of a spatial memory task; two measures of memory acquisition in these animals showed no change (68). NGF increased the survival of axotomized cholinergic neurons, and the use of NGF infusion concomitant with tissue transplant has been examined in axotomized rats (67,69). Long term studies of NGF treatment in the rat have not always shown behavioral recovery (69).

Cerebrovasodilators - The use of indole alkaloids in cognitive disorders is based on their cerebrovascular effects, but such compounds may indeed have other effects as well. Treatment with ergoloid mesylates restored the choline acetyltransferase activity in the brains of aged rats and mice (70), but a clinical study of ergoloid mesylates in patients with mild memory impairment failed to indicate any unequivocal improvement (71). Vinpocetine blocked scopolamine induced amnesia, suggesting that this drug possesses direct or indirect cholinergic activity in addition to its vasodilating actions (72,73). A newer indole derivative, **12**, protected mice against anoxic death, while the corresponding pyrrolidine analog, **13**, was active against scopolamine induced amnesia (74). The substituted tryptamine, **14**, reduced the cerebral edema in rats caused by triethyl tin (75).



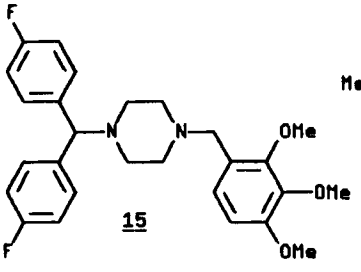
12 $R_1 = R_2 = -Me$

13 $R_1, R_2 = -(CH_2)_4-$

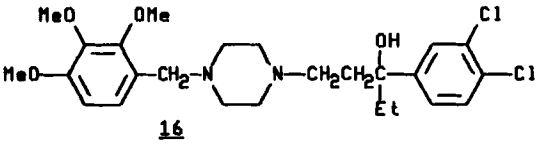


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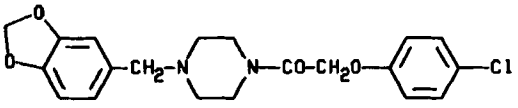
The vasodilator/calcium antagonists, exemplified by flunarizine, remain an approach to the treatment of multiple infarct dementia (76). KB-2796 (**15**) was found to protect mice against memory deficits induced by ECS and hypoxia (77). The QSAR of a series of cerebral vasodilators, of which **16** was the most active, has been discussed (78). Fipexide (**17**), caused some improvement over placebo in geriatric patients with severe cognitive disorders (79).



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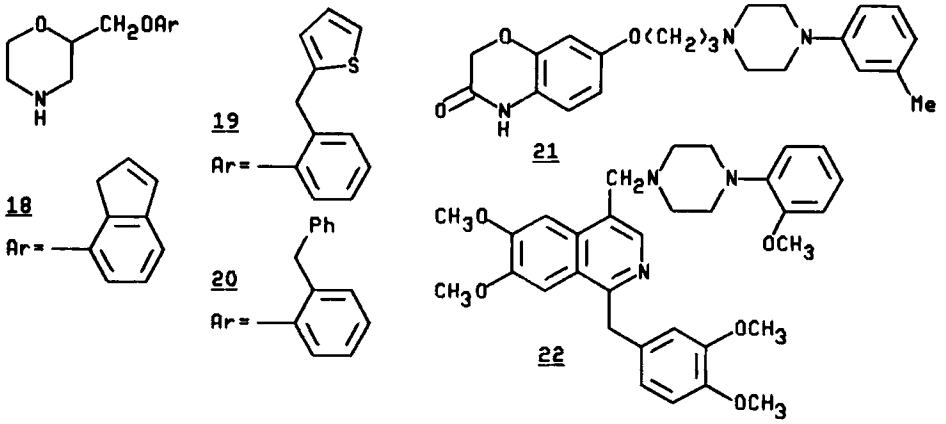


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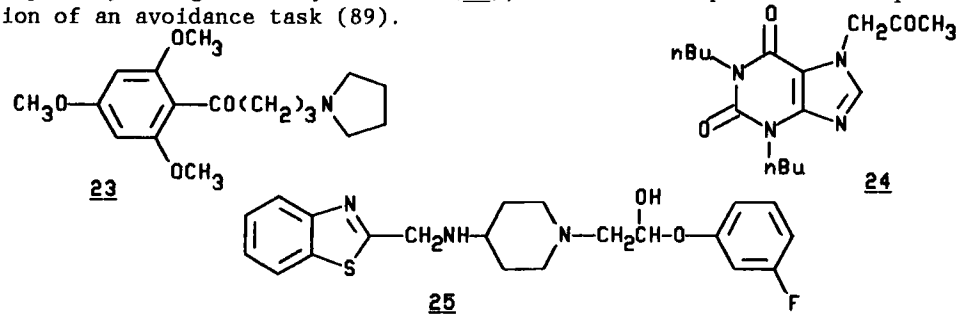


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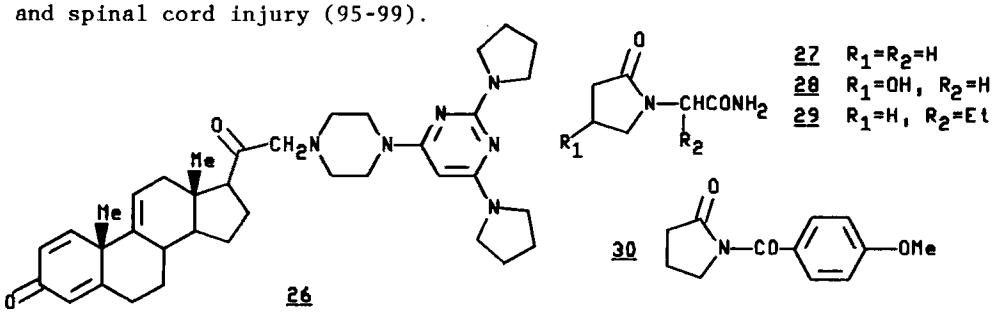
Indeloxazine (**18**) and sulfoxazine (**19**) demonstrated activity against hypoxia induced amnesia, although the related phenyl analog (**20**) did not (80-82). Mice treated with **21** survived over nine times longer than untreated controls under hypoxic conditions (83). In stroke prone SHR rats, chronic administration of Ro 22-4839, **22**, prevented the progress of stroke and cerebral edema, and shortened the acquisition time for new learning in a passive avoidance task (84,85). In patients with multi-infarct dementia, buflomedil (**23**), increased regional cerebral blood flow and scores on psychometric tests (86). Denbufylline, **24**, blocked triethyl tin induced cerebral edema in rats and increased retained learning in an



ischemic gerbil model (87). Forskolin, when injected directly after hypoxia, was effective in blocking memory deficits in a rat model, presumably via effects on cAMP levels (88). Hypoxia induced amnesia was completely antagonized by R 58735 (25), which also improved the acquisition of an avoidance task (89).

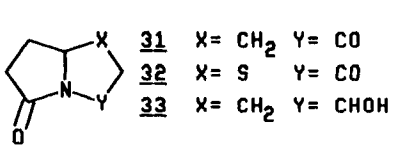


Steroids - Dehydroepiandrosterone increased neuronal survival and differentiation of mouse brain cells in culture, and also enhanced long term memory retention, and reversed amnesia in mice (90,91). Testosterone, in chicks, extended the duration of intermediate memory without affecting short term memory (92). A series of 21-aminosteroids (Lazaroids) was reported as being potent inhibitors of lipid peroxidation (93,94). Of these, U-74006F (26) attenuated post-ischemic and post-hemorrhagic cerebral hypoperfusion in cats, protected gerbils against post-ischemic mortality and neuronal necrosis, protected cats from lesion development following stroke, and enhanced neurological recovery in models of head and spinal cord injury (95-99).

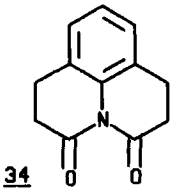


Nootropics - The differences between the nootropics and the psychostimulant and analeptics has been discussed (100). The prototypical nootropic, piracetam (27), combined with lecithin, continues to be tried in SDAT

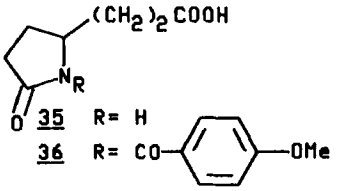
patients, and one model Phase III clinical study of piracetam in demented patients has demonstrated some degree of therapeutic efficacy (101,102). Oxiracetam, **28**, in combination with methamphetamine, significantly increased the avoidance responding in mice over the effects of either drug alone (103). Oxiracetam affected the resting EEG in healthy subjects, but failed to show significant effects in patients with organic brain syndrome (104,105). Etiracetam, **29**, was found to antagonize the amnesia produced by hemicholinium-3 administered intracerebroventricularly (106). Aniracetam, **30**, has been found to augment long term potentiation, a potential mechanism of memory, in hippocampal slices (47,107). Patients with SDAT have been treated chronically with aniracetam with no difference seen between drug and placebo groups (108).



31 X= CH_2 Y= CO
32 X= S Y= CO
33 X= CH_2 Y= CHOH

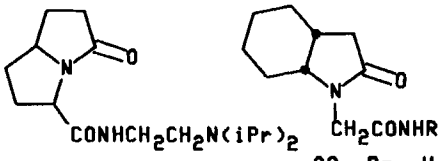


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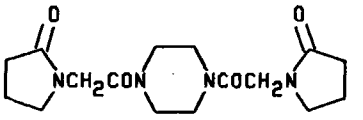
35 R= H
36 R= CO——OMe

In rolziracetam (CI-911, **31**), the 5.5 bicyclic system was reported to be optimal for activity against ECS induced amnesia and that replacement of C-1 with sulfur (**32**), substitution of methyl groups on the ring, or reduction of one of the carbonyl groups (**33**) lowered the activity (109). A benzotricyclic rolziracetam analog, **34**, was active against ECS induced amnesia (110). Rolziracetam undergoes a rapid hydrolysis *in vivo* to the corresponding amido-acid, **35**, which was also highly active against ECS induced amnesia (109,111). Incorporation of the methoxybenzoyl side chain of aniracetam into **35** resulted in CI-933 (**36**), which was more active against ECS induced amnesia (111). The effects of CI-933 on new learning, diazepam induced memory impairments, electrophysiology, brain glucose utilization, and neurochemistry have been reported along with its pharmacokinetics and bioavailability (112-119).

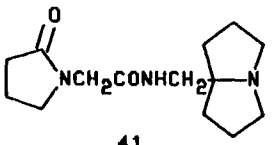


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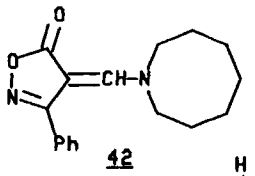
38 R= -H
39 R= $-CH_2CH_2N(iPr)_2$



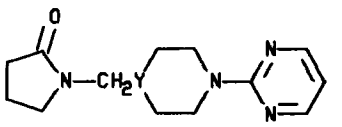
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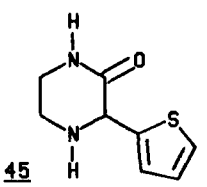
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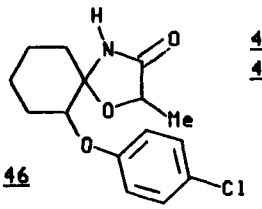
42



43 Y= N
44 Y= CH



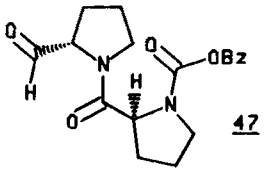
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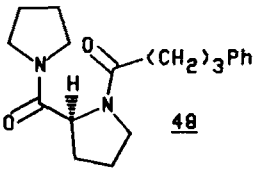
46

A pramiracetam analog, **37**, was recently reported to possess activity against ECS induced amnesia (120). While the piracetam analog, **38**, demonstrated activity against ECS induced amnesia, the corresponding pramiracetam analog, **39**, was less active (121). An analog of pramiracetam, **40**,

has been reported active against ECS induced amnesia, and a pramiracetam analog, **41**, was reported to be active in two models of ischemia (122, 123). An isoxazolone, **42**, demonstrated activity against scopolamine induced amnesia and ECS induced amnesia (124). The piperazine, (**43**) and the related piperidine, **44**, both protected against ECS induced amnesia (125,126). The effects of tenilsetam (CAS 997, **45**) on the EEG in rats has been reported, and in the human the effects are similar to, but more pronounced than, those of piracetam (127,128). CERM 3726, **46**, selectively increased glucose metabolism in the cortex and its behavioral profile resembles that of piracetam (129), while piracetam displayed a more generalized effect upon glucose metabolism (130).



47



48

Prolyl Endopeptidase Inhibitors - Mammalian prolyl endopeptidase (PEP), an enzyme which cleaves peptides at the carboxy side of proline residues, has been identified in hippocampus, striatum, and cortex (131). Nootropics such as aniracetam and pramiracetam have been found to inhibit brain PEP, although with low potency (131). Conversely, Z-prolyl-prolinal (**47**), a potent PEP inhibitor, enhanced new learning and reversed scopolamine induced amnesia in mice (132). SUAM 1221, (**48**), one of a new series of acyl amino acid derivatives, was potent both in *in vitro* PEP inhibition and *in vivo* reversal of scopolamine induced amnesia in rats (133). The PEP inhibitors, and perhaps some nootropics, may be affecting memory by prolonging the lifetime of endogenous neuropeptides critical to the cognitive process.

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Chapter 5. Central Nervous System Actions of Adenosine Agonists and Antagonists

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Introduction - Adenosine is an endogenous purine which, in addition to roles in intermediary metabolism, may exert neuromodulatory actions (for reviews, see 1-6). Adenosine agonists, while not presently used as drugs, have marked and potent sedative-like effects. In addition, the subjective stimulant effects of adenosine antagonists such as caffeine and theophylline are well known. The purpose of this review is to summarize the progress in understanding of the CNS actions and roles of adenosine since the last review of this subject in this series (7).

Adenosine Receptors - Two subtypes of extracellular adenosine receptors, A_1 and A_2, have been identified on the basis of tissue distribution, structure activity relationships (SAR), and physiological effects (8). Adenosine receptors have been distinguished classically by their opposing influences on adenylate cyclase, A_1 receptors mediating inhibition and A_2 receptors mediating stimulation of the enzyme (8). However, recent evidence suggests that second messenger systems other than adenylate cyclase may be involved in the actions of adenosine. For instance, A_1 receptor activation appears to inhibit neurotransmitter release by blocking the uptake or intracellular action of calcium in a cyclic AMP-independent manner (9-11). Effects of adenosine on the phosphatidylinositol system have also been reported (12). Such findings suggest that receptor classification based on SAR may provide a more consistent method for identification of adenosine receptors (8,13). A_2 receptors have been recently divided into high and low affinity subtypes, A_{2a} and A_{2b}, but evidence for distinct roles and characteristics of these proposed subtypes is limited (14,15).

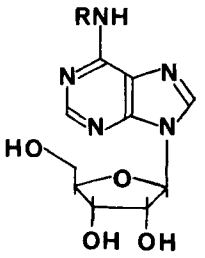
In addition to acting through the extracellular adenosine receptors described above, adenosine inhibits adenylate cyclase via an intracellular "P site" that appears to be located on the catalytic subunit of adenylate cyclase (16). Adenosine concentrations in the high micromolar range are required for agonist actions at the P site, and the role of this site is unknown. Although the P site will not be discussed further in this review, it appears to be an intriguing and largely unexploited target for new drugs.

The Physiological Role of Adenosine - The signal for adenosine production is probably intracellular AMP, produced from ATP in tissues under hypoxic stress (17,18). Large increases in extracellular adenosine can be detected during periods of increased neuronal activity or decreased oxygen supply (19). Free extracellular adenosine has dramatic antihypoxic effects in a wide variety of tissues (5), including the heart, vascular system, kidneys, and the CNS, where it may have a rather general tonic inhibitory effect (4,20). The result of A_1 receptor activation is generally to decrease tissue oxygen demand, whereas A_2 activation generally increases oxygen supply (13).

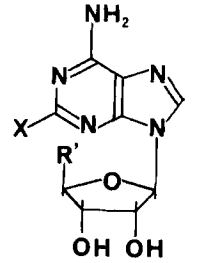
Adenosine Agonists - Most of the information available on the pharmacological actions of adenosines has been generated with a rather small number of stable adenosine analogs whose structures are shown on the following page along with their binding affinities (K_i, nM) at the A_1 and A_{2a} receptors (15). The affinity of adenosine cannot be measured directly because of its rapid degradation by adenosine deaminase, a necessary additive in adenosine receptor binding assays (15). Among agonists, N^6-[(*R*)-1-methyl-2-phenethyl]adenosine (*R*-PIA, **1**), N^6-cyclohexyladenosine (CHA, **2**), and N^6-

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cyclopentyladenosine (CPA, 3) have potent affinities for the A_1 receptor, with at least 100-fold lower affinities for the A_{2a} receptor. The lesser affinity of *S*-PIA (4), the N^6 diastereoisomer of 1, has been used to derive a model of the N^6-binding domain of the A_1 and A_2 receptors (21). 2-Chloroadenosine (2-CLA, 5) displays moderate affinity for both A_1 and A_2 sites and its receptor binding profile is believed to be very close to that of adenosine itself (15). NECA (6) is a potent agonist with high affinity for both A_1 and A_2 sites, while 2-(phenylamino)adenosine (CV-1808, 7) is a moderately potent agonist with 5-fold selectivity for the A_2 receptor over the A_1 receptor (15). The original structure-activity definition of adenosine receptors specifies a potency order *R*-PIA > NECA >> *S*-PIA for A_1 receptors, and NECA > *R*-PIA > *S*-PIA for A_2 receptors (22). However, these criteria can sometimes result in ambiguities, and alternative criteria have been proposed (13,23). The most useful may be a comparison between CPA and N^6-benzyladenosine (BZA, 8), two compounds that are closely matched in physicochemical properties: CPA >> BZA for A_1 receptors, and CPA = BZA for A_2 receptors (13).

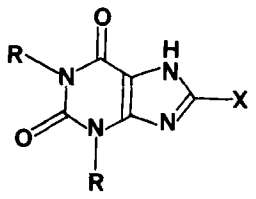


		K_i (nM)	
		A_1	A_2
1	R = (*R*)-$PhCH_2CH(CH_3)$-	1.17	124
2	R = c-C_6H_{11}	1.31	510
3	R = c-C_5H_9	0.59	460
4	R = (*S*)-$PhCH_2CH(CH_3)$-	49	1820
8	R = $PhCH_2$-	120	280

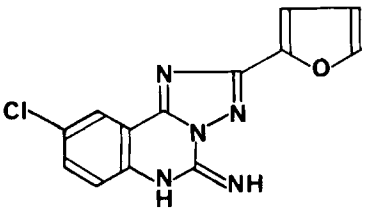


			K_i (nM)	
			A_1	A_2
5	R' = CH_2OH	X = Cl	9.3	63
6	R' = CONHEt	X = H	6.3	10.3
7	R' = CH_2OH	X = NHPh	560	119

Adenosine Antagonists - Adenosine antagonists are also useful tools for the characterization of the roles of adenosine. Although the common xanthines caffeine and theophylline show good solubility and pharmacokinetics, their affinities for A_1 and A_2 receptors are quite weak, and other actions such as phosphodiesterase inhibition and effects on intracellular calcium occur at concentrations only about 10-fold higher than those that block adenosine (1). For this reason, more potent adenosine antagonists have been sought. The first such antagonist, 8-phenyltheophylline (8-PT, 9), showed an excellent separation between adenosine antagonism and phosphodiesterase inhibition, but its poor aqueous solubility made it difficult to use *in vivo* (24). Recent chemical efforts have focused on gaining improved solubility and finding antagonists with selectivity for adenosine receptor subtypes. Several useful antagonists have resulted from this work. 8-Cyclopentyltheophylline (CPT, 10) and its 1,3-dipropyl homolog (CPX, 11) show high potency and selectivity for the A_1 receptor and possess high solubility/affinity ratios (15,25-28). XAC (12) shows good solubility and potent affinity for both A_1 and A_2 receptors (29). PD 115,199 (13) has good A_2 affinity and moderate solubility (30). Non-xanthine adenosine antagonists (for instance, CGS 15943, 14, one of the few A_2-selective antagonists) have also been reported (31). Compounds 10-14 all show antagonist activity on parenteral administration, and 10 and 14 are active orally (25,32).



			K_i (nM)	
			A_1	A_2
9	$R = CH_3$	X = Ph	86	850
10	$R = CH_3$	X = c-C_5H_9	10.9	1440
11	$R = C_3H_7$	X = c-C_5H_9	0.46	340
12	$R = C_3H_7$	X = *p*-$PhOCH_2CO$ -$NHCH_2NH_2$	0.86	27
13	$R = C_3H_7$	X = *p*-$PHSO_2NMe$ -$CH_2CH_2NMe_2$	13.9	15.5



	K_i (nM)	
	A_1	A_2
14	20.3	3.0

Neurochemical and Electrophysiological Effects of Adenosine Agonists and Antagonists

Adenosine's role in the CNS differs qualitatively from the roles of the classical neurotransmitters. Classical neurotransmitters such as acetylcholine are synthesized continuously, stored in vesicles, and released at specific synapses to act locally. In contrast, adenosine acts as a local hormone. It is produced on demand, is probably not stored in vesicles, and is likely to diffuse throughout the local tissue, acting at some distance from its site of production. A_1 receptors are located at extrasynaptic sites on dendritic membranes as well as at synaptic complexes (33). In many cases the information flow of the adenosine system may be retrograde, i.e., adenosine may be produced by post-synaptic cells in response to increases in cellular electrical activity and may act to inhibit neurotransmitter release from incoming excitatory pathways.

Distribution of Adenosine Receptors - The distribution of adenosine A_1 and A_2 receptors in the brain provides some clues about their potential roles. A_1 receptors are widely distributed, with high levels of [^{3}H]CHA binding reported in the human hippocampus, cerebral cortex, and striatum (34). In the rat, A_1 receptors often appear to be located on excitatory nerve terminals (35-36). Inhibition of the release of excitatory neurotransmitters would be consistent with the general role of A_1 receptors in reducing oxygen demand. However, A_1 receptors are located on both pre- and post-synaptic structures in the striatum and other regions (35,37).

In contrast to the A_1 receptor, the high-affinity A_2 receptor (A_{2a}) has a narrow anatomical distribution, with high levels of [^{3}H]NECA binding and A_2-stimulated adenylate cyclase observed mainly in areas that are highly innervated by dopamine such as the striatum, nucleus accumbens, and olfactory tubercle (15,37-39). Low levels of binding are also detected in the cerebral cortex and spinal cord (15,40). The apparent codistribution of A_{2a} and dopamine receptors is especially intriguing in light of the fact that the behavioral effects of A_{2a} receptor activation are largely opposite to those of dopamine agonists (see below), implying that the A_{2a} system may in some way act as a counterbalance to the dopamine system (41).

Stimulation of cyclic AMP accumulation by adenosine in brain slices, an effect mediated by the low-affinity A_2 or A_{2b} receptor, occurs in virtually all areas of grey matter (14). The role of this receptor is presently unknown.

Adenosine deaminase immunoreactivity is localized to discrete neurons in the brains of several species. Areas with populations of adenosine deaminase-positive neurons or glia include hypothalamus, habenula, hippocampus, and spinal cord (42). Whether these cells

constitute a specific population of adenosine-using or adenosine-disposing cells is unclear at present. Immunoactivity for adenosine itself has been localized to specific neurons in the brain and retina (43).

Biochemical Responses to Adenosine - In addition to its effects on the cyclic AMP second messenger system, adenosine also interferes with the calcium second messenger system and inhibits neurotransmitter release, two effects that appear to be closely related and will be discussed together. Neurotransmitters whose release is inhibited by adenosine include norepinephrine, dopamine, serotonin, acetylcholine, glutamate, and probably many peptide neurotransmitters as well (44-47). In contradiction to the initial idea that the role of adenosine would be to inhibit the release of excitatory transmitters, many of the above transmitters can have inhibitory effects on neuronal activity, and in fact the release of the inhibitory neurotransmitter GABA is also inhibited by adenosine (48). In all cases studied to date, the inhibition of release is mediated by an A_1 receptor (49-51), which acts through an inhibitory guanine nucleotide binding protein (44,52). However, despite the fact that the A_1 receptor is known to inhibit adenylate cyclase activity, the inhibition of transmitter release by adenosine is cyclic AMP-independent, since it cannot be reversed by cyclic AMP analogs or forskolin (9). Instead, a great deal of evidence points to the conclusion that adenosine inhibits release by interfering directly with the calcium system. Whether the effect of adenosine is due to inhibition of calcium uptake or interference with calcium's intracellular site of action is still a subject of controversy (10,11). However, adenosine can inhibit calcium currents in the dorsal root ganglion, and this inhibition is direct and not dependent on (for example) hyperpolarization due to opening of potassium channels (53).

Several neurochemical effects of adenosine provide evidence for inhibition of neurotransmitter release *in vivo*. Intraventricular (ICV) administration of 2-CLA and other adenosine analogs causes an inhibition of acetylcholine turnover (as much as 79% in the case of *R*-PIA), an inhibition of high-affinity choline uptake, and an increase in acetylcholine content (7,54). All of these responses are logical consequences of reduced release and hence reduced loss of acetylcholine from presynaptic stores. Intraperitoneal (IP) administration of *R*-PIA and NECA at behaviorally effective doses caused decreases in striatal dopamine release and synthesis (55).

Electrophysiological Effects of Adenosine - Adenosine's effects on electrical activity in the CNS are almost without exception inhibitory. Adenosine depresses the firing of several types of brain neurons, including cerebellar Purkinje cells, cerebral cortical cells, and cells in the locus coeruleus (56,57). The electrically-evoked excitatory postsynaptic potential (EPSP) on CA_1 pyramidal cells in rat hippocampal slices is often used as a model to study adenosine's electrophysiological effects. Although adenosine inhibits transmission by both pre- and postsynaptic actions in this preparation, the presynaptic actions of adenosine occur at lower doses, implying that these actions may be more important (58). In addition, adenosine depressed synaptic excitation to a greater extent than it depressed the excitation caused by iontophoretically applied glutamate, also implying that the presynaptic actions of adenosine may predominate (59).

The postsynaptic effects of adenosine on CA_1 cells have been studied by intracellular recording. Adenosine caused a 7 mV hyperpolarization, which remained when synaptic activity was blocked (60). The actions of adenosine had a reversal potential of around -90 mV (60), and were blocked by 4-aminopyridine but not by tetraethylammonium, implying that the hyperpolarization was caused by activation of the potassium A-current (61). An adenosine-activated potassium conductance was also seen in striatal cells in culture (62). In contrast to these studies, adenosine inhibited potassium currents in rat dorsal root ganglion cells (52). However, at least part of the effect was indirect, being attributable to reduction of a calcium-dependent potassium current, which in turn resulted from an inhibition of calcium influx (52). A calcium-dependent chloride current was also inhibited in these cells. In CA_1 pyramidal cells, adenosine blocked calcium fluxes caused by activation of N-methyl-D-aspartate (NMDA) receptors (58).

Like its presynaptic effects, the postsynaptic effects of adenosine in CA_1 cells appear to be mediated by an A_1 receptor (58). The receptor subtype involved in the postsynaptic effects of adenosine in striatal cells and dorsal root ganglion cells has not been determined; however, the effects of adenosine in both of these cell types require intracellular GTP, indicating that a guanine nucleotide binding protein is involved (52,62).

Effects of Adenosine Antagonists and Adenosine Deaminase - Experiments with adenosine antagonists and adenosine deaminase provide evidence for roles of endogenous adenosine in the CNS. 8-PT and adenosine deaminase both increased the release of acetylcholine, norepinephrine, and serotonin from hippocampal slices, implying that endogenous adenosine tonically inhibits the release of these neurotransmitters (45-47). In hippocampal cells in culture, 8-PT increased the number and size of spontaneous EPSPs and caused bursting activity in some cells (63-65). 8-PT also increased the spontaneous and evoked release of [^{3}H]glutamate from cerebellar granule cells in culture (65). At the mouse neuromuscular junction, 8-PT increased the quantal content of end-plate potentials (66). Microinjection of 2-CLA inhibited striatal release of excitatory amino acids and dopamine measured by *in situ* voltammetry, whereas adenosine deaminase increased release of both classes of neurotransmitter (67).

Since endogenous adenosine inhibits the release of excitatory neurotransmitters, it should also inhibit electrical activity. This has been confirmed in several preparations. When several xanthines were tested for increases in hippocampal excitability, the rank order of potency was the same as for blockade of adenosine's depression of electrical activity and blockade of adenosine-stimulated cyclic AMP accumulation. 8-PT was the most potent excitant in this study (63). 8-PT also enhanced the electrically-evoked EPSP in rat hippocampal slices (64). The adenosine uptake blocker dipyridamole, a potentiator of adenosine, inhibited EPSPs in the rat olfactory cortex, and this effect was blocked by theophylline (68).

Chronic treatment of animals with caffeine or theophylline increases A_1 receptor density in several brain regions, including mouse whole brain, cerebellum, brainstem, and cerebral cortex, as well as rat cerebral cortex and cerebellum (69-73). However, no increase was seen in rat hippocampus or diencephalon. The upregulation of A_1 receptors by adenosine antagonists provides evidence that endogenous adenosine concentrations *in vivo* are high enough to cause some degree of A_1 receptor downregulation, which can be blocked by the antagonists. This supports the proposition that adenosine has a tonic neuromodulatory role in the living animal.

Behavioral Effects of Adenosine Agonists and Antagonists

Sedative-hypnotic, anticonvulsant, and analgesic effects of adenosine agonists are known from the older literature (2-4,6,7). Recent studies have identified new behavioral effects of adenosine agonists and have characterized the receptor subtypes that mediate the effects of adenosine.

Analgesia Produced by Adenosine Agonists - NECA administered intrathecally to mice prolongs response latencies in the tail flick and hot-plate tests at doses that do not alter locomotor behavior (74,75). The reported order of potency for intrathecally-induced analgesia in tail-flick and hot-plate tests in mice is NECA > *R*-PIA > 2-CLA > *S*-PIA > adenosine (76).

Effects of Adenosine Agonists on Sleep and Wakefulness - *R*-PIA administered IP has been reported to decrease waking and increase slow-wave sleep in rats (77). However, EEG changes characteristic of sleep are not always observed following adenosine agonist administration (78). Such findings, coupled with the fact that adenosine agonists do not decrease the latency to sleep, suggest a distinction between a quiet waking state produced by adenosine agonists and the more classical effects of benzodiazepine or barbiturate hypnotics. R-PIA reduces the minimum anesthetic dose of halothane by 50% in rats (79).

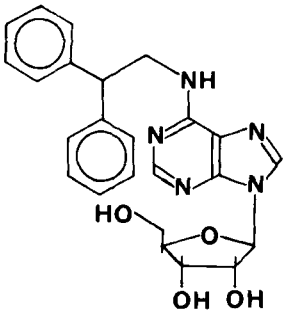
Agonist Effects on Locomotor Activity - Adenosine agonists decrease spontaneous motor behavior in rodents. Adenosine agonists administered ICV produce dose-related inhibition of spontaneous locomotor activity in mice and rats with the potency order NECA > *R*-PIA > *S*-PIA, and these effects are antagonized by the adenosine antagonist caffeine (80-82). Low doses of adenosine agonists administered systemically produce dose-related inhibition of spontaneous locomotor activity in mice, effects that are not accompanied by ataxia and that are blocked by methylxanthine adenosine antagonists, whereas high doses produce profound sedation in which animals are immobile or ataxic but retain the ability to respond to external stimuli (83,84). Although adenosine agonists cause hypothermia, their locomotor inhibitory effects are not reduced by maintaining animals in a warm environment (85).

Unilateral injections of NECA into the striatum of rats had no effect on turning behavior when given alone, but caused turning towards the side of the injection when apomorphine was given subcutaneously (86). NECA was more potent than *R*-PIA, suggesting that this effect was A_2-mediated. Since a similar response to apomorphine is seen with unilateral lesioning of the striatum, these results support a role for the striatal A_2 receptor in supressing the striatal dopamine system. NECA and CLA prevented the self-mutilatory behavior caused by SKF-38393 (dopamine D_1 agonist) and L-DOPA in rats with neonatal 6-hydroxydopamine lesions (87); the order of potencies of different adenosine agonists suggested that an A_2 receptor was involved.

Agonist Effects on Conditioned Behavior - Adenosine agonists administered systemically cause dose-dependent decreases in conditioned behavior. Rats that have been trained to depress a lever to obtain food show decreased responding when adenosine agonists are administered systemically (88-91). These effects are selectively antagonized by methylxanthine adenosine antagonists at doses that do not alter responding when given alone. Caffeine-induced stimulation of responding in rats performing conditioned behavior is blocked by low doses of *R*-PIA (92). Rats trained to depress one lever to obtain food when injected with *R*-PIA and a different lever when injected with saline respond on the *R*-PIA lever when injected with 2-CLA, but lose the ability to discriminate when caffeine is administered in conjunction with *R*-PIA (89,93). Adenosine agonists also reduce conditioned behavior of squirrel and cynomologus monkeys to terminate electric shock (94). The inhibition of conditioned responding by adenosine agonists is seen at doses that do not alter blood pressure. Potency for inhibition of shock-termination responding after IV administration of adenosine agonists in squirrel monkeys is reported to be correlated with affinity for A_2 but not A_1 adenosine receptors (95). *R*-PIA reduces the response-rate increases produced by both caffeine and amphetamine in squirrel monkeys (96). *R*-PIA also impairs the acquisition of a classically-conditioned nictitating membrane response in the rabbit and increases the stimulus threshold necessary for a response in conditioned animals (97).

Antipsychotic-Like Effects of Adenosine Agonists - Because the profile of adenosine agonists in the above-mentioned behavioral tests shares common features with antipsychotic drugs, adenosine agonists have been evaluated in a number of behavioral tests commonly used to identify such agents. Consistent with their lack of brain dopamine antagonist effects, adenosine agonists are weak blockers of apomorphine-induced cage climbing in mice relative to their potency for inhibiting spontaneous locomotion (98). NECA and CV-1808, adenosine agonists with appreciable affinity for A_2 receptors, reduce amphetamine hyperactivity in mice at doses that do not affect motor coordination (a profile also shared by antipsychotic drugs) but other adenosine agonists with preferential affinity for A_1 receptors, such as CPA, CHA and *R*-PIA, reduce the stimulation caused by amphetamine only at ataxic doses (99). Several adenosine agonists were shown to reduce shelf-jump and Sidman conditioned avoidance responding (83), extending previous results obtained with CHA (100). The ability of agonists with high affinity for A_2 receptors to inhibit avoidance with minimal impairment of escape responding is characteristic of available antipsychotics. Although the A_1 agonists reduce avoidance behavior, most inhibit escape responding at the same doses. CI-936 (15), an adenosine agonist with high affinity for both A_1 and A_2 receptors, was found to produce antipsychotic-like effects in a variety

of tests (101). Like available dopamine antagonists, CI-936 inhibits locomotion without producing ataxia and inhibits conditioned avoidance without reducing unconditioned behavior, with a profile of selectivity that equals or exceeds that of available antipsychotics (101). These observations suggest that adenosine agonists with appreciable affinity for A_2 receptors may have utility as novel antipsychotic agents, which may lack the neurological side effects of available antipsychotics.



15

	K_i (nM)	
	A_1	A_2
	6.8	25

These behavioral similarities between adenosine agonists and dopamine antagonists may be related to the presence of adenosine receptors in terminal fields of central dopamine projections (15,37-39) as well as the ability of adenosine agonists to inhibit the release of dopamine from central neurons (55). However, because A_1 but not A_2 receptors have been implicated in the inhibitory effects of adenosine agonists on dopamine release, the selective antipsychotic-like effects of A_2 adenosine agonists may instead be due to functional opposition to the dopamine system rather than inhibition of dopamine release.

Behavioral Effects of Adenosine Antagonists - Since adenosine has sedative effects, it seems plausible that the stimulant effects of caffeine and theophylline could be due to blockade of central adenosine receptors (1). However, caffeine and theophylline are poor tools to test this hypothesis because of their weak affinity for adenosine receptors. More potent and selective adenosine antagonists are now available and their behavioral effects are beginning to be characterized. CPT acts as an A_1-selective adenosine antagonist *in vivo*, blocking the locomotor inhibition caused by the A_1 agonist CPA, but not that caused by the A_2 agonist CV-1808 (25). However, although theophylline causes a large increase in motor activity in mice, CPT by itself has no effect on locomotor activity (25). These results suggest that the gross motor stimulation caused by caffeine and theophylline is not due to blockade of A_1 receptors, but rather may involve A_2 receptor blockade or a non-adenosine mechanism.

Despite the lack of gross motor stimulation by CPT, it does have more subtle behavioral effects. For instance, CPT increases intracranial self-stimulation in rats that have been trained to press a lever to receive electrical stimulation of the medial forebrain bundle (25), an effect seen with stimulant as well as antidepressant drugs. In addition, chronic treatment with caffeine or theophylline causes down-regulation of brain β-adrenergic receptors (70,102,103), an effect that is considered a neurochemical predictor of antidepressant activity. A series of non-xanthine adenosine antagonists has been patented for antidepressant activity based on the swimming (Porsolt) test in mice (104,105). These results indicate that adenosine antagonists may have therapeutic applications as antidepressants or mild stimulants.

Several adenosine antagonists, including XAC and the A_1-selective antagonist CPT, have proconvulsant effects in animal models of epilepsy (106-108). These results support the proposal that adenosine may be an endogenous anticonvulsant (109).

CNS Versus Peripheral Mediation of Behavioral Effects of Adenosine Agonists

The profound cardiovascular effects of systemically administered adenosine agonists cannot be ignored in any consideration of the mechanism of the behavioral effects of these

agents. In particular, adenosine lowers blood pressure by lowering heart rate and force (an A_1 effect), dilating blood vessels (A_2), and inhibiting renin release from the kidney (A_1) (5). Adenosine agonists also produce considerable hypothermia in most animal species (25,110), possibly by a combination of cutaneous vasodilation (A_2) and inhibition of heat production by fat and other peripheral tissues (A_1). Because of these profound peripheral effects of adenosine, a debate has arisen regarding whether some of the CNS and behavioral effects of adenosine might be peripherally mediated. The argument for peripheral mediation of the behavioral effects of adenosine is based on observations such as those of Brodie and colleagues (111). Although pressure injection of *R*-PIA directly into the hippocampus inhibited EPSPs, IP administration of *R*-PIA at doses up to 1000 times those necessary to cause behavioral effects had no effect on EPSPs. After systemic administration of [^{3}H]*R*-PIA, radioactivity could not be detected in the brain by autoradiography or dialysis.

However, considerable evidence also supports the concept that the behavioral actions of adenosine agonists are CNS-mediated. The behavioral effects of centrally administered adenosine agonists are qualitatively similar to their effects on peripheral administration, but occur at much lower doses (80). Although hypotension probably contributes to certain of the behavioral effects of adenosine agonists (especially at high doses), adenosine agonists inhibit locomotion and conditioned behavior at doses that do not decrease blood pressure (80,81,112,113). Systemically administered adenosine agonists have central neurochemical and neurophysiological effects that may logically account for the behavioral effects of these agents, and are not mimicked by hypotensive agents. Although Brodie and colleagues found that [^{3}H]*R*-PIA did not enter the brain, in other studies behaviorally active doses of [^{3}H]CHA and [^{3}H]*R*-PIA have been shown to enter the brain at concentrations that should result in substantial occupancy of brain A_1 receptors (112,114). CHA and *R*-PIA have octanol:water partition coefficients that should permit brain penetration (log P values near 2.0). Finally, the failure of systemically administered adenosine agonists to duplicate certain of the electrophysiological effects of the same agents administered centrally may only indicate that other CNS actions of systemically administered adenosines are more relevant to the behavioral actions of these agents. Further studies will be required before this question can be answered definitively.

Summary

Agents that mimic or block adenosine produce a variety of effects that suggest important CNS actions. Adenosine agonists may have potential utility as novel antipsychotic agents, while adenosine antagonists may have useful stimulant or antidepressant properties.

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Chapter 6. Central Serotonin Receptors: Opportunities for Drug Discovery

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In the past decade there have been significant advances in our understanding of serotonin (5-HT) biochemistry and physiology, and it now seems possible that many CNS diseases result from aberrations in serotonergic systems (1,2). Serotonin may play a role in the etiology of depression, pain, various obsessive-compulsive disorders, obesity, schizophrenia, anxiety, sexual dysfunction, migraine, and various vasospastic disorders (3-5). This review will provide an update on drugs that interact with central 5-HT receptors. This subject was last reviewed in this series in 1986 (6), and in a section of the migraine review of 1987 (7). Recent reviews on 5-HT receptors have appeared (1, 2, 8, 9).

SEROTONIN RECEPTOR HETEROGENEITY

Much of the recent progress in 5-HT research was stimulated by the discovery of multiple 5-HT receptors and by the design of selective ligands. Two seminal discoveries provided the foundation for the recent progress. Gaddum and Picarelli studied 5-HT pharmacology in the guinea-pig ileum and provided evidence for two distinct 5-HT receptor subtypes (10). In 1979, Peroutka and Snyder demonstrated that ^{3}H-5-HT bound with high affinity to a subset of 5-HT receptors (5-HT_1), and spiperone displayed high affinity for a second population of 5-HT receptors (5-HT_2) (11). The 5-HT_1 receptor has now been further subdivided into four separate classes: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, and 5-HT_{1D} (12-15). Although some subsite-selective 5-HT_1 agonists are available, our knowledge regarding these sites is still rudimentary, and more complete characterization awaits discovery of selective 5-HT_1 blockers. Characterization of the 5-HT_2 receptor is progressing due to the availability of several antagonists. For example, ketanserin has high affinity for 5-HT_2 receptors and the compound is now widely used in binding and pharmacology studies (16). Characterization of the 5-HT_3 receptor was made possible by development of selective antagonists (17). Some of the 5-HT_3 antagonists have now been prepared in tritium-labeled form and high affinity, saturable binding sites have been identified in the CNS (*vide infra*) (18,19). Evidence for heterogeneity of 5-HT_3 receptors has appeared, and this is still an area of active exploratory research (17).

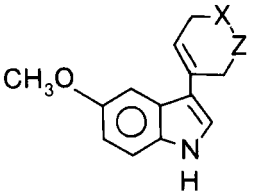
5-HT_1 RECEPTOR LIGANDS

Functional effects mediated by 5-HT_1 receptor subtypes have been actively investigated. In the hippocampus, the 5-HT_{1A} receptor is positively coupled to adenylate cyclase, and electrophysiologically, agonists lead to decreases in spike amplitude in the CA1 region. In whole animals, selective 5-HT_{1A} agonists produce hyperphagia and hypothermia, and stimulate sexual behavior. Moreover, they are

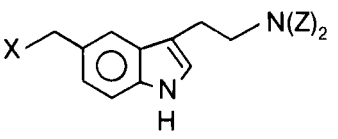
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anxiolytic and cause the 5-HT behavioral syndrome. The 5-HT_{1_B} receptor is less well characterized. In some species it appears to be an autoreceptor, but neuronal cell bodies in the dorsal raphe may use the 5-HT_{1_A} subsite as an autoreceptor. There is controversy regarding the presence of 5-HT_{1_B} receptors in some species, including man. In rat substantia nigra, 5-HT_{1_B} receptors are negatively coupled to adenylate cyclase. Functionally, 5-HT_{1_B} agonists in rodents inhibit aggressive behavior and food intake (1). 5-HT_{1_C} subsites are present in high concentrations in the choroid plexus of rodents, pigs, and man, and are positively coupled to phospholipase C and elevate inositol phosphates (1). Functional rat 5-HT_{1_C} receptors have been cloned and expressed in Xenopus oocytes (20). 5-HT_{1_D} receptors are the most recently described subtype, and no selective ligands are available yet. Metergoline and yohimbine have high affinity for this receptor, which is most concentrated in basal ganglia and is regulated by guanine nucleotides and calcium (15).

Indoles - Since 5-HT itself is selective for 5-HT_1 receptors, it is not surprising that indole analogs often are selective 5-HT_1 agonists (6). RU 24969 (1) is purported to be selective for 5-HT_{1_B} receptors, but the compound also binds with high affinity to 5-HT_{1_A} receptors (21). RU 24969 induces anorexia in free-feeding male rats. Pretreatment with p-chloroamphetamine (PCA), a central 5-HT depletor, does not attenuate the anorexia, suggesting it is mediated via postsynaptic 5-HT_{1_B} receptors (22). RU 28253 (2), an isomer of RU 24969, is also a potent ligand for 5-HT_{1_A} and 5-HT_{1_B} receptors. Methylation of these compounds at position 2 of the indole markedly decreases potency at 5-HT receptors, probably by sterically induced destruction of co-planarity between the indole and tetrahydropyridine rings (21). The acetamido and methanesulfonamido analogs AH-25086B (3) and GR-43175 (4) are 5-HT_1 agonists. Clinical studies with AH-25086B indicated this drug rapidly aborted headache pain after iv administration, and side effects were mild and transient. Its mechanism of action is thought to be selective vasoconstriction of carotid arteriovenous anastomoses. Development of AH-25086B has ceased, but clinical trials are ongoing with oral and iv formulations of GR-43175 (23,24).



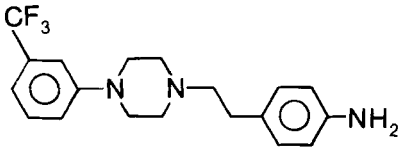
1; X = NH, Z = CH_2
2; X = CH_2, Z = NH



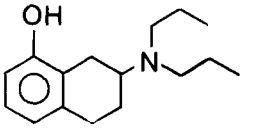
3; X = CH_3NHCO, Z = H
4; X = CH_3NHSO_2, Z = CH_3

Arylpiperazines - 5-HT agonist properties of simple arylpiperazines have been investigated for a number of years. Well-studied compounds include m-chloro- and m-trifluoromethyl-phenylpiperazine, MK 212, quipazine and 1-naphthylpiperazine (2). m-Chlorophenylpiperazine was studied clinically and its 5-HT agonist properties in man were confirmed (25). The piperazine PAPP (5, LY165163) binds to 5-HT_{1_A} sites, and causes hyperphagia and hypothermia in rats. Unlike 8-OH-DPAT (6), it does not produce stereotypy, perhaps because it also blocks dopamine receptors (26). PAPP also binds with reasonable affinity to 5-HT_2 and α_1 receptors; -log K_B values at 5-HT_{1_A}, 5-HT_{1_B}, 5-HT_2 and α receptors were 8.53, 6.48, 8.00 and 6.77, respectively (27). This underscores the

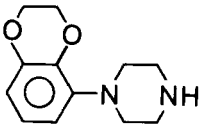
general finding that receptor selectivity has been difficult to achieve with phenylpiperazines.



5

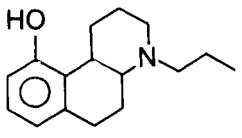


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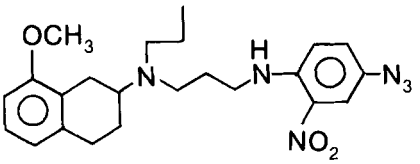


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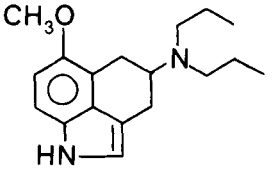
Hyperphagia produced by PAPP is not evident after depletion of central 5-HT stores by PCA (28). This led to the suggestion that this effect is mediated via somatodendritic 5-HT receptors. In contrast, PCA pretreatment had no effect on the hypothermic effects of PAPP or 8-OH-DPAT, indicating this effect may be mediated by postsynaptic 5-HT receptors (28). However, others have observed the opposite and concluded presynaptic receptors were involved in the hypothermic response to 8-OH-DPAT (29). PAPP has been prepared in tritium-labeled form and it binds preferentially to 5-HT_{1A} sites (30). Eltoprazine (7) selectively reduces aggression in animals (31). Eltoprazine has affinity for 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptors. Agonist activity at the 5-HT_{1B} site is thought to be its mechanism of action, since 5-HT_{1A} and 5-HT_2 agonists reduce aggression only at doses that produce other behavioral responses. However, 7 also selectively reduces aggression in species such as monkeys and pigs that may not possess 5-HT_{1B} receptors (31).



8



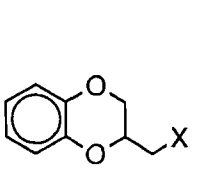
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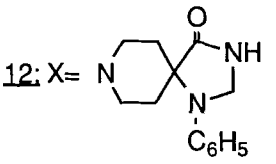
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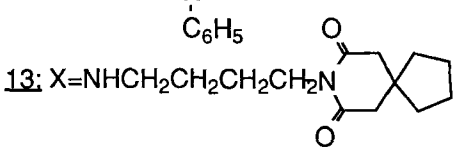
Aminotetralins - 8-OH-DPAT (6) is the prototypic 5-HT_{1A} agonist, and previous 8-OH-DPAT SAR knowledge has been summarized (6). The compound displays a 1000-fold selectivity for 5-HT_{1A} sites relative to other subtypes, and in tritiated form, is a useful ligand for binding studies. In rats, 8-OH-DPAT produces hypothermia and the 5-HT syndrome. The cis-C-1 methylated analog was a potent 5-HT_{1A} agonist; the (+)-enantiomer was equipotent with 8-OH-DPAT whereas the (-)-enantiomer and the trans diastereomer were inactive (32). All four stereoisomers of the C-3-methyl analog of 8-OH-DPAT have been studied, and regardless of stereochemistry, this modification results in either a diminution or destruction of affinity for 5-HT_{1A} receptors (33). The C-4 methylene unit of 8-OH-DPAT may be replaced with an isosteric oxygen without substantially altering its activity (34). The relatively low potency of the octahydrobenzoquinoline 8 relative to 8-OH-DPAT has been explained on conformational grounds (35). 8-OH-DPAT has been used as a template to construct a photoaffinity label for 5-HT_{1A} receptors. The label (9) binds with high affinity (IC_{50} = 6.6 nM vs 8-OH-DPAT) to 5-HT_{1A} sites in rat hippocampus, and has much lower affinity for 5-HT_{1B} or 5-HT_2

receptors. After irradiation, the compound irreversibly blocks 55-60% of 5-HT_{1A} sites, and the receptor appears to be a 63 KD protein with functionally important sulfhydryl groups (36-38). In addition to its importance as a biochemical tool, this agent demonstrates that one of the propyl groups may be modified substantially while maintaining high affinity and selectivity for 5-HT_{1A} receptors. BAY R 1531 (10), an indole analog of 8-OH-DPAT, is an agonist with high affinity for 5-HT_{1A} receptors and displays antidepressant-like activity as well as stimulating sexual behavior is castrated male rats (39). Removal of the methoxy and N-propyl groups from this compound leads to a substantial loss of potency (21).



11: X=$NHCH_2CH_2O(2,6\text{-}(CH_3O)_2C_6H_3)$





13: X=$NHCH_2CH_2CH_2CH_2N$

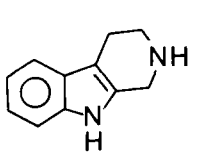
Benzodioxanes - ^{3}H-WB4101 (11), a potent α_1 antagonist, selectively labels 5-HT_{1A} receptors when prazosin is used to obliterate α_1 binding (40). ^{3}H-Spiroxatrine (12) also labels the 5-HT_{1A} site (41), and in the canine basilar artery it is a 5-HT_{1A} antagonist (42). MDL 72832 (13) binds to 5-HT_{1A} sites with high affinity (pIC_{50} = 9.1), and its affinity for α_1 and the other 5-HT receptors was 20 to 1000 fold less. It appears to be a partial agonist (43).

Pyrimidinylpiperazines - The discovery that buspirone has high affinity for 5-HT_{1A} receptors has stimulated intense interest in development of other 5-HT_{1A} agonists as anxiolytic drugs (44, 45). Newer agents of this type (e.g., gepirone and ipsapirone) have greater selectivity for 5-HT_{1A} receptors (relative to dopamine receptors) and may produce their antianxiety effects by either decreasing 5-HT turnover and neuronal firing via activation of 5-HT_{1A} autoreceptors, or by direct action at postsynaptic 5-HT_{1A} receptors (44). These compounds are metabolized to 1-(2-pyrimidinyl)piperazine, which may contribute to some of their activities, although the metabolite has low affinity for 5-HT binding sites (46). A discussion of the newer 5-HT receptor-mediated anxiolytics is included in Chapter 3 (45).

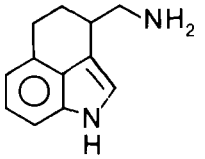
Aryloxypropanolamines - Propranolol, cyanopindolol, pindolol, and alprenolol are antagonists at 5-HT_1 receptors (47). Cyanopindolol is the most potent and the bulk of the activity resides in the (-)-enantiomer (48). ^{125}I-Cyanopindolol is a useful radioligand for labeling 5-HT_1 receptors (49). Functionally, propranolol and cyanopindolol are partial agonists/antagonists in rat brain hippocampus (50).

Miscellaneous structures - Tetrahydro-β-carboline (14) produces stimulus effects in rats by activating 5-HT_{1B} receptors (51). This compound has lower affinity for 5-HT_1 and 5-HT_2 binding sites than 5-HT itself, suggesting 5-HT binds to these receptors in conformations that are different from that of tetrahydro-β-carboline (21). The

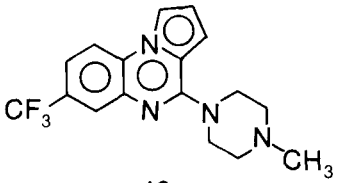
conformationally restrained analog of 5-HT, 15, has relatively low affinity for 5-HT receptors (21). The pyrroloquinoxaline compound CGS 12066B (16) is a 5-HT_{1_B} agonist, and is the most 5-HT_{1_B} selective agent reported to date ($5\text{-HT}_{1_A}/5\text{-HT}_{1_B}$ selectivity = 17) (52).



14



15



16

5-HT_2 RECEPTOR LIGANDS

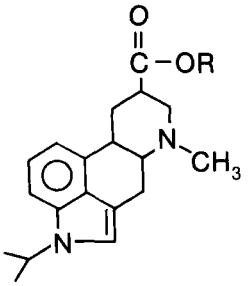
The 5-HT_2 receptor has been more completely characterized than some of the other subtypes due to availability of selective 5-HT_2 antagonists. ^{3}H-Spiperone was the radioligand first used to define 5-HT_2 binding sites, although the compound actually displays higher affinity for D_2 receptors. Most recent studies employ ^{3}H-ketanserin, but this compound is also imperfect because of its high affinity for α_1 and histamine-H_1 receptors (16). It has been proposed that vascular 5-HT_2 receptors exist in two interconvertible forms, and that the 5-HT binding site may be modulated through an allosteric site. The model was developed to explain why ketanserin restores 5-HT-induced contractions that have been depressed by the 5-HT_2 antagonist methysergide. It was suggested that both ketanserin and 5-HT interact with the receptor in a highly active state R, whereas methysergide binds to the low activity state R' (53-56). Whether this model has validity for either peripheral or central 5-HT_2 receptors requires further studies. Effects of 5-HT_2 agonists appear to be mediated by increases in phosphoinositide turnover (57).

The 5-HT_2 receptor mediates a variety of pharmacological effects (2, 4, 5). Agonists at 5-HT_2 receptors in mice produce head twitches, and selective 5-HT_2 antagonists block this effect. Clinically, 5-HT_2 agonists have been associated with hallucinogenic events, and in animals the discriminative stimulus properties of LSD are blocked by 5-HT_2 antagonists. Long-term administration of antidepressant drugs to animals results in down-regulation of central 5-HT_2 receptors, suggesting these receptors may be involved in affective disorders. Contraction of some vascular smooth muscle, including the rat jugular vein, is mediated via 5-HT_2 receptors (8). Animal studies have shown that 5-HT_2 antagonists inhibit cyclic flow variations in stenosed coronary arteries, and 5-HT may mediate a variety of vasospastic and occlusive processes (58). 5-HT_2 blockers may be useful in prophylactic treatment of migraine (7).

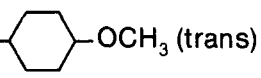
Neuroleptics - K_d values of 17 neuroleptics at 5-HT_{1_A} and 5-HT_2 receptors have been measured using human frontal cortex tissue (59). The compounds were more potent at 5-HT_2 receptors, and the four most potent compounds were spiperone, d-butaclamol, chlorprothixene and ketanserin with K_d values ≤ 0.56 nM. This work revealed no apparent differences between human and rat central 5-HT_2 receptors, and there was no correlation between the neuroleptic doses in humans and their

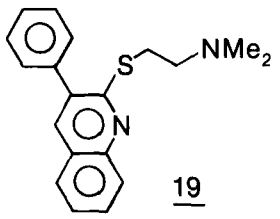
5-HT_2 receptor affinities, suggesting 5-HT_2 antagonism does not mediate their antipsychotic effects (59). Many ketanserin analogs have been investigated (6, 60). Flumezapine and zotepine are potent D_2 and 5-HT_2 antagonists both in vitro and in vivo. In rats, they blocked increases in serum corticosterone elicited by the D_2 agonist pergolide, or the 5-HT_2 agonist quipazine (61). Clozapine was shown to possess 5-HT_2 antagonist properties in man since it blocks MK 212-induced increases in serum cortisol concentrations (62).

Ergolines - Several ergolines are 5-HT_2 blockers, and LSD, mesulergine, methysergide and metergoline represent well-studied compounds. Selectivity is normally difficult to achieve with this class, but the ergoline LY53857 (17) is selective for 5-HT_2 receptors relative to α_1 or D_2 receptors. The compound is a mixture of four diastereomers, all of which individually display nearly equal affinities for 5-HT_2 receptors (63). The indole nitrogen substituent was found to be crucial in maximizing 5-HT_2 affinity, and an isopropyl was optimal (64). Cycloalkyl esters at C-8 produced high affinity compounds at vascular 5-HT_2 receptors, and the cyclopentyl and cyclohexyl esters were most potent. Addition of an oxygen-containing group in the 4-position of the cyclohexyl ring maximized 5-HT_2 receptor affinity (e.g., LY281067, 18). A methyl group on N-6 was also optimal (65). LY53857 enhanced sexual activity in male rats, consistent with other evidence that certain serotonin pathways suppress sexual behavior. LY53857 increased copulatory rate and reduced ejaculatory latency in male rats presented with a sexually receptive female rat (66).



17; R = $CH(CH_3)CH(OH)CH_3$

18; R =  OCH_3 (trans)



19

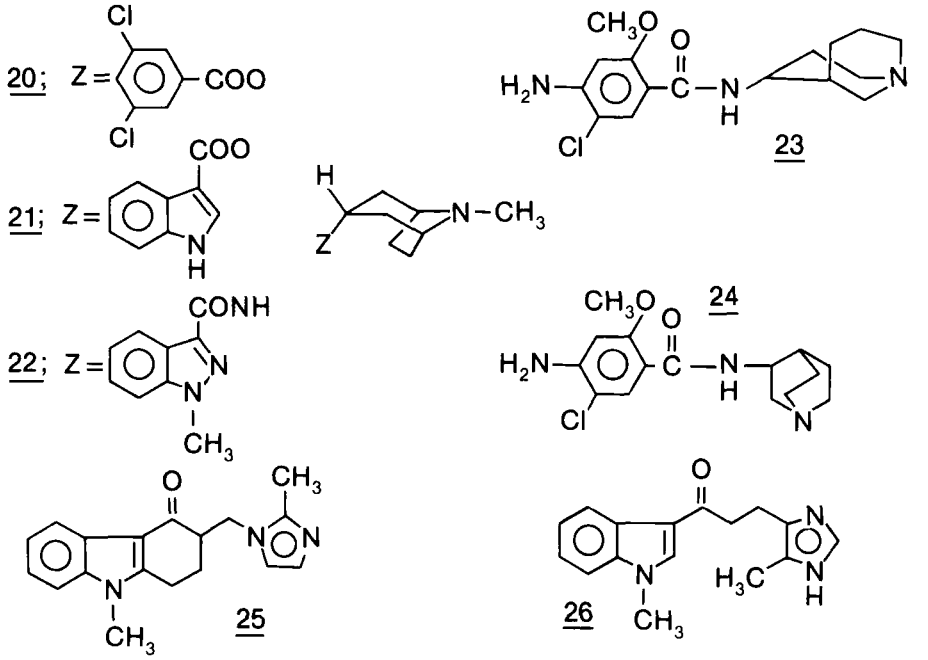
Quinolines - ICI 169,369 (19) represents a new structural class of 5-HT_2 antagonists. The tertiary amine maximized affinity and selectivity for 5-HT_2 receptors. Substitution on both the 3-phenyl and the quinoline rings were explored, and the optimal derivative was the parent, unsubstituted compound (67). It binds most avidly to 5-HT_2, α_1 and 5-HT_1 receptors; K_i values were 1.79×10^{-8}, 5.6×10^{-7} and 1.58×10^{-6} M, respectively. The drug blocks 5-HTP-induced head twitches in mice with an ED_{50} of 1.6 mg/kg (ip), and is in phase II clinical trials for treatment of both psychiatric and cardiovascular diseases (67). ICI 169,369 competes with 5-HT for the 5-HT_2 receptor, and also binds to the purported allosteric site, facilitating conversion of the receptor to the highly active R state (68).

Phenylaminopropanes - 1-(2,5-dimethoxyphenyl)-2-aminopropane derivatives bearing substituents at the 4-position have been studied as 5-HT_2 agonists. One of the most studied analogs is the 4-bromo derivative, DOB. The R enantiomer is 6-fold more potent than the S isomer at 5-HT_2 sites, and the primary amine DOB was more potent than N-methylated derivatives (69). ^{3}H-DOB has been used as a 5-HT_2 agonist radioligand, and it labels a guanyl nucleotide-sensitive state of the 5-HT_2 receptor

in rat frontal cortex (70). The ligand labels only a subset of 5-HT_2 receptors that are labeled with ^{3}H-ketanserin (71). Recently, the ^{125}I analog of DOB was reported as an alternative to ^{3}H-DOB, with a higher specific activity (72).

5-HT_3 RECEPTOR LIGANDS

The neuronal 5-HT_3 receptor mediates a variety of responses to 5-HT. 5-HT produces pain and the wheal and flare response via sensory neurons. In the enteric nervous system and in autonomic neurons, 5-HT produces depolarization and neurotransmitter release (17, 73-74). The 5-HT_3 receptor may be tightly coupled to ion channels which produce depolarization (75). Some workers have hypothesized heterogeneity of 5-HT_3 receptors (73). Confirmation of earlier findings that cocaine produces weak 5-HT_3 receptor blockade led to the identification of numerous antagonists at this receptor including MDL-72222, ICS 205-930, the indazole isostere of ICS 205-930, BRL-24924, zacopride, GR-38032F, and GR-65630 (20-26, respectively) (1, 76-78). These agents terminate pain associated with ongoing migraine headaches, suggesting it is mediated by sensory afferent 5-HT_3 receptors, and several are under development for this indication (7,17). Many are antiemetic and stimulate gastric emptying (79-86), topics explored in detail in Chapter 21 (87).



ICS 205-930 and MDL 72222 are impotent as blockers of agonist-induced head twitches, forepaw treading, and tremor in rats suggesting they have low affinity for central 5-HT_2 receptors in vivo (88). Some evidence for central effects of these compounds has appeared using nontraditional models for schizophrenia and anxiety. For example, when dopamine is directly infused into the rat mesolimbic nucleus accumbens, a biphasic hyperactivity is produced that is blocked by clinically effective neuroleptics. Zacopride, GR-38032F and ICS 205-903 also block this dopamine-mediated hyperactivity (89). In some models of

anxiety such as intruder threat in marmosets and light/dark discrimination in mice, 5-HT_3 blockers produce anxiolytic effects (90). Moreover, GR-38032F has anxiolytic effects in cynomolgous monkeys (91). Injection of a neurokinin agonist into the midbrain area of rats results in activation of the mesolimbic dopamine system. Behaviorally, this is manifest as hyperactivity, and GR-38032F reduces the neurokinin-induced increase in dopamine metabolism and blocks behavioral hyperactivity (92). Two of the 5-HT_3 blockers were prepared in tritium-labeled form, and specific binding sites in the CNS have been demonstrated. ^{3}H-GR-65630 binds with high affinity to rat entorhinal cortex, and affinity of a variety of 5-HT_3 antagonists to this binding site appeared to correlate well with their potencies in rat isolated vagus nerve (18). Binding sites were also found in NG 108-15 neuroblastoma-glioma cells with tritium-labeled ICS 205-930 (19). Whether these binding sites represent functional 5-HT_3 receptors must still be established (93).

CONCLUSIONS

During the past several years the existence of multiple 5-HT receptors has become apparent, providing new opportunities for drug discovery. The availability of drugs that are selective for certain 5-HT receptor subtypes is allowing characterization of 5-HT receptor physiology in animals. Moreover, as more clinical data are obtained using these new tools, we will hopefully be able to treat various CNS diseases more effectively, as well as gaining a greater understanding of 5-HT receptor function in humans.

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Section II - Cardiopulmonary and Vascular Agents

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Chapter 7. Antihypertensive Agents

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Introduction - The recent introduction of new classes of antihypertensive agents has provided the clinician with greater flexibility in the treatment of essential hypertension (1). Calcium entry blockers, angiotensin converting enzyme (ACE) inhibitors, and α_1-adrenoceptor blocking drugs are being regarded as first choice agents by some physicians (2). Such alternatives have initiated a trend away from the "step-care" approach to a strategy where drug selection is strongly influenced by individual factors such as angina, heart failure or diabetes (3).

In a critical review of intervention trials in hypertension, it has been claimed that only a few patients with mild hypertension benefit from treatment (4). As a way to avoid overtreatment in this group, ambulatory blood pressure monitoring is being used to identify truly normotensive subjects (5). In general, results from trials of mild to moderate hypertensives have shown that blood pressure control has little or no effect on the mortality associated with coronary heart disease (CHD), although two large trials have suggested some reduction among male nonsmokers (6). A J-shaped relationship between mortality from myocardial infarction and treated diastolic pressure has been suggested (7,8), underscoring the potential harm in aggressively reducing blood pressure in hypertensives with ischemic heart disease. A reduction in the incidence of strokes with the control of blood pressure continues to be shown (9).

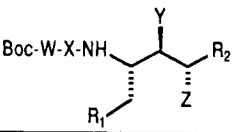
Based on the suggestion that essential hypertension is an insulin-resistant state (10), weight reduction as a method for blood pressure control (11-13) has received more attention. The possibility that an endogenous digitalis-like factor may be activated in hypertension has been reviewed (14), although a direct connection with cardiac and vasoconstrictor mechanisms in hypertension has not been shown.

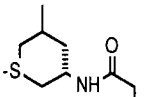
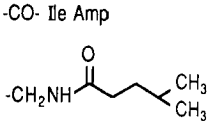
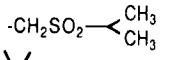
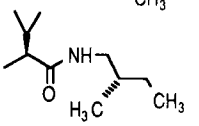
Renin-Angiotensin System - Recent advances in the biochemistry of the renin-angiotensin system have been reviewed (15) and the clinical aspects and implications of the pharmacologic intervention described (16,17). The cellular organization of the brain renin-angiotensin system has been summarized and a unified model of intracellular angiotensin production proposed (18). Evidence is accumulating that the vascular wall renin-angiotensin system is functionally important, although the molecular biological techniques used in these studies must continue to be critically examined from the standpoint of specificity and sensitivity (19,20).

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Renin Inhibitors - Manipulation of the renin-angiotensin system and the blockade of renin, in particular, currently constitute the major areas of investigation for novel antihypertensive agents (17). In support of this approach, active immunization against human renin reduced blood pressure in sodium depleted marmosets, thereby establishing the feasibility of long-term control of blood pressure through renin inhibition (21). The thesis was also substantiated by results obtained from the acute administration of (3S,4S)-4-amino-5-cyclohexyl-3-hydroxy pentanoic acid (ACHPA) containing renin inhibitors. Such compounds were as effective as an ACE inhibitor in chronically controlling blood pressure in renal hypertensive dogs (22). Excellent state-of-the-art reviews on renin inhibitors (23,24) and their therapeutic potential (25) have appeared. Proposals for the rational design of such inhibitors based on the X-ray structure of related aspartic acid proteases have been made (26,27).

The principal approach to developing useful drugs from current lead structures is to maximize potency, reduce molecular size, and decrease the number of amide bonds. Important modifications include both the transition state moiety and the P_2' and P_3' functionalities, which are positions important in defining species specificity (28). The use of this strategy coupled with molecular modeling has yielded compounds, such as 1-9 (29-37), which have *in vitro* nM potency against human renin. However, the challenges to decrease hepatic clearance (38), stabilize against metabolic instability (39), and improve bioavailability (39-41) remain to be achieved.

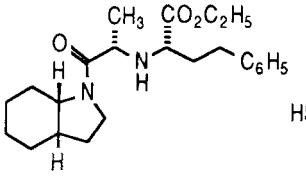


	W	X	Y	Z	R_1	R_2
1	Phe	Leu	OH	OH	cC_6H_{11}	$-iC_4H_9$
2	Phe	His	OH	H	cC_6H_{11}	
3	1-Nal	His	OH	H	iC_3H_7	$-S-cC_6H_{11}$ 
4	Phe	His	OH	F_2	iC_3H_7	-CO- Ile Amp
5	(H_3CO) Tyr	His	OH	H	cC_6H_{11}	
6	Phe	Leu	O	F_2	cC_6H_{11}	C_2F_5
7	Phe	His	OH	H	cC_6H_{11}	
8	Phe	His	OH	OH	iC_3H_7	
9	Phe	Phe	O	F_2	iC_3H_7	-CO-Leu-Phe-NH_2

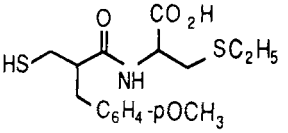
ACE Inhibitors - The angiotensin I converting enzyme (ACE) inhibitors, captopril and enalapril, have become valuable additions to the therapy for hypertension. Their success has been a strong stimulus for continued research in the renin-angiotensin area, and novel ACE inhibitors continue to appear. Those in advanced development include perindolpril and its

diacid metabolite, S-9780 (42,43), ramipril (44), cilazapril (45-47), spirapril (48), zofenopril (49), and fosfenopril (fosinopril) (49)

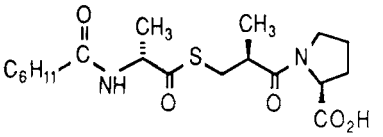
Among recent examples, the replacement of the proline in enalapril with a saturated indole-2-carboxylic acid moiety has given CI 907 (indolapril, 10) which is equipotent to standard agents both *in vitro* and *in vivo* (50). Among a series of N-mercaptoalkanoyl derivatives of sulfur containing amino acids, 11 was the most active example *in vitro* and in the range of captopril (51,52). MC-838, 12, an example in which the mercapto group is protected as a stable thiol ester, was less active than captopril *in vitro*; however, in SHR, a profile of slow onset and long duration with potency equivalent to captopril was seen (53).



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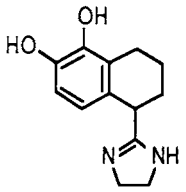


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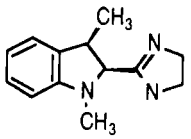
AII Antagonists - Little progress has been made toward a bioavailable agent capable of blocking the final step in the renin angiotensin cascade, i.e. activation of the angiotensin II receptor. However, modifications of AII continue with the aim of defining the role of each amino acid and eliminating agonist activity. From these studies, examples have been found which behave as potent antagonists with low intrinsic activity (54,55).

α_2-Adrenoceptor Agonists - Further biochemical studies with the endogenous brain factor, clonidine displacing substance (CDS), continue to show its similarities to clonidine. In human platelets CDS inhibits epinephrine-induced aggregation and potentiates the effects of collagen and ADP, thus suggesting a role in the regulation of platelet action (56).

The most potent full agonist, 13, of the α_2 adrenoceptor has been resolved and the absolute configuration of the more active enantiomer established as R by single crystal X-ray analysis (57). An α_2 agonist/-antagonist reversal based on stereochemistry has been observed in a series of (indolin-2-yl)imidazolines. The *cis* isomer, 14, is an α_2 agonist equal in potency to clonidine, while the *trans* is a moderately potent α_2 antagonist (58).



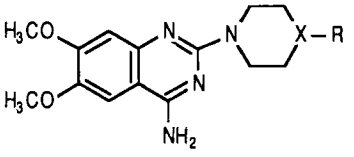
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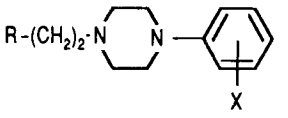


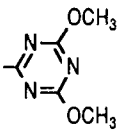
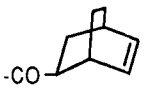
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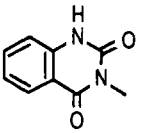
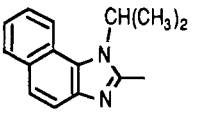
α-Adrenoceptor Antagonists - The clinical pharmacology of doxazosin (59,60) and terazosin has been reviewed with particular regard to essential hypertension (61). These studies and others continue to demonstrate the clinical efficacy (62,63) of the prazosin class. The optical isomers of terazosin have been prepared; evaluation by radioligand methods

indicated the l-isomer possessed higher affinity for the α_1 receptor (64). New series structurally related to prazosin are exemplified by 15 and 16. The former, although exhibiting high *in vitro* affinity for the α_1 receptor, is less active than prazosin as an oral antihypertensive agent in SHR (65). The latter piperidino analog of prazosin showed high *in vitro* affinity and sustained antihypertensive activity in SHR (66). The effect of the α_1-adrenoceptor antagonist SM 2470, 17, on plasma cholesterol level is due to inhibition of cholesterol absorption secondary to a reduction in cholesterol solubilization (67). SGB 1534, 18, has been compared to prazosin in the dog; both drugs decreased preload and afterload via α_1-adrenoceptor blockade in the peripheral vasculature (68). The indoramine analog L15848, 19, lowered blood pressure in renal hypertensive and normal dogs, but in the rat was 100X less potent than prazosin (69).



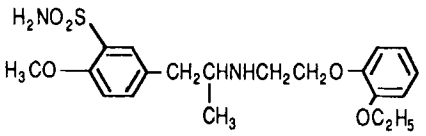


	X	R
15	N	
16	CH	$-CH_2CONHC_2H_5$
17	N	

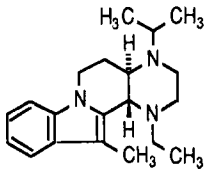
	R	X
18		2-OCH_3
19		3-CF_3

The effect of stereochemistry on α_1-adrenoceptor antagonism continues to receive attention. For example, the R enantiomer, 20, of YM-12617 showed both greater affinity and selectivity for the α_1 as compared to the α_2 receptor. Predictably, the hypotensive activity of the isomers reflected their α_1 potencies (70).

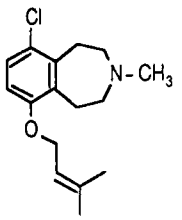
The previously reported AY-28,228 (aliprosin, 21), has been defined as an α_1-adrenoceptor antagonist which falls between prazosin and ketanserin in terms of potency (71). The compound has good bioavailability with no tachycardia nor tolerance displayed on repeated administration (72). The pharmacology of the selective post-junctional α_2-adrenoceptor antagonist, SKF-104078 (22), has been described (73).



20



21

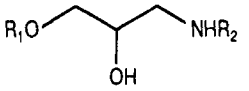


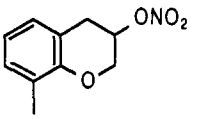
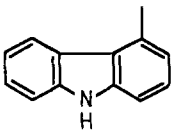
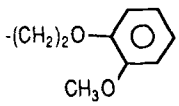
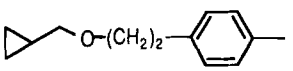
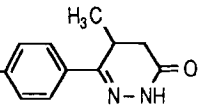
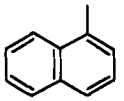
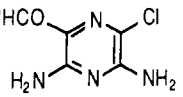
22

β-Adrenoceptor Antagonists - The structure-function relationships with the hamster β_2 adrenoceptor (β_2AR) have revealed that ligands bind to the receptor at its rhodopsin-like core (74). Single amino acid substitutions or deletion of stretches of amino acids from several of the hydrophobic and hydrophilic domains have identified specific amino acid residues required for ligand binding (75-78).

Clinical studies with various β-adrenergic antagonists continue (79-82) with emphasis on blood lipid effects (83,84) and the advantages of intrinsic sympathomimetic activity (85). Three long-term trials including the HAPPY (Heart Attack Primary Prevention in Hypertension) trial (86) have failed to show a β-blocker-derived protective effect against coronary artery disease (87).

K-351 (nipradilol, 23), a dual acting α,β-blocking/vasodilating agent, suppressed renin release induced by renal sympathetic nerve stimulation (88) and had beneficial effects in the ischemic canine heart (89). The β-adrenoceptor antagonist/vasodilator, carvedilol, 24, reduced blood pressure in hypertensive animals and inhibited isoproterenol-induced tachycardia over a similar dose range (90). SKF-95018, 25, a vasodilator/β-blocker, increased hindquarter blood flow in anesthetized rats and inhibited isoproterenol-induced tachycardia (91). ICI-147,798 (26) has both diuretic and β-adrenoceptor properties (92); however, it is not clear whether the molecule is metabolized *in vivo* to the individual pharmacologic entities.

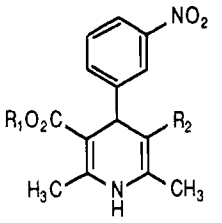


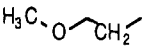
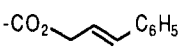
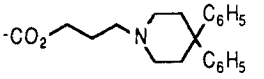
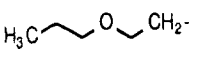
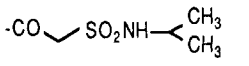
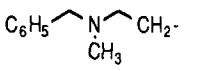
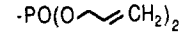
	R_1	R_2
23		$-CH(CH_3)_2$
24		$-(CH_2)_2O-$ 
25		$-(CH_2)_2CONH-$ 
26		$-(CH_2)_2NHCOC(CH_3)_2NHCO-$ 

The synthesis and structure-activity relationships of the β_1 selective agent betaxolol have been reported (93).

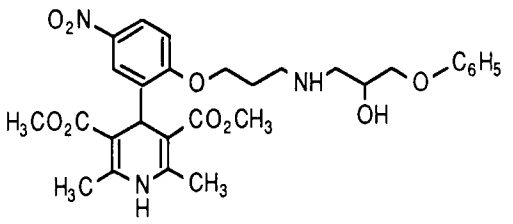
Calcium Antagonists - The interaction of calcium channel blockers with voltage operated calcium channels (VOC's or L-channels) has been reviewed (94) and their status in the treatment of hypertension (95,96) summarized. The pharmacological profile of amlodipine (97) in comparison with other calcium entry blockers has been reported.

The existence of distinct types of Ca^{+2} channels has been proposed; only one of which is susceptible to blockade by classical calcium antagonists (98,99). This dihydropyridine sensitive calcium channel from rabbit transverse tubule membranes has been purified and found to consist of three noncovalently linked classes of subunits, and a model for their interaction has been proposed (100). The complete amino acid sequence of the dihydropyridine binding protein of this complex has been predicted by sequence analyses of DNA complementary to the messenger RNA (101).

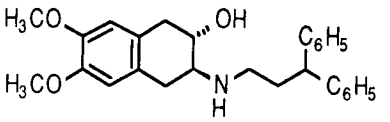


	R_1	R_2
27		
28		
29		
30		

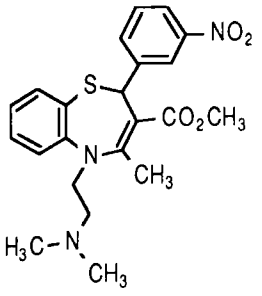
Further support has been provided for the proposed bioactive conformation of the nifedipine class of calcium entry blockers (102). Structural modifications among the dihydropyridines continue to be concentrated on the ester and aryl substituents with the aim of discovering examples having a slower onset and longer duration of action. Results with FRC-8653 (27) are encouraging; in spontaneous hypertensive rats, this compound exhibited an antihypertensive effect of slow onset with a duration of over seven hours (103). Similar long-lasting effects also were observed in dogs with B-844-39 (28) (104). More extensive structural modifications of the ester moiety are found in 29, Ro 18-301 (105), and 30 (106). Further attempts to generate a dual acting calcium channel/β-adrenoceptor antagonist resulted in YM-16151-1 (31) and 32. The former was more potent than diltiazem as a calcium channel blocker and equipotent to propranolol as a β-blocker (107); the latter was similar in profile to verapamil with modest β-blocking activity (108). A nifedipine-diltiazem structural hybrid, 33, was found to interact competitively with both binding sites (109). Among non-dihydropyridines which inhibit voltage dependent calcium channels are MCI 176 (34) (110,111) and the sesquiterpinoid alismol (35) (112).



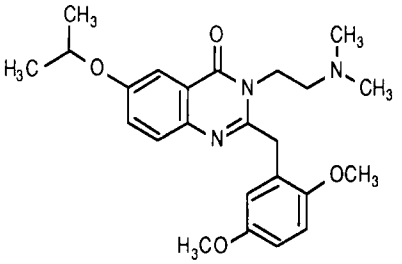
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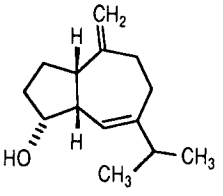
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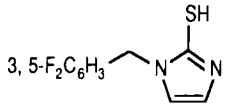


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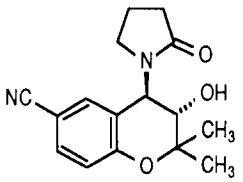
Miscellaneous Agents - Structure-activity relationships were developed using a modified Hansch analysis for a series of aralkyl-imidazole-2-thiones as dopamine β-hydroxylase inhibitors (113,114). One of the more promising examples, SKF 102691 (36), was acutely active orally in SHR and attenuated the development of hypertension when given chronically (115).

The emergence of potassium channel activators such as BRL-34915 (37), and the recognition that pinacidil, minoxidil, and nicorandil also may interact with this channel, indicate a possible new direction in the application of ion channel modulators to cardiovascular disease. The effect of pinacidil on the potassium channel from vascular smooth muscle has been reviewed (116) and the results from human trials with BRL-34915 have been reported (117).

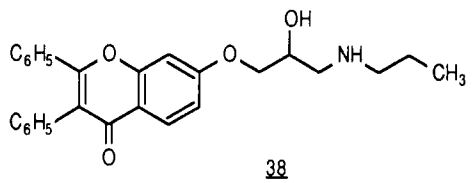
A new class of neuronal catecholamine depleting agents of the diphenylchromonoxypropanolamine type, exemplified by 38, has been described; although within the β-adrenoceptor antagonist structural class, β-blocking activity was not observed (118).



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Chapter 8. Pulmonary and Antiallergy Agents

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Introduction - The prevalence of pulmonary and allergic diseases continues to stimulate a broad range of research into new therapies for these conditions. The potential involvement of leukotrienes in the pathogenesis of asthma has focused attention on the search for agents that antagonize the actions of these potent biological substances and/or inhibit 5-lipoxygenase. Several peptide leukotriene antagonists have progressed to the clinic and early results regarding their activity in preventing LTD_4-induced bronchoconstriction in man and their efficacy in treating asthmatics have appeared (1,2). Platelet activating factor (PAF) has been implicated in airway hyperreactivity associated with asthma suggesting the potential utility of PAF antagonists in the treatment of asthma (3). Other approaches being pursued to discover drugs for asthma and allergic diseases include: mediator release inhibitors, thromboxane antagonists and biosynthesis inhibitors, non-sedating H_1 antihistamines and bronchodilators. Evidence that the proteolytic enzyme elastase plays a major role in lung injury in chronic pulmonary diseases such as emphysema has promoted the search for specific elastase inhibitors.

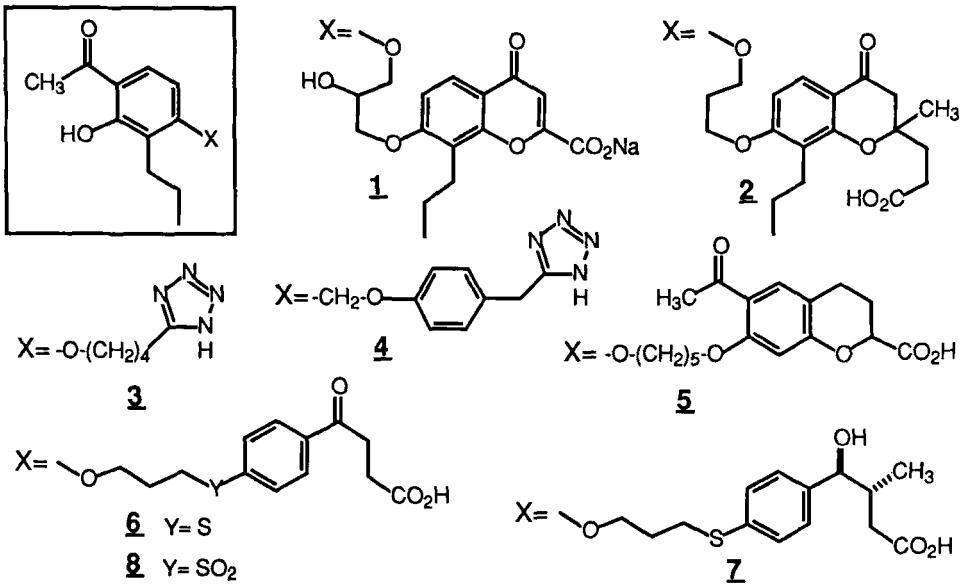
LEUKOTRIENES

The biological effects of leukotrienes, their potential involvement in diseases (4-7) and the discovery and evaluation of antagonists and biosynthesis inhibitors (8-10) were actively investigated. Leukotrienes were detected in sputum of patients with chronic bronchitis (11) and LTC_4 levels were found elevated in the blood of children undergoing an acute asthmatic attack (12). A direct effect on airway smooth muscle was suggested for LTD_4-induced bronchoconstriction in man (13). Activation of the LTD_4 receptor on RBL-1 cells was shown to induce phosphatidyl inositol hydrolysis which was G-protein regulated (14). The successful cloning of 5-lipoxygenase and LTA_4-hydrolase, two key enzymes in the biosynthetic pathway leading to the leukotrienes, has been reported (15-17). An interesting mechanism for regulating leukotriene production was suggested for sodium diclofenac. In addition to being a potent cyclooxygenase inhibitor, it enhanced the spontaneous uptake of arachidonic acid into the cellular triacylglycerol pool thereby reducing the availability of arachidonic acid for metabolism to leukotrienes (18).

Leukotriene Antagonists - FPL-55712 (**1**) has been the prototype for the development of several leukotriene antagonists. SC-39070 (**2**) displayed selective antagonism of LTD_4 with a $pA_2 = 8.2$ on guinea pig ileum. A dose of 10 mg/kg po blocked LTD_4-induced bronchoconstriction in guinea pigs for 20 hours (19). The structure-activity relationships (SAR) in the series leading to LY171,883 (**3**) showed that the 2-propyl-3-hydroxy-4-acetylphenoxy unit was important for activity and a 4-7 methylene unit spacer was optimal between the phenoxy and tetrazole units (20,21). A single 400 mg dose of **3** in normal subjects caused a rightward shift in the LTD_4 dose response curve for FEV_1, 4.6-fold and for $Vmax_{30}$, 6.3-fold (1). In the first long term study of a LT antagonist in asthmatic patients, **3**, given 400 mg twice a day reduced symptom scores, decreased the use of β_2 agonist and increased FEV_1 (2, 22). The SAR leading to LY 163,443 (**4**) was reported (23) and a series leading to Ro23-3544 (**5**) was described (24).

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Using a hyperreactive rat model, two LTD_4 antagonists, L-647,438 (6) and L-649,923 (7) were evaluated after oral administration and found to inhibit antigen-induced dyspnea with ED_{50} values of 5.75 and 1.3 mg/kg, respectively (25). A longer duration of action was observed for 7 (>6 hrs) compared to 6 (<4 hrs). In man, a 3.8-fold shift in the airway dose-response curve to inhaled LTD_4 was observed 1 h after ingestion of a 1 g dose of 7, whereas no effect on histamine-induced bronchoconstriction occurred (26). In mild asthmatics with known early and late responses to antigen challenge, the same dose produced only a small protective effect against the early phase response and no effect on the late response (27). L-649,923 (7) was poorly tolerated and adverse effects included abdominal discomfort and diarrhea. The aerosol administered LT antagonist, L-648,051 (8), was evaluated in a trial with nine normal subjects and only partially inhibited LTD_4-induced bronchoconstriction (2).



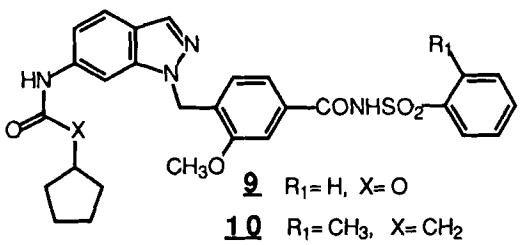
The potent and selective LT antagonist ICI-198,615 (9) demonstrated competitive antagonism of LTD_4 and LTE_4 contractile effects on guinea pig trachea and parenchymal lung strips with pA_2 values of 10.1 to 9.5 (28) and inhibited ^{3}[H]-LTD_4 binding to guinea pig lung receptors with a K_i of 0.27 nM (29). Against LTD_4 induced dyspnea in the guinea pig, 9 was effective by iv, po, or aerosol administration and the pharmacological half-life of the compound after oral administration was in excess of 16 hrs (30). Further development of this series provided an even more potent analog 10 (31). SKF-104353 (11) with stereochemistry similar to LTD_4, had a K_i of 5 nM whereas the enantiomer SKF-104373, was about 35-fold less potent (32). Further characterization showed that 11 inhibited LTD_4 binding to human lung membranes with a K_i of 10 nM. It antagonized LTD_4-induced contraction of guinea pig trachea with a pA_2 of 8.6 but was without effect on LTC_4 elicited contractions. On isolated human bronchial tissue, the compound antagonized contractions induced by LTD_4 with pA_2=8.2 and LTC_4 with pK_b=8.3. Aerosol administered compound inhibited LTD_4-induced bronchoconstriction in anesthetized guinea pigs. Additional pharmacology of 11 has been reported (33).

By combining structural features of 1-alkylimidazoles, known thromboxane synthase inhibitors and pyrido[2,1-b]quinazoline amides with activity as LT antagonists, a series of compounds with dual activity was developed (34). Compound 12 inhibited SRS-A-induced contraction of guinea pig ileum (IC_{50}=1.0 μM) and inhibited TXA_2 synthase (IC_{50}=0.2 μM). It was orally active in reducing bronchoconstriction induced by antigen

(ID_{50}=50 mg/kg), PAF (ID_{50}=13 mg/kg) and histamine (ID_{50}=39 mg/kg). This compound was also active in the rat PCA model but was less potent than the parent carboxylic acid **13**.

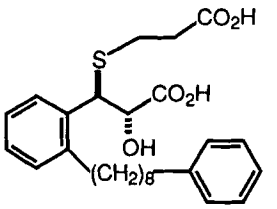
Compounds have been discovered which inhibit leukotriene production and antagonize their actions. The SAR leading to REV-5901 (**14**) was reported (35). It inhibited 5-LO in rat neutrophils with IC_{50} of 0.12 μM and blocked LTC_4-induced contraction of guinea pig lung parenchyma with IC_{50} of 3.6 μM. At 10 mg/kg id, **14** inhibited antigen-induced bronchoconstriction in actively immunized guinea pigs. This compound also inhibited leukotriene release from human lung tissue at 1-10 μM and antigen-induced histamine release at 10 μM (36). In man, **14** provided no protection against histamine- or LTD_4-induced bronchoconstriction (2).

Compound **15**, a 5-LO inhibitor and LTD_4 antagonist was developed from a previous series (37). Two phenylephrine derivatives **16** and **17**, inhibited 5-LO with IC_{50}'s of 2.4 and 19.6 μM and LTD_4-induced bronchoconstriction in guinea pig with ED_{50}'s of 56 and 36 mg/kg po (38). Against LTD_4-induced bronchoconstriction in guinea pigs, WY-48252 (**18**) had an ED_{50} of 0.1 mg/kg by intragastric administration and a pharmacological half-life of 5 hrs (39). This compound antagonized LTD_4-induced contraction of guinea pig trachea with a pKb of 7.6 and also inhibited 5-LO and cyclooxygenase in rat neutrophils with an IC_{50} of 4.6 μM and 3.3 μM, respectively.

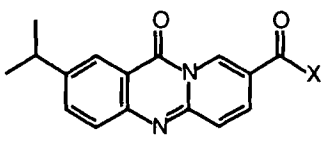


9 R_1= H, X= O

10 R_1= CH_3, X= CH_2

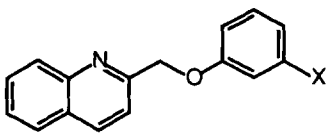


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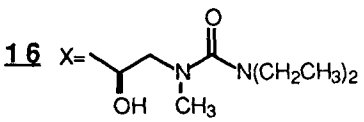


12 X= HN-$(CH_2)_4$-N

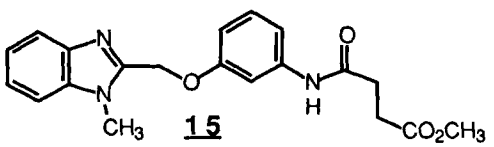
13 X= OH



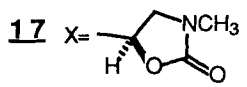
14 X= $CHOH(CH_2)_4CH_3$



16 X=



15



17 X=

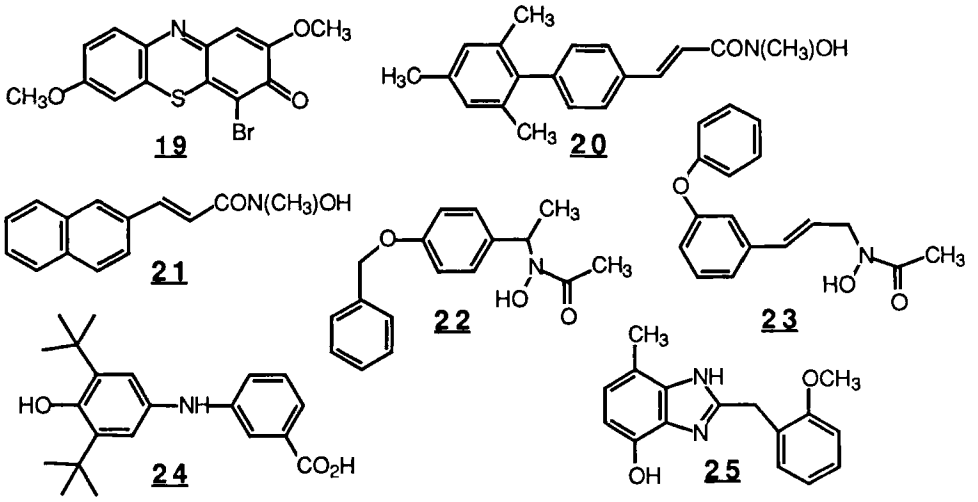
18 X= $NHSO_2CF_3$

Inhibitors of Leukotriene Biosynthesis - A mechanism for the reaction catalyzed by soybean lipoxygenase has been proposed involving concerted deprotonation and electrophilic addition of Fe(III) to carbon, resulting in an organoiron intermediate that reacts by oxygen insertion (40). A chemical model for this process has been demonstrated by the oxygen insertion reaction of allylic tin compounds in the presence of $FeBr_3$ at low temperature (41).

L-651,392 (19) blocked LTB_4 formation in ionophore-challenged rat polymorphonuclear leukocytes with an IC_{50} of 60 nM (42). It inhibited antigen-induced dyspnea in a hyperreactive rat model with ED_{50} of 0.8 mg/kg po (29) and at 5 mg/kg po inhibited early and late pulmonary responses to allergen-induced bronchoconstriction in squirrel monkeys (43).

A series of arylalkyl- and arylalkenyl hydroxamic acids was found to be potent *in vitro* inhibitors of 5-LO (44). For example, the biphenylpropenyl hydroxamate **20** inhibited RBL-1 supernatant 5-LO with IC_{50} of 22 nM. The hydroxamic acid **21** was shown to inhibit leukotriene biosynthesis *in vivo* in a rat peritoneal anaphylaxis model when administered ip, ED_{50} = 0.2 mg/kg; however, it provided only 66% inhibition at 100 mg/kg po (45). Pharmacokinetic evaluation of **21** demonstrated that it was rapidly metabolized to the corresponding inactive carboxylate (iv $T_{1/2}$ <5 min). Structural modifications were examined and 2-arylpropionyl hydroxamates were found to be less prone to this metabolism (45). Moving the alkylaryl group to the nitrogen provided a series of hydroxamates with improved oral activity, as demonstrated for A-63162 (22) with an ED_{50} of 8 mg/kg po in a rat anaphylaxis model (46). From another series of hydroxamate compounds, BW-A4C (23) inhibited LTB_4 synthesis in human PMNL with an IC_{50} of 0.1 μM; in the rat, 50 mg/kg po provided prolonged inhibition of ionophore-stimulated LTB_4 production in an *ex vivo* whole blood assay (47,48). BW A4C (23) also blocked antigen-induced bronchospasm in anesthetized guinea pigs.

S-26431 (24) was reported to be an orally effective 5-LO inhibitor (49). From a series of 1H-benzimidazol-4-ols, compound **25** inhibited 5-LO with an IC_{50} of 3.4 μM and significantly inhibited the release of SRS-A in a rat passive peritoneal model of anaphylaxis at 200 μM ip (50). Nafazatrom given orally (2 x 3 g) was ineffective against antigen-induced bronchoconstriction in man (51). This result was rationalized by the fact that plasma concentrations of drug were too low for effective leukotriene biosynthesis inhibition.

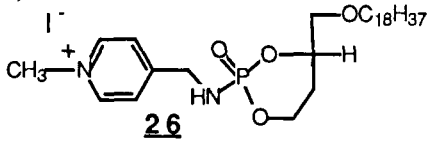


PLATELET ACTIVATING FACTOR (PAF)

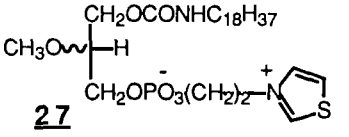
Reviews have appeared summarizing the potential pathophysiological role of PAF (3,52,53) and the properties of PAF antagonists (54). PAF was reported to potentiate the contraction caused by LTD_4 in guinea pig trachea (55). Studies using guinea pig trachea, bronchus, and parenchyma indicated that PAF is not a direct agonist of bronchoconstriction (56). Several compounds from different drug classes afforded protection in PAF-induced

lethality in mice, suggesting that leukotrienes are not the sole mediators of PAF effects (57). PAF induced neutrophil aggregation occurs by two mechanisms: one dependent on and one independent of LTB_4 (58). A screening technique for PAF antagonists using neutrophil elastase release and microtiter plate technology was described (59).

PAF Antagonists - The cyclic analog **26** had similar potency as CV-3988 (**27**) for inhibition of PAF-induced rabbit platelet aggregation with an IC_{50} of 2-4 μM (60). A series of ether-chain homologs of PAF, from C1 to C20 was synthesized and their agonist activity was found to be related to the hydrophobicity of the chain with C_{16}-C_{18} providing maximum activity (61). Replacement of the ether oxygen with methylene or sulfur or by an ester function dramatically decreased potency. The 2-methyl analog of lyso-PAF and 2-methoxy-PAF were found to have no effect on rabbit platelets and no hypotensive effects in rats (62).

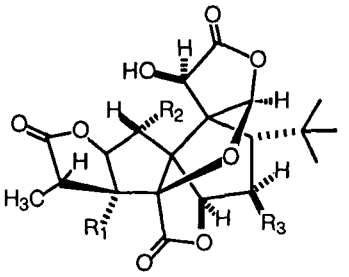


26



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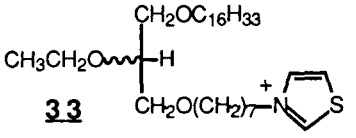
Ginkgolide B (BN 52021) (**29**), a natural product isolated from *Ginkgo biloba* leaves, is an antagonist of PAF which was used to elucidate the pathophysiological role of PAF in guinea pig passive anaphylaxis models (63). An X-ray crystal analysis of the monohydrate was reported (64) and the first total synthesis accomplished (65). BN 52063, a mixture of Ginkgolides A (**28**), B (**29**), and C (**30**) was used in the first study demonstrating efficacy in man of a PAF antagonist against the immediate response to inhaled allergen challenge in asthmatics (66). SRI-63-441 (**31**) was shown to antagonize lung responses to PAF but was inactive against antigen-induced bronchoconstriction in Rhesus monkeys (67). Pharmacological characterization of CV-6209 (**32**) showed it to be more active than **27**, ONO-6240 (**33**), **29**, and the anxiolylic etizolam (68). Differences in the abilities of PAF antagonists to prevent the actions of PAF on platelet and non-platelet tissues has led to the suggestion that there is more than one type of PAF receptor (69,70). L-652,731 (**34**), **27**, triazolam and alprazolam exhibited inhibition of PAF-induced aggregation with IC_{50}'s of 0.2, 0.1, 1.5, and 6.5 μM, respectively and serotonin secretion from rabbit platelets with IC_{50}'s of 0.05, 0.15, 0.6 and 2.5 μM, respectively (70). L-652,731 (**34**) was effective in antagonizing PAF effects in rats (71) and blocked pulmonary hypertension in sheep induced by PAF but not by complement (72).



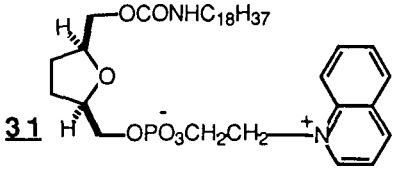
28 R_1= OH, R_2=R_3= H

29 R_1= R_2= OH, R_3= H

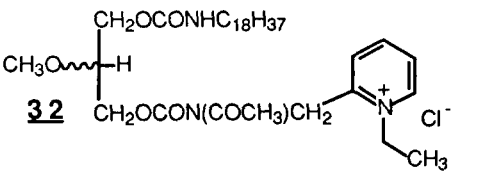
30 R_1=R_2=R_3= OH



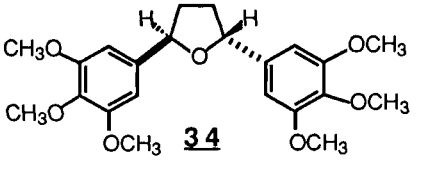
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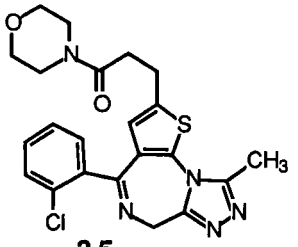
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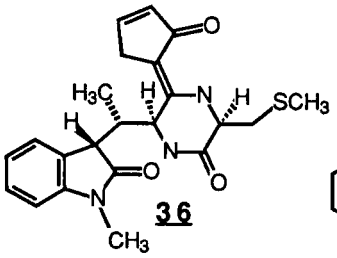
34

Etizolam was found to be a potent PAF antagonist with an IC_{50} of 1.3 μg/ml (73). Related compounds, brotizolam and triazolam inhibited PAF-induced human platelet aggregation with IC_{50}'s of 0.54 and 7.6 μM, respectively and inhibited intrathoracic aggregation of platelets in anesthetized guinea pigs induced by iv PAF with ED_{50}'s = 5 and 50 mg/kg po, respectively (74). Brotizolam also inhibited bronchoconstriction, systemic hypotension and the lethal effect of iv PAF in guinea pigs. Benzodiazepines lacking the triazole ring had little or no effect against PAF *in vitro*. WEB 2086 (**35**) was reported to block the effects of PAF in guinea pig lungs but not the anaphylactic response to antigen (75). In another study, **35** was shown to be more effective in blocking antigen-induced bronchoconstriction in passively sensitized guinea pigs than actively sensitized animals (76). This compound also blocked PAF-induced changes in isolated rat lung (77).

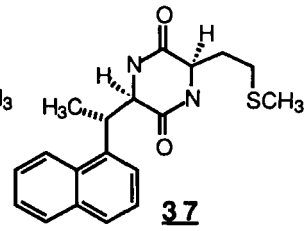
The structure of FR-900452 (**36**) a specific inhibitor of PAF-induced rabbit platelet aggregation with an IC_{50} of 0.37 μM, has been deduced by X-ray crystal analysis of the C20,21 dihydro derivative (78). A series of simplified analogs of **36** was synthesized and **37** had an IC_{50} of 0.18 μM (79). Another diketopiperazine derivative **38**, had an IC_{50} of 0.69 μM (80). The sesquiterpene, L-652,469 (**39**) was reported to be a PAF antagonist and Ca^{+2} blocker with a K_i 1-4 μM for both [^{3}H]PAF and [^{3}H]nitrendipine binding (81). It inhibited rabbit platelet aggregation and was also orally active in inhibiting PAF-induced rat paw edema and the first phase of carrageenan-induced rat paw edema. An association of PAF receptors and calcium channels was suggested by the inhibition of PAF-induced platelet activation by calcium channel blockers, diltiazem and verapamil (82).



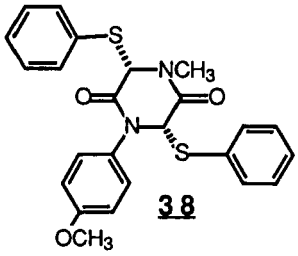
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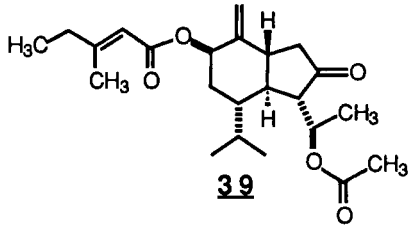
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37



38

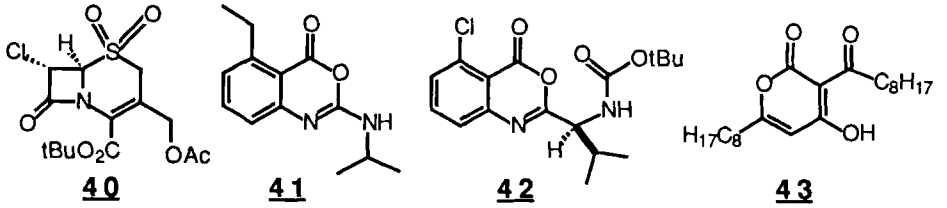


39

MISCELLANEOUS AGENTS

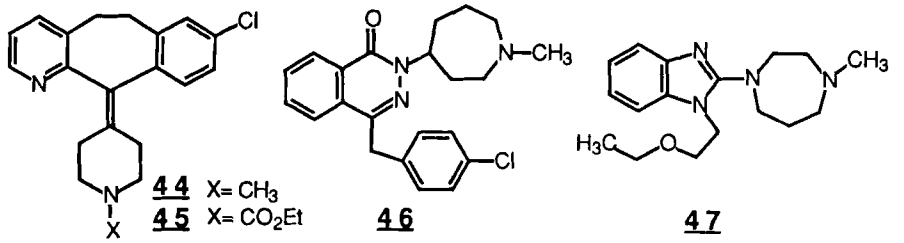
Inhibitors of Elastase - Inhibition of the proteolytic enzyme elastase as a biochemically based approach to develop agents to treat chronic pulmonary diseases such as emphysema has been reviewed (83-85). Biochemical efficacy of $alpha_1$-antitrypsin replacement therapy in deficient humans was demonstrated (86). Human leukocyte elastase (HLE) has been crystallized and data concerning its tertiary structure should be forthcoming (87). The amino acid sequence of HLE is 43% homologous to porcine pancreatic elastase (88). A crystallographic study of the complex of the irreversible beta-lactam inhibitor **40** and porcine pancreatic elastase has been reported (89). Two covalent bonds between inhibitor and enzyme are formed and a mechanism for this inhibition was discussed. A kinetic study of peptidyl trifluoromethyl ketone inhibitors of HLE revealed a slow-binding inhibition

involving reaction of a serine residue in the enzyme with the trifluoromethyl ketone to form a hemiacetal intermediate complex (90). The inhibitor Z-Lys(Z)-Val-Pro-Val-CF_3 had a K_i of 0.1 nM. A study of various 2-(alkylamino)-benzoxazinones led to **41** with K_i of 0.94 nM (91). The benzoxazinone derivative **42** is a competitive, slow-binding, inhibitor of HLE with K_i of 0.5 nM (92). Evaluation of a series of 2-pyrone analogues of elasnin led to **43** with a K_i of 4.6 μM for HLE (93).



40 **41** **42** **43**

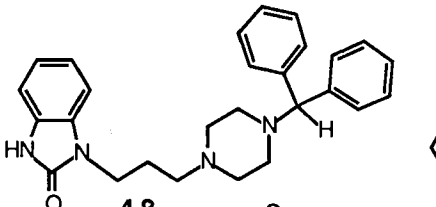
Antihistamines - The role of histamine in some of the manifestations of allergic reactions is well known; however, its role in pulmonary airway disease is controversial and as yet ill-defined. Terfenadine was tested in asthmatics, at 60, 120 and 180 mg and shifted the bronchoconstrictive response to histamine 14.8, 22.9, and 34-fold but had no effect on methacholine challenge (94). In a clinical study of the response of the nasal mucosa, topical administration of azatadine (**44**) was effective against histamine-challenge, but not cold dry air, suggesting different mechanisms of mediator release by these conditions (95). Modification of **44** led to loratidine (**45**) which was devoid of sedating activity and retained antihistamine activity in animal models (96,97). In man loratidine did not affect coordination, visual acuity, memory, or mood (98). The potent histamine H_1 receptor antagonist, azelastine (**46**) was reported to have activity as a leukotriene antagonist and also inhibited ionophore-induced release of leukotrienes from human PMNL (99). LY-188695 (KB-2413) (**47**) was found to have potent oral activity against histamine-induced mortality in guinea pigs, ED_{50} of 4.4 μg/kg (100). In histamine challenged guinea pigs, **47** was active as an aerosol (101) and was effective against antigen-challenge in guinea pigs (102).



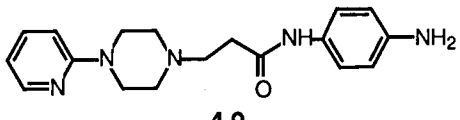
44 X= CH_3
45 X= CO_2Et
46 **47**

Mediator Release Inhibitors - Disodium cromoglycate (cromolyn) remains an important antiallergic agent (103). Recent investigations suggested that cromolyn acts as an anti-PAF agent (3) and/or that it inhibits activation of inflammatory cells by decreasing the formation of complement and IgG receptors and preventing oxidative burst (104). A review of the mast cell's role in bronchospasm described the case for mast cell heterogeneity and indicated that interactions of other cells with mast cells may be very important (105). Oxatomide (**48**) was shown to be more potent than cromolyn, ketotifen or tranilast in inhibiting the release of PGD_2 or LTC_4 from guinea pig lung fragments and rat peritoneal cells stimulated with ionophore or antigen (106). The 1-(2-pyridinyl)-piperazine **49** showed activity in rat PCA, histamine-induced bronchospasm in the guinea pig and rat mesenteric mast cell degranulation induced by compound 48/80 (107).

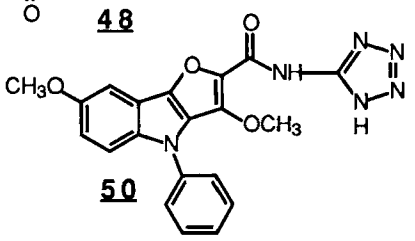
CI-922 (**50**) prevented antigen-induced secretion of histamine and LT from actively sensitized human basophils and guinea pig cells and tissues with an IC_{50} <15 μM (108). REV 2871 (**51**) was reported as an orally effective inhibitor of mediator release with an irreversible inhibition mechanism; **51** also inhibited PAF-induced histamine release (109).



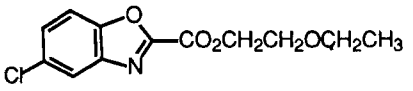
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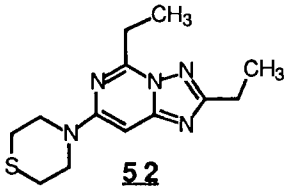


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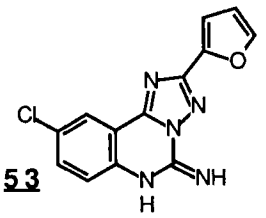


51

Bronchodilators - R-836 (**52**) is a relatively potent bronchodilator, active in several guinea pig models of bronchoconstriction with the potential for less side effects compared to theophyline (110). CGS 15943 (**53**) is a non-xanthine adenosine antagonist having no phosphodiesterase inhibitory activity (111).

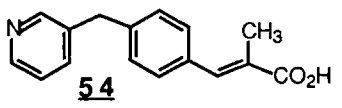


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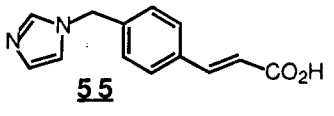


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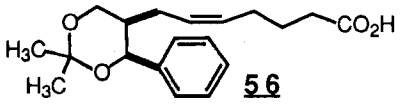
Thromboxane Antagonists and Biosynthesis Inhibitors - Although this type of agent was initially thought to be useful for cardiovascular diseases, there is now evidence that either biosynthetic inhibitors or receptor blockers could be useful in asthma. OKY-1581 (**54**) was shown to block bronchoconstrictor responses to arachidonic acid in the cat but not to PGH_2 (112). OKY-046 (**55**), a thromboxane synthesis inhibitor, inhibited IgE and ionophore induced histamine release (113). In a two week clinical trial in asthmatics, with a 400 mg dose of **55** , bronchial response to LTD_4 was assessed before and after dosing. PD_{20} values were significantly reduced after dosing and the compound showed no direct bronchodilating effect. It was concluded that the drug can diminish bronchial hyper-reactivity.



54



55



56

The phenyldioxanealkenoic carboxylate **56** is a specific, competitive thromboxane A_2 receptor antagonist (114) and showed pA_2= 6.3, 6.84, and 6.58 for antagonism of U46619 -induced contraction of isolated rabbit and rat aorta, and guinea pig trachea, respectively. At 5 mg/kg iv, **56** inhibited bronchoconstriction induced by U46619 in anesthetized guinea pigs for over 2 hr.

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2. N. Barnes, J. Evans, J. Zakrzewski, P. Piper, J. Costello, International Conference on Drugs Affecting Leukotrienes and Lipoxygenase Products; A Potential Fulfilled?, IBC Technical Services Ltd.: London, Sept. 30-Oct.1 (1987).
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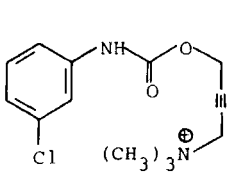
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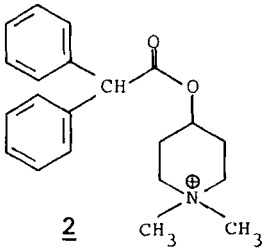
Chapter 9. Peripheral Actions of Selective Muscarinic Agonists and Antagonists

G. Mihm and B. Wetzel, DR. KARL THOMAE GMBH,
Biberach an der Riss, West Germany

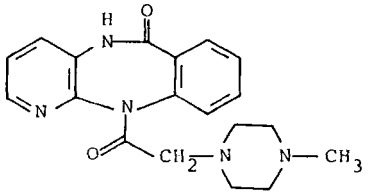
Introduction - Interest in agents with a selective action at peripheral muscarinic receptors (mAChr) and pioneering work towards receptor subclassification (1-5) was initiated by the discovery of the first selective agonists, such as McN-A 343 (1), and selective antagonists, such as 4-DAMP (2).



1



2



3

However, it was not until the synthesis of the antiulcer-drug pirenzepine (PZ, 3) that the concept of heterogeneity of the muscarinic system was generally accepted (6,7).

The progress which has been made in understanding the muscarinic system is reflected by several symposia dealing with the general pharmacology of the muscarinic system (8), muscarinic receptor subtypes in the GI-tract (9) and heart (10). Reviews have been published on muscarinic receptor subtypes (11,12), subtypes in CNS (13), cardiovascular system (14) and lung (15).

Receptor Subtypes and Structure - M_1-receptors are now commonly defined as binding sites with high affinity for PZ and are mainly located in neuronal tissue. In contrast, M_2-receptors are defined as binding sites with a low affinity for 3. In the periphery they are predominantly located in the effector cells of the heart, smooth muscle and glands. Recent results based on the properties of novel selective antagonists provide further evidence for heterogeneity of the M_2-receptor system. Thus the mAChr in the glands might represent a distinct receptor subtype (16-18). Concomitantly several proposals for receptor nomenclature have been made (11, 16-19). However, to date there is no common, generally accepted nomenclature for the different binding sites with low affinity for PZ. In this review these sites will therefore be subdivided into cardiac, glandular and ileal M_2-receptors.

The concept of heterogeneity of the muscarinic system, relying mainly on the different binding behaviour of

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selective ligands, has recently been confirmed by the elucidation of structural and molecular properties of mAChr-subtypes. The results of these studies have been summarized in several reviews (20-23).

In order to obtain information on the question whether heterogenous binding behaviour of mAChr-subtypes is the consequence of intrinsic properties of the receptor proteins or coupling to effector mechanisms, detergent solubilized mAChrs have been studied. Thus solubilization of the low affinity PZ binding site from rat myocardium results in increased PZ binding and suggests an influence of the membrane environments on PZ binding (24). Conversely the high affinity PZ binding site from rat cortex can be solubilized without change of affinity (25). A similar behaviour has been observed for the M_1-receptor of bovine cortex and the M_2-receptor from pons medulla (26).

The apparent molecular masses of the purified mAChrs from brain or heart range from 70000 to 95800 daltons (20, 27,28). An extensive study of the cardiac mAChr revealed a molecular mass of 50000-60000 daltons for the protein portion alone and 68000-71000 daltons including carbohydrate (29).

The amino acid composition of porcine brain and heart mAChrs have been determined. The two receptors showed sequence differences (29,30) which have been confirmed by the cloning and sequencing of the mAChrs from pig brain (31) and heart (32,33). Expression of the cDNA produces functional mAChr in Xenopus oocytes which differ in their binding behaviour towards selective antagonists. Thus the brain receptor displays high affinity for PZ (M_1-type) (31) whereas the cardiac receptor shows higher affinity for AF-DX 116, the prototype of an M_2-antagonist (34).

In the rat four functional mAChr genes encoding proteins of 460, 466, 478 and 589 amino acids have been defined (35), supporting the hypothesis of a further heterogeneity of the muscarinic system.

Based on hydropathy data of the receptor proteins a model of the receptor has been proposed (23), containing seven transmembrane spanning segments. The acidic residues, responsible for ligand binding are suggested to be located within the first three transmembrane helices (36).

M_1-Antagonists - The pharmacological properties and therapeutic efficacy of pirenzepine, the first M_1-antagonist introduced into ulcer-therapy, have been reviewed (37). PZ significantly reduces the relapse rate of duodenal ulcers (38) as well as symptoms of reflux oesophagitis (39) without cholinergic side-effects. Simultaneous blockade of M_1- and H_2-receptors by administration of a combination of 50 mg 3 plus 150 mg ranitidine resulted in a significant suppression of meal-stimulated acid secretion without effects on gastrin release. It has been suggested that this may be useful in the treatment of the Zollinger-Ellison syndrome (40). There has been some debate on the reasons for the

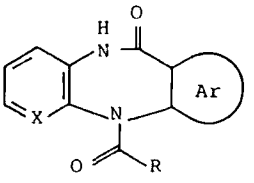
selective inhibition by 3 of gastric acid secretion (41). In addition to this activity, PZ exerts cytoprotective effects and enhances gastric mucosal defenses presumably via an improvement of the gastric mucosal microcirculation (42).

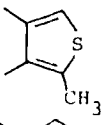
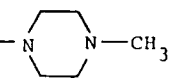
Despite its tricyclic structure with close resemblance to psychotropic agents like imipramine, 3 is devoid of any antidepressive activity (43). The structural requirements for selectivity towards the muscarinic receptor have been discussed as well as those for M_1-selectivity. Thus the amide bond in the central ring of PZ is essential for selective actions at the mAChr (44). On the other hand receptor selectivity within the muscarinic system is mainly influenced by the side chain. Only groups with restricted conformational flexibility exhibit M_1-selectivity (45).

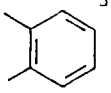
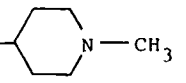
In man, PZ (10-20 mg/kg i.v.) results in reduction of airway resistance, mainly in the peripheral airways (46,47). This corresponds with experiments in rabbits, where 75% of the muscarinic binding sites in peripheral lung are of the M_1-type (48). The inhibitory action of 3 on vagally induced bronchoconstriction seemed to be the result of blockade of M_1-receptors located on peribronchial, parasympathetic ganglia (49). Conversely, the muscarinic receptors of smooth muscles and tracheal mucosa in the airways of several species have been shown to exhibit only low affinity for PZ both *in vitro* (50) and in receptor binding studies (51,52).

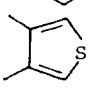
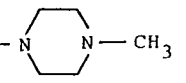
PZ has been used to identify muscarinic receptors involved in GABA-release in guinea pig small intestine (53) and ACh-release in guinea pig ileum (54) as M_1-receptors.

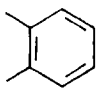
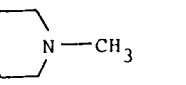
Several compounds structurally related to 3 and with a similar pharmacological profile have been described. Telenzepine (4) is now in clinical trials. Its inhibitory action on acid secretion is significantly stronger than the action of 3. However the clinical application of 4 may be limited by the closely related inhibition of salivation (55). In addition to its higher potency as compared to 3, UH-AH 37 (5) has an improved bioavailability (56). DF-754 (6) and DF-545 (7) are other PZ-analogs with similar antiulcer activity (57).



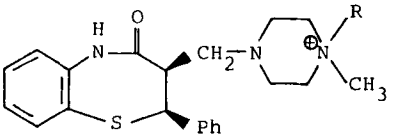
X = CH Ar =  R =  4

= C-Cl =  =  5

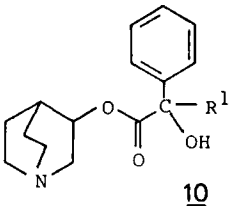
= N =  =  6

= CH =  =  7

Structurally unrelated to tricyclic PZ-analogs are the M_1-antagonists BTM 1086 (8) or BTM 1073 (9). In competi-



R = H : 8
R = C_4H_9: 9



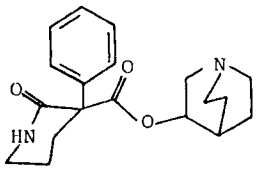
R^1 = H, CH_3, Et,

10

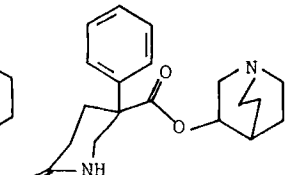
tion experiments using ^{3}H-QNB as radioligand, 8 exhibits higher affinity to mAChr of the cortex than to ileal M_2-receptors. The quaternary compound 9 shows a decreased selectivity (58).

The quinuclidine derivatives 10 display preferential affinity to M_1-receptors of the brain in contrast to cardiac M_2-receptors (59). Selectivity and affinity are strongly dependent on the stereochemistry at the chiral centers. Thus the RR-enantiomer of 10 (R^1 = CH_3) proved to be the most active compound with a 25-fold higher affinity toward the M_1-receptor (60).

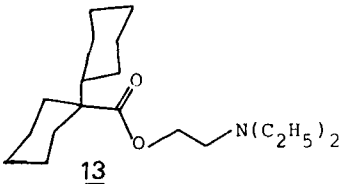
M_1-selectivity has also been claimed for the phenylpiperidones DA 5508 (11) and DA 5591 (12). Competition



11



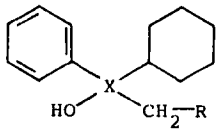
12

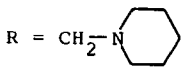


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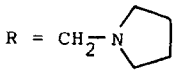
experiments reveal a 36 and 62 fold higher affinity at the M_1-receptor for 11 and 12, respectively (61).

Dicyclomine (13) and benzhexol (14) also exhibit a higher affinity to M_1-receptor sites compared to the M_2-receptors of the myocardium (62,63). However, in contrast to 3, analysis of the binding curves of 13 and 14 for the cortical receptor indicates positive cooperative binding behaviour. Moreover 13 and 14 also show remarkable affinity for the glandular M_2-receptor (63). A similar

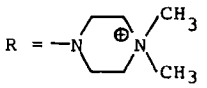




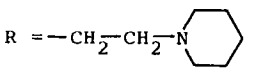
14, X = C



15, X = C ; 16, X = Si ;

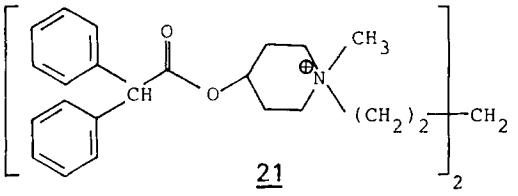


17, X = C ; 18, X = Si ;



19, X = C ; 20, X = Si

selectivity profile has been observed using the derivatives 15-20 of the antiparkinson drugs difenidol and procyclidine. Despite of their M_1-activity 14-20 have been valuable tools for the further subclassification of the M_2-receptor system. They exhibit higher affinity for the ileal M_2-receptors compared to those of the heart (19,64, 65); 18 was about six times more potent in urinary bladder preparations than in ileal or tracheal smooth muscle (65).

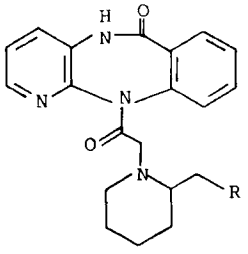


21

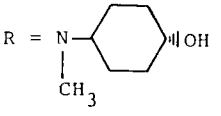
Finally, 4-DAMP and its dimer (21) represent further examples of compounds with high affinity for the ileal M_2-receptors (66).

In addition to their importance for the subclassification of the M_2-receptor system, 17-20 also show different behavior on M_1-receptors of hippocampus and cervical ganglia. Therefore it has been suggested that the M_1-receptor system itself is heterogenous (19).

M2-Antagonists - AF-DX 116 (22) was the first competitive, cardioselective M_2-antagonist (8,16,67). In competition experiments this compound shows high affinity to the cardiac mAChr ($K_D \sim 115$ nM), intermediate affinity to M_1-receptors in neuronal tissue ($K_D \sim 700$ nM) and low affinity to the receptors in the glands ($K_D \sim 3200$ nM), thus providing evidence for heterogeneity of the M_2-system (16). Pharmacological studies confirmed this cardioselectivity (67). AF-DX 116 is currently in phase II clinical trials for the treatment of AV-block or sinus bradycardia. The competitive action of tricyclic compounds such as 22 or its derivative AF-DX 237 (23) clearly distinguishes

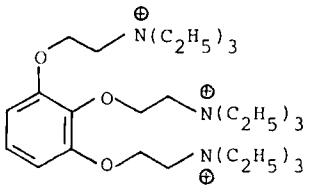


R = NEt_2 : 22

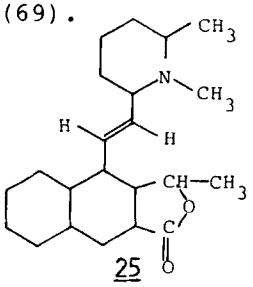


23

these compounds from gallamine (24), an allosteric modulator of receptor binding with pronounced cardioselectivity (10,16, 68). Conformational calculations trying to elucidate the structural requirements for M_2-selectivity in this class of compounds have been published (69).



24

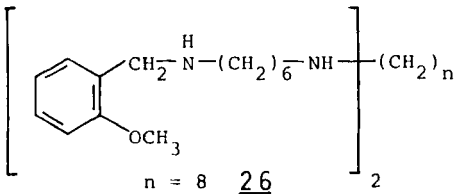


25

AF-DX 116 was used in competition experiments to identify the receptors in bovine tracheal smooth muscle as M_2-cardiac receptors (70). In functional studies however, 22 possesses a lower affinity to tracheal smooth muscles in comparison to cardiac receptors (67).

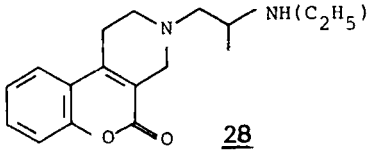
Himbacine (25), an alkaloid isolated from the bark of galbulimima species, is another cardioselective muscarinic antagonist with 10fold higher activity at the atrial mAChr in comparison to the ileum (71).

Recently a novel class of compounds has been described exhibiting selective affinity to the cardiac mAChr (72). The pA_2-values of methoctramine (26) or BHC-9C (27) on smooth muscle preparations are two orders of magnitude lower than in the heart and in vivo experiments have confirmed these findings (73, 74). However at higher concentrations (1 µM) allosteric interactions could be observed (75).



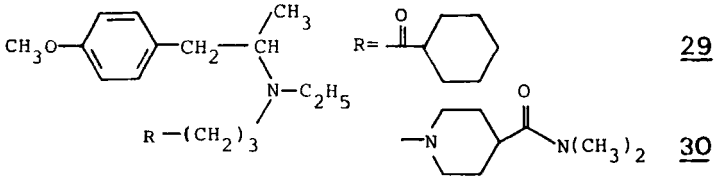
n = 8 26
n = 9 27

CI 923 (28) has been claimed to act selectively at the tracheal smooth muscle (ID_{50} = 210 µg/kg) as compared to salivary glands (ID_{50} = 1232 µg/kg) (76). In vitro experiments revealed a 3-4 fold higher activity of 28 at tracheal smooth muscle than at ileum and urinary bladder (65).



28

The specific action of secoverine (29) or idaverine (30) on smooth muscles cannot be explained by selective actions on mAChr-subtypes. It has been shown that the compounds are not able to differentiate between M_1- and M_2-receptors in competition experiments as well as in in vivo and in vitro studies (77-79).



29

30

Constriction as well as relaxation of vascular smooth muscle can be elicited by ACh, depending on the presence of an intact endothelium (14). The vascular muscarinic receptor mediating endothelium dependent relaxation has been shown to exhibit rather low affinity for PZ both in vitro (80,81) and in competition experiments (80,82,83). However species differences (84) as well as prejunctional M_2-receptors and postjunctional M_1-receptors have been observed (85).

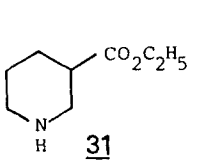
AF-DX 116 (67,86), 4-DAMP and gallamine (81) have been used both in vivo and in vitro to further characterize the

vascular mAChr. The results indicate differences between this receptor and the cardiac mAChr.

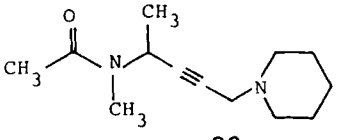
Muscarinic Agonists - Research on muscarinic agonists is mainly focussing on their action in the CNS with the hope of treating patients suffering from Alzheimer's disease (see Chapter 4). Thus, newer data on peripheral actions of agonists are relatively sparse, mainly due to the lack of selective agents. In addition, the interpretation of their action is difficult because factors such as receptor reserve or receptor-effector coupling mechanisms play an important role.

Non-selective peripheral agonists are currently used as stimulants of the smooth muscle of the GI-tract and the urinary bladder as well as for the treatment of glaucoma. It has been suggested that cardioselective muscarinic agonists might be protective for patients with coronary artery disease (87).

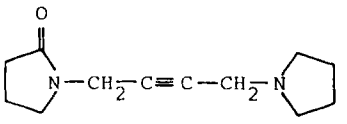
R-Ethylnipecotinate (31) behaves as an agonist for the M_2-receptor subtype and as an antagonist at M_1-sites (88). A mixed agonist/antagonist activity is also shown by



31



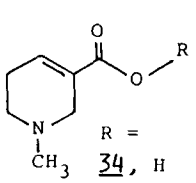
32



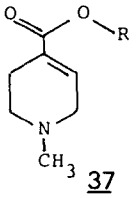
33

BM 5 (32), acting as a presynaptic antagonist and postsynaptic agonist (89). However, no selectivity within the muscarinic system could be observed. In addition to 32, several other analogs of oxotremorine (33) have been used to elucidate the possibility of achieving selective muscarinic actions even in the absence of distinct receptor subtypes (90).

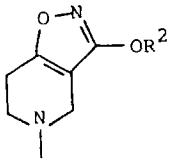
A series of arecaidine (34) and isoarecaidine esters (37) has been studied for their ability to stimulate ganglionic M_1-receptors. The propargylderivative (APE, 35)



R =
34, H
35, $CH_2-C\equiv CH$
36, $CH(C_6H_5)-C\equiv CH$



37



38

proved to be one of the most potent compounds (91). No differentiation between M_1 and M_2 could be observed. On the other hand, introduction of a phenyl group (36) leads to potent antagonistic activity at M_2-receptors (92).

The arecoline-bioisosteres (38) exhibit strong activity at ileal mAChr; however no data about their M_1/M_2-selectivity are available (93). McN-A 343 1, frequently used as an M_1-selective agonist, has been shown to be not selective in terms of affinity (94). The previously reported selectivity of 1 may be due to intrinsic efficacy or tissue receptor reserve.

Receptor-Effector-Coupling - Stimulation of the mAChr results in activation of various second messenger systems including inhibition of adenylate cyclase, hydrolysis of phosphoinositides (PI) or modulation of ion conductances (95,96). However, to date there is no clear evidence for a selective coupling of one second messenger system to a distinct mAChr-subtype (95).

Conclusion - The discovery of various selective ligands, the recent elucidation of the amino acid sequence of several receptor subtypes, and the increasing knowledge about the mechanisms of signal transduction have led to a better understanding of the muscarinic system. It can be anticipated that this will result in the synthesis of new compounds with interesting selectivity profiles as well as valuable therapeutics. These might be beneficial in the treatment of various diseases where the use of anticholinergic compounds is often limited by their side effects. Thus, cardioselective M_2-antagonists could be used for the long term treatment of patients with sinus bradycardia or AV blockade and might replace pacemaker implantation for some patients, whereas compounds with a selective action at smooth muscle receptors might be useful spasmolytics in the GI-tract and the airways.

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Chapter 10. Vasopressin Antagonists

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Introduction - Vasopressin (1) or one of its phylogenetic precursors is found in all vertebrates and participates in the regulation of cardiovascular homeostasis. The identification of receptor-selective antagonists has furthered the characterization of the physiological and pathophysiological roles of vasopressin (1,2). The purpose of this chapter is to 1) present the rationale for a therapeutic interest in vasopressin antagonists, 2) describe the current status of animal models for the discovery of antagonists and 3) summarize the structural features present in antagonists.

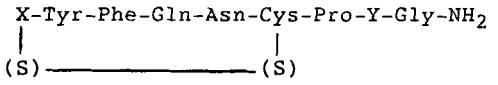
Pharmacology of Vasopressin - Although vasopressin has been implicated in many biological processes, its principal physiological action is the regulation of body fluid tonicity and volume (3). These actions are mediated by two types of receptors. The antidiuretic or V_2 receptor, is located on the basolateral membranes of renal collecting duct epithelia and is coupled to adenylate cyclase via stimulatory guanine nucleotide regulatory proteins (4,5). Activation of V_2 receptors leads to intracellular accumulation of cAMP, an increase in the water permeability of the mucosal membrane surface, and an increase in transepithelial reabsorption of water in the presence of an osmotic gradient (6). Recent evidence suggests the presence of an extrarenal V_2 type receptor located in the peripheral vasculature (7,8). This receptor is apparently stimulated by pharmacological doses of V_2 agonists and is associated with vasodilation, flushing and release of clotting factors (Factor VIIIc, Von Willebrand's Factor) in plasma (8).

A second receptor subtype, the vasopressor or V_1 receptor, is located in the vasculature (9) and liver (10) and was recently identified in the adenohypophysis (11), the brain hippocampus (12), and seminal vesicles (13) of mammals. V_1 receptors mediate activation of phosphatidylinositol bisphosphate hydrolysis, which, via the formation of inositol 1,4,5-trisphosphate, causes an increase in free cytosolic calcium (14). V_1 receptors are not coupled to adenylate cyclase but can inhibit cAMP and cGMP formation (15,16). Stimulation of V_1 receptors leads to an array of physiological effects, including vasoconstriction (9), stimulation of glycogenolysis and gluconeogenesis (17), lipogenesis (18,19), aggregation of platelets (20), and a variety of activities in the central nervous system (21).

While the pressor activity of vasopressin is not thought to play a major role in the regulation of normal blood pressure or the control of normal circulatory hemodynamics (22), stimulation (or lack of stimulation) of V_1 or V_2 receptors has been suggested to contribute to a spectrum of pathophysiological processes. Stimulation of peripheral and/or central V_1 receptors has been suggested to

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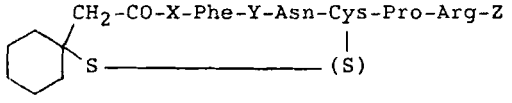
contribute to the development and/or maintenance of hypertension, congestive heart failure, adrenal insufficiency and brain edema in experimental animals (6). Stimulation of V_2 receptors can produce inappropriate fluid retention leading to edema and hyponatremia in experimental animals and in humans (23). Insufficient V_2 receptor activity results in a condition of diabetes insipidus and compensatory thirst or polydypsia (24).



compound	X	Y
1	Cys	Arg
2	Mpr*	D-Arg
3	Cys	Lys

*Mpr = mercaptopropionic acid

Clinical Studies with Vasopressin Agonists and Antagonists - Vasopressin and analogs have been studied for a variety of clinical applications. V_2 agonists (AVP, 1; dDAVP, 2) are used to stimulate renal concentrating ability and ameliorate polyuric conditions (25). High doses of the V_2 selective agonist dDAVP are used to stimulate production of clotting factors in mild hemophilia and in uremic bleeding disorders (8,26). Stimulation of V_1 receptors with AVP or LVP (3) is used to staunch bleeding along the gastrointestinal tract (27,28). Vasopressin receptor antagonists have also been studied for potential clinical applications, although none are currently approved for general use. The "Manning Antagonist", [Pmp^1,$Tyr(Me)^2$]AVP, (4), a potent in vivo pressor antagonist (29) and a V_1 receptor antagonist (30), has been evaluated for efficacy in hypertension (31), congestive heart failure (32,33) and end-stage renal disease (34); antihypertensive and vasodilatory activity was only demonstrable in the most severely ill patients with congestive heart failure and end-stage renal disease. Vasopressin antagonist 4 was effective in reversing vasoconstriction associated with administration of exogenous vasopressin and cigarette smoking (34,35). The combined results of these studies do not support a major pathophysiological role of V_1 receptor stimulation in human hypertension, congestive heart failure and renal failure.



compound	X	Y	Z
4	Tyr(Me)	Gln	Gly-NH_2
5	D-Tyr(Et)	Val	NH_2

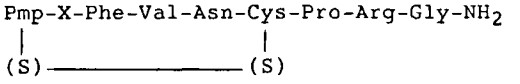
Another V_1 receptor antagonist, (1-deaminocysteine, 2-O-ethyl-D-tyrosine, 3-isoleucine, 4-threonine, 8-ornithine)vasopressin (36), has been studied for efficacy in primary dysmenorrhea (37). It is

not clear if the clinical pharmacology of this antagonist is the result of its V_1 or oxytocin (OT) receptor blockade. This molecule, as well as analog **4**, is also a potent antagonist at OT receptors.

SK&F 101926, (**5**), a potent V_2 receptor antagonist (38,39), was recently tested in human volunteers and shown to be a full antidiuretic agonist (40,41). This result was surprising since **5** was a potent *in vivo* antagonist, with no apparent agonist activity, in rat (38,42), dog (6) and squirrel monkey (43). Similar results were obtained *in vitro* in studies at the renal receptor in rat, pig, dog, monkey and human tissue (6). The hypothesis that V_2 receptor antagonists might be therapeutically beneficial in hypertension and various hyponatremic states awaits the development of clinically-effective V_2 antagonists.

Pharmacologic Models - *In vitro* vasopressin binding and stimulation of vasopressin-dependent adenylate cyclase in animal and human renal membranes and microdissected renal tubules have been employed for the design of vasopressin receptor antagonists (6,44). Unfortunately, *in vitro* adenylate cyclase determinations have proved to be particularly insensitive to low level partial agonist activity; indeed, adenylate cyclase determinations in human renal membranes and in isolated tubule segments failed to predict the *in vivo* agonist activity of SK&F 101926 (6,41). Differences in sensitivity have been observed between plasma membrane and isolated tubule or cultured cell preparations (45). Thus, the value of current *in vitro* assays for V_2 receptor activity is primarily as an index of affinity of particular structures for V_2 receptors. While *in vitro* agonist activity is likely reflected in antidiuresis *in vivo*, *in vitro* antagonism does not reliably predict diuresis (V_2 antagonism) *in vivo*.

The vast majority of *in vivo* structure-activity data has been developed in rat (6). Somewhat late in the evolution of vasopressin antagonists it was discovered that several key substitutions that afford potent antagonists in rat lead to minimal V_2 antagonist activity in dog and monkey (39,42). Differences in apparent receptor recognition patterns, as exemplified in compounds **6-9**, have been reported for most species studied (6). In addition, the various rat, dog, pig and



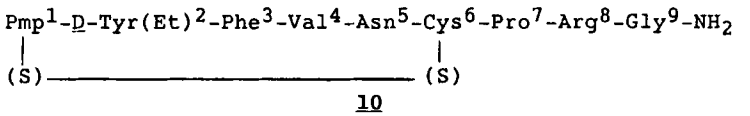
Compound	X	V_2 Receptor K_i (nM) Rat	Pig	Human
6	D-Tyr(Et) ($desGly^9$)	0.4	3.0	2.3
7	D-Phe	5.9	47	23
8	Leu	84	2260	1830
9	D-Leu (Ile^4)	100	4000	1100

squirrel monkey models do not predict the agonist activity of SK&F 101926 encountered in humans. In contrast, in the rhesus monkey, SK&F 101926 behaves as a full agonist (46). Recently, cyclooxygenase blockade has been shown to increase the sensitivity to the antidiuretic activity of vasopressin and the partial agonist activity of vasopressin antagonists in dogs (6,47). This has led to the hypothesis that endogenous

prostaglandin tone may be a critical factor in determining the sensitivity of vasopressin V_2 receptor/effector coupling in experimental animals and man (41,45).

Structural Features of Vasopressin V_2 Receptor Antagonists - It is not clear whether the agonist activity observed with SK&F 101926 (5) is the result of intrinsic activity at the renal V_2 receptor or is produced by other vasopressin-related actions such as vascular V_1 receptor mediated changes in prostaglandin tone (48), or perhaps interaction with some as yet uncharacterized receptor. SK&F 101926, like most V_2 receptor antagonists, is actually non-selective in that it is a potent antagonist of V_1, V_2 and OT receptors. If the clinically observed agonism is the result of intrinsic agonist activity, the problem would be simplified if one could use the extensive SAR for V_2 receptor agonists to predict the residual intrinsic activity in antagonists. However, a number of reports have appeared which indicate that agonists and antagonists at the V_2 receptor have substantively different pharmacophores (49,50,51). Those chemical features which contribute to partial agonist activity can potentially be inferred from evaluation in the newly discovered indomethacin-pretreated, water-loaded dog (52) and rhesus monkey (46) models.

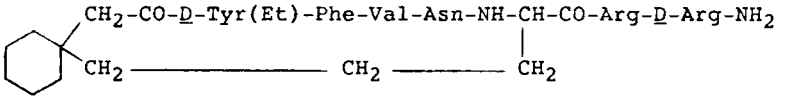
The key structural features compatible with potent V_2 receptor antagonists of the general structure 10 (53) are presented below along with any partial agonist information determined in either the dog or rhesus monkey models. Finally, those areas in which agonist and antagonist pharmacophores appear to differ will also be discussed.



10

Position One - The presence of the bulky ß,ß-cyclopentamethylene-ß-mercaptopropionic acid group (Pmp) at position one is a key feature of almost all V_2 antagonists. The size of the ring may vary somewhat with retention of antagonist activity; however, optimal activity is found with either cyclopentane or cyclohexane functionality (54). Recently, it has been shown that substitution of the non-cyclic ß,ß-diethyl-ß-mercaptopropionic acid at position one in D-Tyr(Et)^2 analogs affords molecules with antagonist activity comparable to the Pmp-substituted analog (55).

Disulfide Bond - Substitution of methylene groups for the sulfur atoms depicted in structure 10 affords potent antagonist molecules (52,56). These "dicarba" analogs, such as SK&F 105494 (11), exhibit less partial agonist activity than their disulfide congeners in dogs (52) and in rhesus monkeys (46), in contrast to the fact that "dicarba" substitution in agonists results in analogs which retain significant agonist activity (57). Very recently, linear analogs of 10, e.g. compounds 12 and 13, have been reported to display unexpectedly potent antagonist activity in rats (58). Key issues with respect to the linear analogs relate to species specifity and the potential for the linear molecules to represent a novel antagonist pharmacophore different from that presented by the cyclic antagonists.



11

R-CH_2-CO-D-Tyr(Et)-Phe-Val-Asn-Abu-Pro-Arg-Arg-NH_2

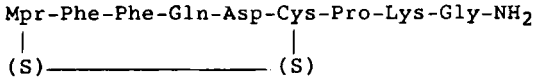
12: R = adamantyl
13: R = methyl

Position Two - The antagonist pharmacophore requirements at position two appear to be highly species dependent. In rat, analogs of 10 with alkyl sidechain containing amino acids (e.g. D-Leu, D-Ile, D-cyclohexylalanine, Leu) at position two retain significant antagonist activity both in vitro and in vivo (42,54,59). Comparable in vitro evaluation in porcine and human renal medullary tissue revealed that those molecules are only weak antagonists (42). Generally speaking, D-amino acid substitutions at position two which have aromatic sidechains, e.g. D-Tyr, D- or L-Tyr(alkyl), D-Phe, D-Trp, result in potent antagonists in all species tested (54). At the human V_2 receptor, the most potent antagonists are those which have a D-Tyr(Et)^2 substitution (42). In contrast to arginine vasopressin itself, monoiodination of the D-Tyr^2 residue in antagonists affords analogs which retain antagonist activity and thus can be used as radiolabelled probes (60).

Position Three - There appears to be an almost absolute requirement for L-phenylalanine in position three of antagonists based on structure 10. Good antagonist activity is retained with close phenylalanine congeners, e.g. Tyr(Me) and Phe(p-Et), substituted at position three. However, all other substitutions at position three result in analogs with a 2-3 order of magnitude reduction in receptor affinity/antagonist activity, independent of the species tested (51). In agonists, Phe^3 can be substituted by an aliphatic amino acid, e.g. Ile^3-AVP (arginine vasotocin) is equipotent with vasopressin at the receptor (57).

Position Four - A wide variety of L-amino acids can be substituted at position four with the retention of good antagonist activity (61,62,63). The list of acceptable substitutions includes Ala, Abu, Val, α-MeVal, Ile, Nle, Thr, Ser, Cha, and Lys. In agonists, Gln^4 can be replaced by aliphatic amino acids (57), as well as by α-MeVal (51) with retention of activity.

Position Five - As with position three, the antagonist pharmacophore requirements about residue five are very stringent. Substitution of amino acids other than asparagine uniformly results in a drastic reduction in antagonist activity (51). Previously, a bicyclic analog of vasopressin (14), modified at residues two, five and eight, was reported to be a V_2 antagonist (64); however, further development of this lead into more potent antagonists has not been reported.

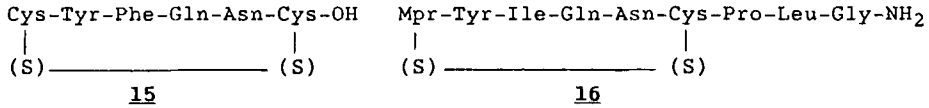


14

Position Six - Only a few substituents have been made at position six since the intact cyclic hexapeptide ring displayed in compound **10** was believed, until very recently, to be required for antagonist activity (58). The D-Cys^6 substitution is reported to result in analogs which retain antagonist activity (65). Agonist SAR indicates that the D-Cys^6 substitution affords an analog with a three log reduction in V_2 receptor affinity (57).

Carboxy Terminal Tripeptide - A variety of substitutions and deletions can be made at positions seven, eight and nine with retention of good antagonist activity (49,66,67). Amino acids such as D-Pro, N-MeAla, Sar, Ala, Gly, and Aib (α-methylalanine) can be substituted for proline at position seven with retention of V2 antagonist activity (50). A series of position nine-modified peptides, which include substitutions of Ala, Orn, Ser, Val, Arg, Tyr, Phe, Ile, Thr, D-Ala, Pro, and Eda (ethylene diamine), have been reported to retain significant V2 antagonist activity (67). Thus, the pharmacophore requirements for the C-terminal tripeptide portion of antagonists are very flexible as long as a basic residue is present (49,67). The position of the basic residue, the number of basic residues and the overall size can also be varied with the retention of potent antagonist activity. The minimum element necessary for antagonist activity is a basic residue attached directly to Cys^6 (49,68). Modifications of the natural Pro-Arg-Gly-NH_2 tripeptide, such as Pro-Gln-Arg-NH_2 (69), Arg-Gly-NH_2 (66,67), Arg-Arg-NH_2 (70), Arg-Gly-OH (66), Arg-NH_2 (49), Arg-OH (66), Gln-Arg-NH_2 and Arg-Gln-NH_2 (69), all result in active molecules. The normal carboxamido terminus is also not required for presentation of the antagonist pharmacophore, since potent analogs result from placement of diamines such as putrescine or cadaverine as the carboxy terminal residue (49,68). Analogs that contain Pro-Arg-NH-$(CH_2)_2$-NH_2 as the C-terminus are the first examples of vasopressin antagonists to demonstrate oral activity (67). All of the above results are in contrast to agonist SAR which exhibits very stringent requirements in the tripeptide portion in order to retain agonist activity (51,57).

Spectroscopic and Modelling Studies - Conformational aspects of the neurohypophsial hormones were reviewed recently (71). In particular, newly reported X-ray crystallographic structures of pressinoic acid **15** (72) and deaminooxytocin **16** (73) confirm the presence of ß-turns, encompassing residues 2-5, in the these molecules. Currently, no X-ray



15 **16**

structures for vasopressin antagonists have been reported. However, information is available which suggests that antagonists and agonists might share similar conformational features about position four. Strikingly similar SAR in both agonists and antagonists for a conformationally-constrained series of amino acids (Val>α-MeVal>Cle) indicates similar pharmacophore elements about position four and thus potentially similar molecular conformations (51). NMR-based conformational analysis of vasopressin antagonists is complicated by conformational exchange, even at -100 °C (74). Partial CD spectra of

antagonists indicate a strong peak of negative ellipticity at 238 nm which might correlate with the presence of γ-turns (75).

Conclusions - The therapeutic utility of V_1 and V_1/OT receptor antagonists is being evaluated in clinical studies. At present, therapeutic benefit appears to be limited to primary dysmenorrhea (37) and very selective cardiovascular abnormalities (35). The therapeutic utility of V_2 receptor antagonists has not been tested since the only V_2 antagonist to be evaluated in man to date exhibited agonist activity (40). Identification of a clinical candidate, lacking agonist activity, may result from the use of new animal models such as the rhesus monkey.

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Chapter 11. Atrial Natriuretic Peptide

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Introduction - Atrial natriuretic peptide (ANP) is a recently discovered hormone which is synthesized, stored in and released from atrial cardiocytes. Several biological properties have been ascribed to ANP, including effects on fluid, electrolyte and blood pressure homeostasis. ANP is the most potent diuretic substance known and exhibits a ceiling for natriuresis which is similar to hydrochlorothiazide. ANP has vasorelaxant properties and through its interactions with other hormonal systems, inhibits steroidogenesis and the secretion of renin and vasopressin.

This chapter presents a summary of experimental results which should be useful to the medicinal chemist for designing ANP analogs with increased potency or extended duration of action. We have restricted the discussion to ANP receptors, second messenger systems, metabolism and structure-activity relationships. The reader is referred to several reviews (1-5) for accounts of recent advances in other areas of ANP research.

Various numbering systems for the amino acid sequence of ANP have been employed by different research groups. For ease of reference, we have transcribed all ANP analogs to the numbering system based on the 126-amino acid prohormone, ANP(1-126), such that the circulating 28-amino acid peptide is ANP(99-126), 1.

```
99                      105                 110
                         ┌──────────────────────
Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg

        115                     121                 126
──────────────────────────────┐
Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr
```

1

Rat, rabbit and mouse ANPs have the amino acid sequence shown above, whereas the human, bovine, porcine and canine peptides contain Met at position 110.

ANP Receptors - Specific high affinity receptors for ANP are present in several target tissues such as kidney, vascular smooth muscle, adrenal, brain and lung (6-10). Affinity cross-linking studies have revealed the presence of one or more ANP binding proteins in rabbit and bovine aortic smooth muscle (11-13), bovine aortic endothelial cells (14), rat and bovine adrenal (15-17), rat kidney cortex (18), rat renal glomeruli (19), rabbit lung (20) and human placenta (21). One class of ANP binding sites is associated with stimulation of particulate guanylate cyclase activity, while the other is not coupled to any known second

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messenger system (14,22). Although the proportion of cyclase-linked and non-linked binding sites varies depending on the cells or tissues, the cyclase-linked receptors generally constitute only 1-30% of the total ANP binding sites (14,20,23-25).

ANP binding proteins have been isolated from bovine adrenocortical cells (26), bovine aortic smooth muscle cells (27), and bovine lung (28), and appear to be disulfide-linked dimers of approximately 125-140 kDa with a subunit M_r of 60-70 kDa. In contrast, other ANP binding proteins have been isolated which copurify with guanylate cyclase activity. These receptors are nonreducible polypeptides with M_r in the range of 110-180 kDa, and have been purified from rat lung (29), bovine adrenocortical cells (28,30), and rat adrenocortical carcinoma cells (31). Two-dimensional peptide mapping of the purified cyclase-linked and non-linked ANP receptors from bovine adrenocortical cells revealed that the profiles of the two proteins were more than 90% different (26).

With a rabbit reticulocyte lysate system, the primary translation product of bovine lung ANP receptor mRNA has a M_r of 58 kDa (32). The bovine aortic smooth muscle (BASM) non-cyclase linked receptor ('C-ANP receptor') has been expressed in frog oocytes, and has a M_r of 65 kDa (33). This molecular weight is larger than the 56 kDa primary translation product predicted by the complementary DNA (cDNA) and observed in a cell-free translation system, suggesting that the protein is glycosylated. The cDNAs from BASM and a human kidney library encode highly conserved receptors comprising an extracellular domain (436 amino acids), a transmembrane domain (23 amino acids) and a short, cytoplasmic tail (37 amino acids) (34). Specific antibodies have been raised against the bovine 'C-ANP receptor' in rabbits (35). Rat kidney cortex contains a guanylate cyclase-linked ANP receptor that has been identified as a 140-kDa glycoprotein (36). The nucleotide sequence of the cDNA encoding this receptor consists of 2895 base-pairs. Mouse antibodies have been prepared against the rat kidney ANP receptor (36).

ANP Receptor-Effector Coupling - ANP has been shown to increase intracellular cGMP levels and to stimulate particulate guanylate cyclase activity in various target tissues (37,38). Recently, it was reported that activation of particulate guanylate cyclase activity by ANP *in vitro* is potentiated by ATP, with Mg^{2+} as a required divalent cation (39). ATP increased the maximal velocity of the cyclase without changing the affinity for GTP or ANP. Non-phosphorylating ATP analogs also enhanced ANP-stimulated guanylate cyclase activity. The data suggest that an allosteric ATP binding site might be involved in the activation of particulate guanylate cyclase activity by ANP.

Evidence is accumulating that ANP receptor binding influences the phosphorylation of membrane proteins in various target tissues. ANP has been shown to inhibit the phosphorylation of two proteins of 78 kDa and 240 kDa in plasma membranes from bovine adrenal cortex (40). In addition, cGMP inhibited the phosphorylation of the same 78 kDa-species. Immunoblotting with a specific antiserum indicated that this protein may be protein kinase C. Pretreatment of rat aortic smooth muscle cells with phorbol dibutyrate, an activator of protein kinase C, attenuated ANP-stimulated cGMP accumulation (41). In another study using rat adrenocortical carcinoma cells, ANP-stimulated cGMP accumulation was decreased in the presence of phorbol 12-myristate 13-acetate (PMA), a known activator of protein kinase C (42). PMA-mediated inhibition of the ANP effect was blocked by 1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine, a protein kinase C inhibitor.

Metabolism of ANP - Administration of ANP to rats (43-45), dogs (46) and humans (47) indicates that the peptide has a biological half-life of a few minutes or less. This observation has prompted a number of research groups to investigate the mechanism of metabolic clearance for ANP. Several organs including the kidney, liver and muscle participate in the clearance of ANP in humans (48,49). In rats, the kidneys, intestine and muscle are responsible for the clearance of ANP (50). The heart and lungs do not appear to metabolize ANP in either species.

ANP(99-126) remains intact after a 60-min incubation with whole blood or plasma in vitro (51). The same peptide is degraded by an isolated mesenteric artery preparation, although the identity of the products has not been reported (51). The rate of clearance of ANP(103-126) in anephric rats is approximately two-fold lower than that in normal animals (44,45), and degradation, rather than excretion, accounts for 59% of its elimination from the circulation. Incubation of ANP(99-126), ANP(103-126) and acetyl-ANP(103-126)-NH_2 with a rabbit kidney brush border membrane preparation results in degradation of the peptides and loss of biological activity (52,53). The rate-limiting step in the inactivation of these peptides is cleavage of the Cys105-Phe106 peptide bond. Interestingly, ANP(103-126) and acetyl-ANP(103-126)-NH_2 are cleaved at multiple bonds, whereas cleavage of ANP(99-126) results in a single product. Membranes prepared from rat kidney cortex degrade ANP(99-126) similarly (54). Degradation of ANP by kidney membranes is inhibited by 1,10-phenanthroline, EDTA and phosphoramidon, indicating that the enzyme responsible for proteolysis is a metalloendoprotease (54,55). The pattern of hydrolysis of Met110-ANP(99-126) by pig kidney microvillar membranes was similar to that observed with purified endopeptidase 24.11 (55). In contrast to the results obtained with partially purified membrane preparations, the major degradation products produced after incubation of ANP(105-126)-NH_2 with a rat kidney homogenate were ANP(105-125), ANP(105-124) and ANP(105-123) (56). When ^{125}I-ANP was injected intravenously into rats, an initial degradation product was radiolabelled ANP cleaved at the Ile110-Asp111 peptide bond (57).

STRUCTURE-ACTIVITY RELATIONSHIPS

Role of the Ring Structure - ANP contains a 17-amino acid ring, formed by a disulfide bridge between Cys105 and Cys121. Several laboratories have investigated the requirement for this ring in the interaction of ANP with its receptor and in eliciting biological responses. Early studies demonstrated that ANP lost its natriuretic and vasorelaxant properties when the cyclic conformation was destroyed by reduction and carboxymethylation of the disulfide bridge (58), or by cleavage of the Asp111-Arg112 peptide bond within the ring (59). However, linear analogs with full biological efficacy have been described. ANPs with acetamidomethylated thiol groups at Cys105,121 exhibited 100% vasorelaxation, although their potencies had decreased by 1-2 orders of magnitude when compared to parent cyclic compounds (6). Another linear analog, ANP(106-125), possessed receptor binding and smooth muscle relaxant activities, but was 100-1000 times less potent than the cyclic analog, ANP(103-125) (60). Met110-ANP(106-120) also retained dose-dependent diuretic and natriuretic effects, but had no hypotensive activity (61).

ANP analogs in which the disulfide bridge at Cys105,121 was replaced by CH_2-CH_2 had natriuretic properties and exhibited spasmolytic activity in rat aorta and chick rectum assays (62). These ethylene-linked cyclic analogs were 2 to 50-fold less potent than

reference compounds containing the disulfide bridge. Another analog, Met110-ANP(105-121)-NH_2, has been synthesized in which cystine was substituted with 8-aminocaprylic acid, resulting in replacement of the disulfide with a CH_2-CH_2-CH_2 bridge (61). This analog had somewhat higher potency than the parent analog for diuresis and natriuresis. The propylene-bridged analog may have greater potency than the ethylene analog because it better approximates the interatomic distance spanned by the disulfide bridge. Substitution with D-Cys105 or D-Cys121 reduces the potency of ANP to approximately 25% of the L-Cys105,121 cyclized molecule (63).

Analogs of ANP(105-126), 2, have been synthesized in which Cys105 was replaced with 2-mercaptoacetic acid (Mac), 3; 3-mercaptopropionic acid (Mpr), 4; or 4-mercaptobutyric acid (Mbu), 5, (64). For inhibition



		R	n
2	Cys105-ANP(105-126)	NH_2	1
3	Mac105-ANP(105-126)	H	0
4	Mpr105-ANP(105-126)	H	1
5	Mbu105-ANP(105-126)	H	2

of aldosterone production in bovine zona glomerulosa and of ^{125}I-ANP(101-126) binding to cell membranes from the same tissue, 4 and 5 were about 10-fold less potent and 3 was 20-fold less potent than ANP(103-126). In a rabbit aorta vasorelaxation assay, ANP(103-126), 4 and 5 were about equipotent, in contrast to 2 and 3 which were 3-5-fold less potent.

Reduction in the ring size of ANP by deletion of residues lacking functional side chains also decreases biological activity significantly. des[Gly120]ANP(103-126), des[Leu119,Gly120]ANP(103-126) and des[Gly118,120,Leu119]ANP(103-126) had 20, 4 and 0.4% and 3, 0.3 and 0.3%, respectively, of the activity of ANP(103-126) in vasorelaxant and aldosterone suppression bioassays (65). In another study, des[Gly107] Met110-ANP(105-126) had 2 and 0.2% spasmolytic activity in chick rectum and rat aorta, but was devoid of natriuretic activity when compared to Met110-ANP(105-126) (62). The smooth muscle relaxant potencies of analogs with multiple deletions, des[Phe106,Gly107,108]Met110-ANP(105-126) and des[Ala115,Gln116,Ser117,Gly118,120,Leu119]Met110-ANP(105-126), were reduced by a factor of 10,000 when compared to Met110-ANP(105-126) (62). Thus, the data indicate that the cyclic primary structure of ANP is not a requirement for activity, but assists in placing the peptide in a conformation which facilitates interactions between critical endo- and exocyclic residues of the molecule and functional domains of the receptor.

Effect of Chain Length - The effects of systematic deletion of residues from the amino or carboxy termini of ANP have been investigated. In one study, serial deletion from the amino terminus demonstrated that

ANP(101-126), ANP(102-126), ANP(103-126) and ANP(105-126) relaxed rabbit aorta with relative potencies of 1.0, 0.5, 0.2 and 0.15 when compared to ANP(99-126) (66). Another study showed that ANP(102-126) was equipotent with ANP(101-126) in eliciting vasorelaxation, whereas ANP(103-126) was 10-fold less potent (67). Further deletion of Ser103 and Ser104 modestly decreased or had no significant effect on biological activity in several assays (23,66,68). Interestingly, Met110-ANP(105-126) has been reported to be more potent than Met110-ANP(99-126) in both chick rectum and rat aorta smooth muscle assays (62,69,70).

Although a small increase in potency is observed with elimination of Tyr126 (23,66), further deletions at the carboxyl end of ANP reduce activity dramatically. Deletion of Arg125-Tyr126 resulted in a loss of activity which was less than 1 order of magnitude for vasorelaxation in an ANP(103-126) series (66) and almost 2 orders of magnitude for vascular cGMP accumulation in an ANP(102-126) series (23). Further truncation of Phe124-Tyr126 produced ANP(103-123), whose potency for vasorelaxation differed when tested in rat as compared to rabbit aorta. ANP(103-123) has full vasorelaxant activity in rabbit aorta but is 100 times less potent than ANP(103-125) (66,71). In contrast, ANP(103-125) was only twice as potent as ANP(103-123) in producing relaxation of rat aortic rings (71). Finally, deletion of Ser123 from ANP(103-123) to produce ANP(103-122) results in no further loss of potency in a rabbit aorta relaxation assay (66).

Contribution of Amino Acid Side Chains to Activity - To investigate the contribution of specific amino acid side chains to the biological activity of ANP(101-126), systematic substitution of each residue with Ala was made (72). These derivatives were tested for vasorelaxant activity using rabbit aorta and rabbit renal artery. Analogs containing Ala substitutions at Asn122, Ser123 and Phe124 retained high potencies, indicating that the side chains of these residues were not critical for activity. Derivatives in which the N- or C-terminal Arg residues were replaced by Ala retained high potency provided that at least one cationic site was retained at either terminus. Thus, one end of ANP may lie in close proximity to the other end of the molecule. Deletions of the side chains of Phe106, Ile113 and Leu119 decreased vasorelaxant potency about 20 to 50-fold, indicating that the respective hydrophobic side chains play an important role in determining activity. In contrast, the hydrophilic side chains of Arg109 and Gln116 seemed to contribute very little to vasorelaxant potency. Other analogs, in which Gly residues at positions 114, 118 and 120 were replaced with Ala, had 1 to 2-fold greater potency than ANP(101-126).

Another series of ANP analogs has been made in which each optically active amino acid of ANP(103-125)-NH_2 was substituted with Gly. Native Gly residues were substituted with Ala in this series of replacements. These were tested for biological activity in a rabbit aorta relaxation assay (Figure 1) (73). Deletion of the side chains of Cys105-Cys121, Phe106 and Ile113 reduced activity 500-, 500- and 800-fold, respectively. Removal of the side chain from Arg109, Ile110, Arg112, Ser117 or Leu119 decreased activity moderately, by 10 to 60-fold. Based on these results, a model of ANP(103-125)-NH_2 has been constructed in which two functional sites are proposed: (a) Phe106-Cys105-Cys121-Gly120-Leu119-Gly118-Ser117-Gln116, and (b) Arg109-Ile110-Asp111-Arg112-Ile113. These two regions are connected by two short flexible portions of the molecule consisting of Gly107-Gly108 and Gly114-Ala115. It was postulated that Arg109-Ile113 has an α-helical conformation, whereas Ser117-Gly120 has an extended structure. In

addition, the side chains of Phe106, Leu119, and Ser117 might form a hydrophobic core within the molecule.

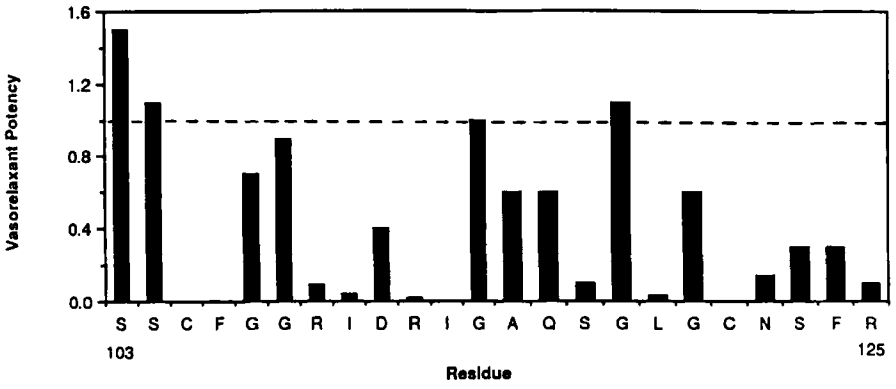
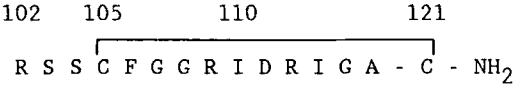


Figure 1 (with permission, reference 73)

Systematic substitution of D-enantiomers throughout the ANP molecule has also been used to probe the contributions of the side chains of specific residues for eliciting biological activity (63). ANP(101-126)-OH, ANP(103-124)-NH_2 and ANP(104-124)-NH_2 were used as reference compounds and Gly was replaced with D-Ala. D-enantiomer substitution of exocyclic residues Ser104, Asn122, Ser123 and Phe124 reduced activity modestly to 37, 34, 88 and 42%, respectively, of the reference analogs. In contrast, D-amino acid substitution within the ring produced a wide range of changes in vasorelaxant activity. Analogs with D-amino acid replacement of Gln116 or Gly at positions 107, 114, 118 or 120 retained high potency (range = 68-165% activity). D-substitution of Cys105, Gly108, Arg109, Ile113, Ala115, Ser117, Leu119 and Cys121 produced greater reductions in activity to 10-30% of the reference analog. D-Phe106, D-Ile110, D-Asp111 and D-Arg112 analogs exhibited the greatest loss of vasorelaxant activity to 1-2% of their reference compounds.

Receptor-Selective Analogs - Analogs which are selective for the non-guanylate cyclase-linked ANP receptor have been synthesized and their biological activities examined. des[Gln116,Ser117,Gly118,Leu119, Gly120]ANP(102-121)-NH_2, 6, an analog of ANP in which a portion of the ring structure has been deleted, binds with high affinity to approximately 99% of ANP receptors in an isolated perfused rat kidney (24).



6

The analog itself has no activity in an isolated perfused rat kidney and does not antagonize any of the known renal hemodynamic and natriuretic actions of ANP. In intact anesthetized rats, sodium excretion increased after administration of the analog with an accompanying elevation in endogenous immunoreactive ANP. As the majority of the renal ANP receptors are not coupled to guanylate cyclase, it is postulated that this class of receptors (termed the 'C-ANP receptor') may serve as peripheral storage-clearance binding sites to modulate plasma levels of ANP.

A truncated, linear ANP analog, des[Cys105,121]ANP(104-126), 7, displaces bound ^{125}I-ANP(103-126) selectively from only non-cyclase linked ANP binding sites in rabbit lung membranes but with the same potency as ANP(103-126) (20).

```
104                 110             120         126
 S - F G G R I D R I G A Q S G L G - N S F R Y
```

7

The octapeptide, ANP(106-113)-NH_2, 8, is the smallest known fragment of ANP that retains high affinity for the non-cyclase linked 'C-ANP receptor' in cultured vascular cells (74).

```
106          113
 F G G R I D R I - NH2
```

8

Phosphorylated Analogs - ANP(96-126), ANP(99-126), ANP(101-126), ANP(102-126) and a proANP analog can be phosphorylated at Ser104 by cAMP-dependent protein kinase (75,76). Phosphorylated ANP(96-126) and ANP(101-126) are more effective than the respective dephospho-peptides in stimulating Na/K/Cl cotransport in cultured smooth muscle cells (75). Phosphorylated and thiophosphorylated ANP(99-126) are less potent than the unphosphorylated peptide in displacing bound ^{125}I-ANP(99-126) from rabbit lung membranes and in vasorelaxant activity (76). ProANP secreted by rat neonatal cardiocytes is phosphorylated within the N-terminal portion of the molecule (77), whereas rat atria contain proANP phosphorylated within the C-terminal portion at Ser104 (78).

Summary - Substantial progress towards the purification of guanylate cyclase-linked and non-linked ANP receptors has been reported by several laboratories. Efforts to sequence both types of ANP receptors continue and the complete sequence of at least one of these receptor proteins is expected within the next year. More complete characterization of these receptor proteins, examination of the physical and functional relationship between the two receptors and establishing a biological and/or clearance function for the non-cyclase-linked receptor can also be anticipated.

The design of analogs which are selective for the non-cyclase-linked receptor has provided a useful tool for discriminating between the two types of ANP receptors. Structure-activity studies have established that the guanylate cyclase-linked receptor exhibits rigid structural requirements for binding, while binding to the non-linked receptor is relatively insensitive to structural alterations. The design of ANP antagonists to aid in elucidating the biological role of this novel peptide hormone is eagerly awaited.

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Chapter 12. Plasminogen Activators

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Introduction - In addition to its uses in deep vein thrombosis, pulmonary embolism, and peripheral arterial occlusion, thrombolytic therapy is rapidly becoming the treatment of choice for acute myocardial infarction (MI) when diagnosed early. Three thrombolytic agents, streptokinase, urokinase and alteplase (tissue plasminogen activator, recombinant) are approved in the U.S. and in addition a fourth (APSAC) in Europe. Many new thrombolytic agents are currently in preclinical and clinical development. The agents are large proteins and all work by activating the plasma zymogen plasminogen to plasmin which cleaves fibrin. Streptokinase is a bacterial protein, urokinase and alteplase are human proteins and APSAC is a chemically modified complex of streptokinase and plasminogen. Specific plasma and platelet borne inhibitors have been identified which are thought to modulate the activity of these drugs in vivo. Alteplase binds fibrin directly and streptokinase and APSAC bind to fibrin indirectly through plasminogen. However, only alteplase is dramatically activated by fibrin and alteplase degrades comparatively less fibrinogen in vivo compared to urokinase, streptokinase or APSAC.

CLINICAL AND PRECLINICAL RESULTS

First Generation Plasminogen Activators and Their Clinical Use in Myocardial Infarction
A number of reviews have dealt with the large number of studies of streptokinase and urokinase in preclinical animal models(1,2). Treatment of patients with intravenous streptokinase following acute myocardial infarction has been shown to reduce mortality, (3-5) reduce infarct size (6,7), and improve left ventricular function (4) even though intravenous streptokinase has been shown to be relatively ineffective in inducing prompt coronary artery thrombolysis (8,9). Early presentation has been shown to be critical to achieve a greater mortality benefit (3). Data on long-term effects are conflicting (10,11) but favor sustained benefit. There are little or no data to date regarding the efficacy of intracoronary or intravenous urokinase beyond coronary thrombolysis (12,13), i.e., no controlled trial showing reduced infarct size, improved ventricular function or reduced mortality. One study showed reduced left ventricular end diastolic volume, an important prognostic variable, following intracoronary urokinase (14).

Second Generation Plasminogen Activators: Preclinical data - Preclinical data have shown tissue plasminogen activator (alteplase) and anisoylated plasminogen streptokinase activator complex (APSAC) to be effective thrombolytic agents in vitro, and in animal models of myocardial infarction (15). Single chain urokinase (scu-PA, prourokinase) synthesized in mammalian cells has been shown to be an effective thrombolytic in a rabbit model (16,17). Nonglycosylated scu-PA made in E. coli has been shown to reopen occluded coronary arteries in baboons and dogs with little loss of fibrinogen (18,19), whereas urokinase was shown to have similar efficacy but caused almost complete defibrinogenation (18). Polyethylene glycol modification has been shown to extend the half life of urokinase in dogs with an increase in fibrinogen consumption over urokinase (20).

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Second Generation Plasminogen Activators: Their Clinical Use in Myocardial Infarction

Early trials with a tissue plasminogen activator utilized a two-chain molecule produced in tissue culture by a pilot scale roller bottle process (9,21-23). Subsequently, a predominantly single-chain molecule called alteplase, produced by a commercial scale suspension culture process, was introduced into clinical trials (24,25). The latter molecule is cleared from the circulation more quickly (26), and the dose had to be adjusted upward (from 80 to 100 mg) (25). Fibrinogen sparing was noted in all trials (21-28) and was present with both roller bottle (21-23,26-28) and suspension culture molecules (24-26). Studies at 150 mg revealed more rapid coronary thrombolysis (25), but an increased risk of toxicity from intracranial bleeding as well (29,30). Studies at 100 mg have shown that early treatment with alteplase can reduce infarct size (31), improve left ventricular function (31-34), reduce the incidence of congestive heart failure (32), and reduce mortality (31). Studies involving treatment of patients up to six hours after onset of infarction showed comparable thrombolytic benefit when alteplase was used to treat patients with early or late presentation (9). Three additional multicenter trials have shown that following coronary thrombolysis with alteplase emergency coronary angioplasty need not be performed, and indeed may be deleterious (35,36).

Trials focusing on coronary thrombolysis have shown APSAC to be comparable to streptokinase with regard to overall ability to lyse thrombi in coronary arteries (37,38). A recently reported controlled study with APSAC showed improved ventricular function (39), but a second study failed to confirm this (40). Clear evidence of reduced mortality has been observed (41,42) in APSAC treated patients. Though predicted to be fibrinogen sparing in preclinical models (43), APSAC has not proven to be fibrinogen sparing in clinical evaluation (44-46).

Scu-PA has been studied in only a small number of patients (47,48). There are little data on coronary thrombolysis and none on ventricular function or mortality. Fibrinogen sparing has been observed in most but not all patients (47,48). Some synergy between scu-PA and alteplase has been reported in a very small number of patients (49).

Use of Plasminogen Activators in Indications Other Than MI: Preclinical - Studies in several models of cerebral thromboembolic occlusion have demonstrated a potential role for thrombolytic agents for this indication. Rabbit (50), baboon (51), and dog (52-54) models have responded to local administration of urokinase. Rabbit (55) and rat (56) models have responded to systemic alteplase administration.

Alteplase has been shown to have a positive effect in a non-thrombotic model of myocardial ischemia (57); the effect was ascribed to its lytic effect on non-imageable microthrombi. This was recently contradicted by a second study (58).

Models of retinal vein thrombosis in the cat (59) and posterior penetrating eye injuries in the rabbit (60) have also been used to test the efficacy of thrombolysis with urokinase. Intraoccular administration of urokinase was effective in opening occluded retinal veins (60). Alteplase was shown to be effective in reversing eye damage when introduced intraocularly in rabbit models (61-63). The opening of thrombosed arterial grafts has been studied in dogs using alteplase or urokinase alone (64) or in combination with aspirin (65), and alone in limb reimplantations in rats (66). Streptokinase was shown to decrease tissue damage following experimental frostbite in rabbits (67).

Use of Plasminogen Activators in Indications Other than MI: Clinical - Much recent activity in thrombolytic clinical research has focused on alteplase. An angiographically-controlled multicenter trial showed excellent efficacy in pulmonary embolism (68,69), which was confirmed by a European study (70) and alteplase was shown to be superior to urokinase in a third trial (71). Favorable results were reported in a recently completed trial with urokinase in massive pulmonary embolism (72). Initial efficacy with APSAC in major pulmonary embolism has also been reported (73). A placebo-controlled pilot

study of alteplase in unstable angina showed a favorable angiographic and clinical effect (74); a second placebo-controlled study showed a beneficial effect on pacing threshold (75). Preliminary data suggest that alteplase will be effective in peripheral arterial occlusion (76,77) and stroke (78,79). Valvular (80,81) and ventricular thrombi (82) have also been successfully treated with alteplase (81) or urokinase (80,82).

PHARMACOKINETICS AND PHARMACODYNAMICS

Clearance Mechanisms - The principal clearance mechanism for alteplase appears to reside in the liver (83,84), possibly in hepatocytes which have been shown to have the ability to take up and degrade alteplase (85). The A chain (amino acids 1-275) and B chain (amino acids 276-527) of alteplase have been separated by limited reduction and the patterns of elimination were compared in rats (86). The half lives of intact alteplase and the A and B chains were 2.3, 1.0 and 5.7 min., respectively, suggesting that the clearance domain might reside in the A chain. The presence of a high mannose oligosaccharide at residue 117 appears to contribute to the rapid clearance of alteplase (87,88).

Urokinase has been shown to bind to a number of cell types and enhance plasmin and plasminogen binding to these cells (89,90). The amino terminal region of urokinase has been implicated in this cell binding activity (91,92). Alteplase has been shown to bind tightly to endothelial cells (93-95). The bound alteplase was shown to be protected from inhibition by the fast acting plasminogen activator inhibitor (PAI-1) and was still able to activate plasminogen (94). Though displaced by added urokinase, alteplase bound to endothelial cells was not effectively displaced by fibronectin, epidermal growth factor or plasminogen, all of which have known receptors on endothelial cells (94).

Alternative Drug Delivery - The intramuscular administration of alteplase with the use of absorption enhancers has been shown to result in significant plasma concentrations (96,97). These concentrations also caused thrombolysis in a dog coronary artery model with continuing release into the circulation for six hours (98).

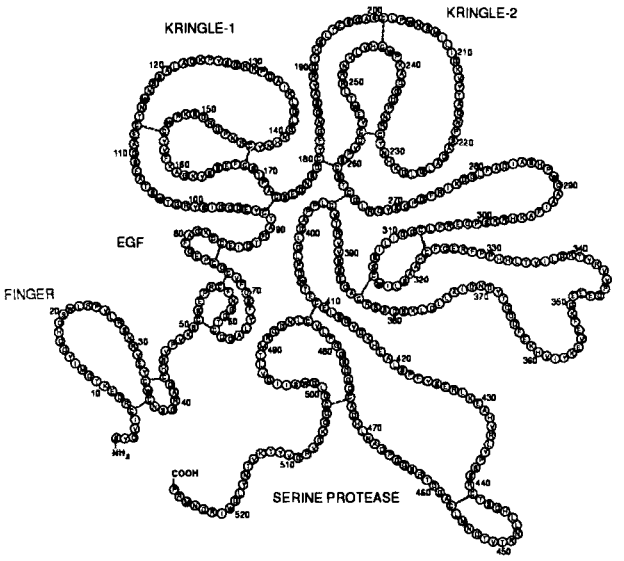
In Vivo Inhibition of Plasminogen Activators - PAI-1 from endothelial cells and platelets is normally present in plasma at pharmacologically insignificant concentrations. When induced by endotoxin, PAI-1 completely inhibited ex vivo measurements of alteplase; however, in vivo thrombolysis was not affected (98,99). Reactivation of the complex during circulation was postulated to explain the results (99). Though local concentrations of PAI-1 in the vicinity of a thrombus may play a role in lysis rate or rethrombosis, in animals with normal plasma concentrations of fast acting inhibitor, there appears to be little complex accumulation during treatment with pharmacologic doses of alteplase (99).

NEW THROMBOLYTIC AGENTS - ALTEPLASE AND UROKINASE ANALOGS

Domain Deletions - Alteplase, 1, is a complex multidomain protein (100,101). At the N-terminus there are two domains: a so-called "finger" region, homologous to the finger domains of fibronectin (102), and a "growth factor" region, homologous to epidermal growth factor (103). A pair of "kringle" structures, homologous to similar motifs in urokinase, plasminogen, prothrombin, and Factor XII comprise the central two domains (104), and the C-terminus of the molecule consists of a trypsin-like serine protease (100,105).

Alteplase has at least two functionally important (and clinically relevant) biochemical properties, namely, fibrin binding and fibrin-stimulated proteolysis of the Arg561-Val562 peptide bond of plasminogen. Several groups have carried out deletion mutagenesis studies, producing and characterizing alteplase analogs lacking one or more domains (106-112). The aim of this work was to assign functional properties to these regions and thereby address the problem of how the five domains of alteplase act in

concert to produce its unique properties. Experiments employing convenient natural restriction sites to construct deletion analogs showed that kringle two and the finger were the two domains most responsible for fibrin binding. The same studies also implicated kringle two as the domain responsible for fibrin stimulation and lysine binding (106-108). Other studies, in which newly-introduced interdomain restriction sites (109) or precise oligonucleotide-directed deletions were used (110-112), have confirmed these results. Deletion mutagenesis studies have also shown that an alteplase variant lacking the two kringle domains is less reactive with PAI-1 (110). Finally, it should be noted that a natural, finger-deleted variant of alteplase exists, produced from the wild type gene via alternative splicing in Detroit 562 cells (113). The physiological significance of such splicing variants, or whether they even exist in vivo, is unknown.



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Glycosylation Mutants - Alteplase contains four potential N-linked glycosylation sites at asparagines 117, 184, 218, and 448 (103,114). Asparagine 218 is not glycosylated, presumably because proline 219 blocks utilization of this site. Each of the three remaining sites exhibits a different pattern of glycosylation. At position 117 all molecules are modified with a "simple" high mannose-type sugar (115,116). At position 184 only approximately half of the alteplase molecules are glycosylated, but when this has occurred it is with a "complex"-type of carbohydrate (114-116). The site at asparagine 448 is fully glycosylated with a complex sugar (115,116).

Several groups have attempted to modify the properties of alteplase by removing one or more of the glycosylation sites, usually by substituting glutamine for the asparagine. Alternatively, glycosidases such as Endo H (which cleaves high mannose type sugars) or N-glycanase have been used to modify the alteplase glycosylation pattern enzymatically, or biosynthesis in the presence of tunicamycin has been used to suppress N-linked glycosylation completely (117,118). These experiments have shown that removal of the sugar moiety at residue 117 or 448 changes the pharmacokinetic properties of alteplase, but does not otherwise affect its biochemical behavior (87,88,119). The absence of glycosylation at 184 is correlated with increased in vitro clot lysis activity (111,120,121), but the presence or absence of a sugar at this position does not seem to affect pharmacokinetics.

Single Chain Analogs - Almost all trypsin-like serine proteases are synthesized as inactive zymogen precursors. These molecules are converted to catalytically active enzymes by an obligatory proteolytic cleavage that causes a conformational change

(involving the new N-terminus) and a resultant ordering of the active site residues (122). Alteplase is unique among this family of serine proteases in that it does not require a proteolytic cleavage to activate the zymogen form. Rather, alteplase can be converted to the fully active conformation upon binding to fibrin, without any accompanying covalent modification.

Alteplase contains an arginine at residue 275, a position analogous to the activation clip site of trypsinogen, and the Arg275-Ile276 peptide bond in alteplase is readily cleaved by serine proteases such as plasmin that have trypsin-like specificity. However, uncleaved ("one-chain") alteplase was found to have full plasminogen activating activity in the presence of fibrin (123,124). An analog of alteplase, Glu275, was made which was intrinsically resistant to cleavage by plasmin. [Glu275]alteplase also exhibited full specific activity in the presence of fibrin (124). When assayed in the absence of fibrin, one-chain alteplase was observed to have approximately 20 times less plasminogen activating activity than two-chain alteplase (124). A similar difference in activity between one- and two-chain urokinase, a fibrin-independent plasminogen activator, has been reported to be due to primarily a difference in turnover number (kcat) (125).

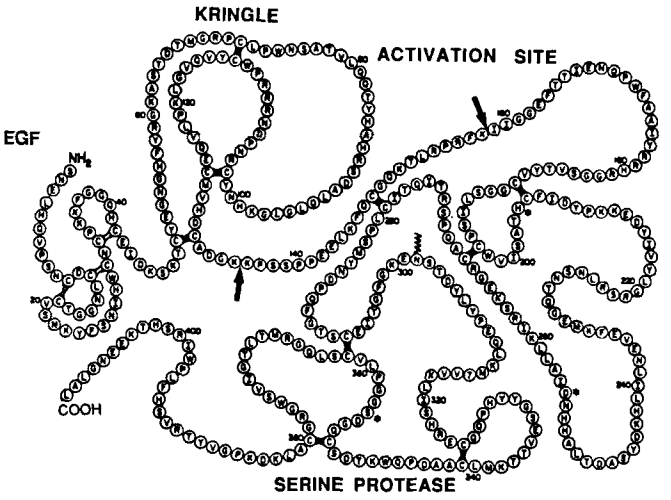
Other position 275 alteplase analogs have been reported: Gly275 (126); Asp275 (127); His275, Lys275, Thr275 (128); and all 19 possible substitutions (129). Two groups reported that Lys 275 alteplase was plasmin sensitive (128,129); however there is disagreement as to whether the His275 analog is plasmin resistant (129) or plasmin sensitive (128). All other position 275 analogs exhibited properties virtually identical to the prototypical Glu275 analog except Cys275, which had significantly lower specific activity (129). This reduced activity may have been due to improper protein disulfides (i.e., misfolding) or mixed disulfide formation resulting from the extra protein thiol introduced by the Cys275 mutation. Several other properties of Glu275 alteplase are also noteworthy: increased binding to fibrin, relative to two-chain alteplase (124,130); increased sensitivity to plasmin cleavage at Lys277 (131); decreased plasma clearance rate (132).

Other Analogs - Position 277, a lysine residue of alteplase, has been mutated to isoleucine (133,134). The resultant alteplase analogs do not appear to behave differently from the parent molecule. Glu275 alteplase is more sensitive to plasmin inactivation than is alteplase, but the Glu275, Ile277 double analog is partially protected from plasmin inactivation, implicating position 277 in the inactivation (131). This may play a role in the regulation of alteplase activity in vivo, as has been proposed for the thrombin-catalyzed inactivation of scu-PA via a clip at Arg156 (135). Deletion of the three C-terminal amino acid residues Met525 Arg526 Pro527 leads to an alteplase analog with significantly increased specific activity (120).

A limited number of single-chain urokinase, **2**, analogs have also been made. Lys158 was replaced with Glu or Gly and it was shown that these single-chain urokinase analogs, which could not be converted to the two chain form by plasmin, had a significantly lower specific activity in a plasminogen activating assay (136). Other analogs, in which the Arg 156 and Lys 158 were replaced by Thr, showed similar properties (137).

NEW THROMBOLYTIC AGENTS - HYBRID MOLECULES

Urokinase - Alteplase Hybrids - Hybrid molecules between proteolytic domains from plasminogen activators and amino terminal domains from proteins which bind to fibrin (e.g. plasminogen, alteplase) or non-binding proteins (e.g. urokinase) have been used to further dissect the role of the various domains. Several distinct approaches have been used to produce these hybrid molecules. Limited reduction and affinity chromatography (138) have been used to isolate the amino terminal domains of plasmin and the protease domain of urokinase. These domains were then mixed and reoxidized and a hybrid was isolated containing Lys-78 to Arg-561 of plasmin disulfide-bonded to Ile-159 to Leu-411 of urokinase. The amidolytic activity of the hybrid was ~ 1/2 of that of the high



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molecular weight form of urokinase on a IU/mg basis. The hybrid did bind to fibrin and also exhibited a 5-fold enhancement of plasminogen activating activity in the presence of soluble fibrin. A similar approach was taken to prepare a hybrid containing the plasminogen amino terminal and the alteplase protease domain (139). This hybrid had about 40% of the amidolytic activity of alteplase. The plasminogen activating activity was difficult to quantify due to non-parallel dose-response curves, but seemed to be slightly less than that for intact alteplase. The hybrid bound fibrin, but not as well as alteplase.

Chimeric proteins have also been made by recombinant DNA techniques. The similarity in the domains of alteplase and urokinase has led several groups to insert domains from one plasminogen activator into the other protein with or without corresponding deletions. A chimeric protein containing Ser-1 to Thr-263 from alteplase attached to Leu-144 to Leu-411 of urokinase was constructed by fusing the appropriate coding sequences from the cDNAs for these proteins (140,141). The single chain form of the molecule was expressed and characterized. Similar to scu-PA, this molecule had little amidolytic activity until conversion to a two-chain form by plasmin. Both the single-chain and two-chain forms of the protein bound to fibrin to a limited degree and their plasminogen activating activity was enhanced by fibrin peptides. Both forms were inferior to alteplase in both fibrin binding and fibrin stimulation, suggesting the importance of interdomain communication in these functions. The hybrid had decreased inhibition by PAI-1 when compared to alteplase or urokinase (141).

A series of alteplase/urokinase chimeras, all of which contain the urokinase protease domain, have been produced (137). All recombinant activators which contained the 'activation' site at Lys-158 in urokinase and could be converted to the two-chain form were active when expressed in mammalian cells. Further analysis (142) of the protein containing residues Ser-1 to Tyr-67 of alteplase attached to Lys-135 to Leu-411 of scu-PA demonstrated that the protein was similar to scu-PA in that it did not bind fibrin. In a plasminogen activating assay the catalytic constants were quite similar although scu-PA exhibited a Michaelis constant ~ 3-fold lower than that of the hybrid. However, the presence of a cysteine residue at position 366 (which is not present in the natural protein) as well as an "unpaired" cysteine at position 50 could lead to non-native disulfide bond formation and misfolding of some of the hybrid molecules. Further studies will be necessary to resolve this possibility.

When the growth factor and kringle domains of urokinase were inserted either before kringle one or after kringle two of full-length alteplase, or used to replace the finger and growth factor domains of alteplase (143), it was found that all proteins had similar amidolytic activity. All three hybrid proteins had somewhat reduced fibrin binding activity, and the plasminogen activating activity was not stimulated to the same degree as with alteplase. A comparison of the three hybrids suggested that the finger/growth factor regions were important in fibrin binding and in fibrin stimulation. The insertion of the urokinase domains after kringle two of alteplase also interfered with fibrin binding and stimulation, but to a lesser extent than removal of the alteplase finger and growth factor domains and to a greater extent than insertion of the urokinase domains before kringle one of alteplase.

Antibody - Plasminogen Activator Conjugates - An alternative manner in which to alter the characteristics of a plasminogen activator is to covalently couple the activator to antibodies of known specificity. Both anti-fibrinogen antibodies (144) and monoclonal antibodies raised against the amino-terminal residues of the beta chain of fibrin (145) have been chemically cross-linked to urokinase. The conjugates retained amidolytic activity. When the antibody conjugates were compared to urokinase at an equivalent amidolytic activity, a significantly lower amount of antibody conjugate was required to produce an equivalent amount of plasmin as measured in an 125I-fibrin release assay. The same result was obtained with a Fab' fragment - urokinase conjugate. The beta chain amino-terminal peptide completely abolished the enhancement of activity (146). In analogous experiments using alteplase, the conjugate was 2-10 fold more effective than alteplase alone if equal amidolytic activities were infused in an *in vivo* rabbit thrombolysis model (147).

Covalent linkage of alteplase to the beta chain fibrin specific antibody has also been accomplished via recombinant DNA technology. (148) The cDNA for the heavy chain from the beta specific monoclonal antibody was cloned and linked to the coding sequences for the serine protease domain of alteplase. The chimeric gene was expressed in a myeloma cell line which had lost the ability to produce the heavy chain, but still retained expression of the light chain of the antibody. The recombinant protein was purified by affinity chromatography, and presumably contained the correct association of two light chains and two recombinant heavy chains as judged by its molecular weight. The recombinant alteplase-antibody hybrid bound fibrin, although less well than the antibody alone, and retained amidolytic and plasminogen activating activity although to a lesser extent than in free alteplase.

SUMMARY

Far broader use of thrombolytic therapy in myocardial infarction as well as other indications should be expected in the near future as the recently published clinical data becomes widely appreciated. The probable increase in the use of this class of agents will inevitably lead to a number of new agents under investigation in the clinic. The next generation of thrombolytic agents may have greater efficacy and potentially a wider therapeutic ratio than the recently marketed second generation agents.

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Section III - Chemotherapeutic Agents

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Chapter 13. Beta-Lactam Antibiotics

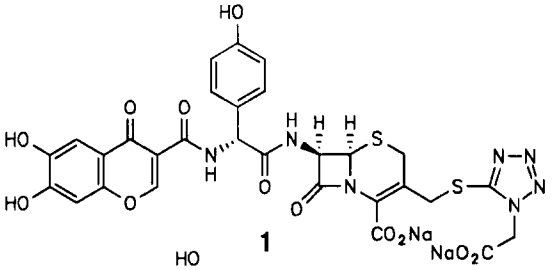
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Introduction - In the two years since the previous review of β-lactam antibiotics appeared in these reports (1), market introductions of imipenem/cilastatin and aztreonam, the first representatives of the carbapenem and monobactam classes of antibacterial agents were made. The chemistry of the β-lactam antibiotics published during 1986 was recently reviewed (2); thus developments reported in 1987 will be the primary subject of this chapter. The proceedings of a Mayo Clinic symposium on antimicrobial agents was published and a journal supplement presenting a perspective on classifications, microbiological studies, antibacterial effects, pharmacokinetics and therapeutic applications of cephalosporins appeared (3,4). The results of a task force evaluation of the factors which affect antibiotic resistance worldwide was published (5). A review of chromosomal cephalosporinases responsible for resistance to the newer β-lactam antibiotics appeared (6), and the genetic basis for antimicrobial resistance in *Staphylococcus aureus* was reported (7). A discussion of human pharmacokinetic properties of the cephalosporins (8), a report on the interaction of monobactams with bacterial enzymes (9), and an extensive review of the enantioselective synthesis of carbapenem antibiotics were also published (10). Progress on crystallographic studies of the β-lactamases from *B. cereus* was presented (11), and a review on the biosynthesis of sulfur-containing β-lactam antibiotics was published (12).

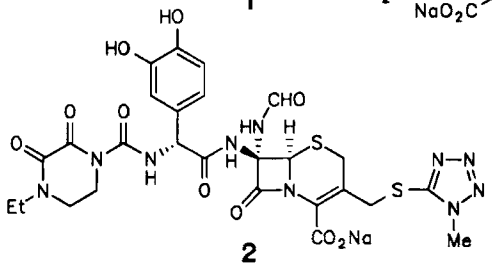
Cephalosporins and Carbacephalosporins - Detailed evaluations of ceforanide and cefmenoxime were published (13,14). The mechanism of action of the potent antipseudomonal agent E-0702 (1) was attributed to active transport into bacterial cells *via* the *tonB*-dependent iron transport system facilitated by similarity of the catechol moiety in E-0702 to a natural iron-siderophore (15). Synthesis of a series of 7α-formamido-cephalosporins which have β-lactamase stability comparable to the cephamycins while maintaining broad antibacterial activity was reported (16). A catechol effect might also contribute to the excellent Gram-negative and antipseudomonal spectrum observed for 2 in this series (17,18). Third generation cephalosporins bearing a quaternary nitrogen heterocyclic group in the C-3 side-chain have also shown extended Gram-negative activity. The synthesis and SAR which led to the selection of the antipseudomonal agents DQ-2522 (3) and DQ-2556 (4) for further development was discussed (19). Evaluation of ME-1228 (5) indicated superior activity to cefpirome and ceftazidime against clinical isolates of *Enterobacteriacae* acceptable pharmacokinetics ($t_{1/2}$=25 min in rats) and low toxicity (20,21). The 6,7-dihydroxyisoquinolinium substituted derivative 6 (BO-1341, L-658,310) had an MIC_{90}=0.78 mcg/mL against 151 clinical isolates of *P. aeruginosa*, but 6 is only weakly active against *S. aureus* (22,23). *In vitro* and *in vivo* analysis of E-1040 (7) also indicate excellent antipseudomonal activity for this parenteral agent and a broad spectrum superior to either ceftazidime or cefotaxime against anaerobes (24-26). E-1040 was at least as stable as ceftazidime and cefmenoxime to hydrolysis by β-lactamases

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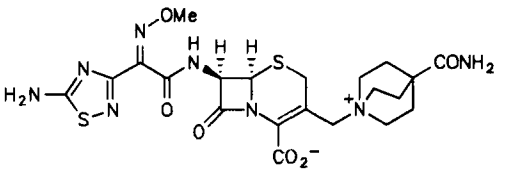
(27), and results of phase I clinical trials show it to be well tolerated at doses of 2.0 g/day (28). Synthesis and SAR for the antipseudomonal carbacephems KT-4380 (8) and the catechol O-methyl transferase resistant KT-4697 (9) was published (29,30). Antipseudomonal activity was also reported for a series of isothiazole tautomers of cefotetan (31), and for the 2-methyloxacephalosporin 10 (OCP-9-176, L-656,575) (32,33). The new parenteral cephalosporin ICI-194,008 (11) has exceptional stability to Type-I β-lactamases and inhibited 90% of 70 MRSA isolates (34). The synthesis and SAR which identified the cefadroxil analog BMY-28100 (12) was published (35). This oral agent has an antibacterial spectrum and β-lactamase stability better than cephalexin and cefaclor (36,37), high urinary recovery (82% in mice), and it is 45% protein bound in human serum (38). An olefinic substituent at C-3 also increases oral activity in derivatives bearing aminothiazolyl-oximino side-chains. The parenteral agent BMY-28232 (13) shows activity similar to cefuroxime and its prodrug esters BMY-28271 (14) and BMY-28257 (15) have very good (60-70%) bioavailability (39). FK-482 (16) has a spectrum superior to cefixime (FK-027) which includes methicillin-resistant *S. aureus* (MRSA) activity, and pharmacokinetic studies of the free acid have encouraged further development (40-42). The synthesis and SAR which lead to the selection of the orally active 3-unsubstituted cephalosporin 7432-S (17) for clinical trials was described (43). Preparation (44) of cefpodoxime (18, CS-807) a broad spectrum prodrug ester of the parenteral cephalosporin R-3763 was published (45,46); good bioavailability and low toxicity were seen in phase I trials (47,48). A number of laboratory studies of the carbacephem (19, LY-



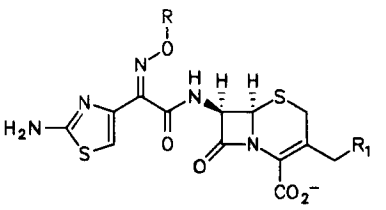
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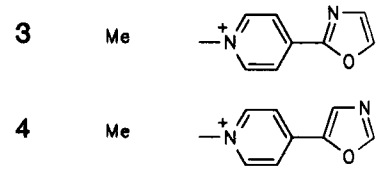
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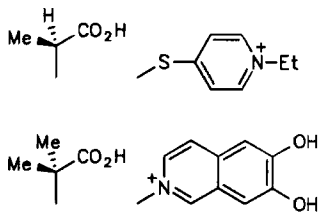


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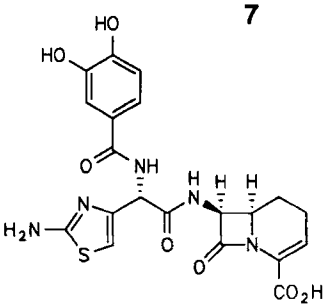
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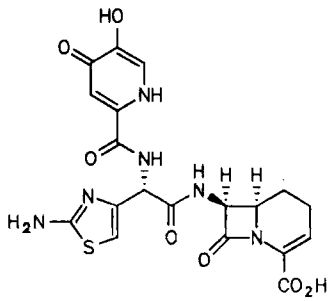


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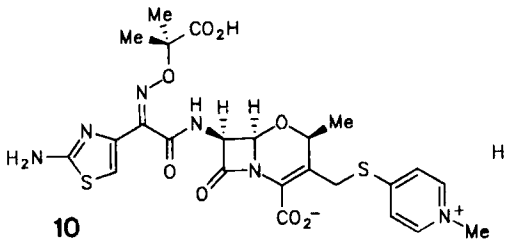


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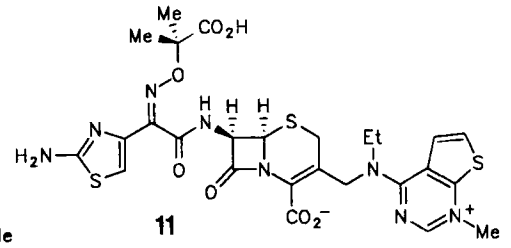


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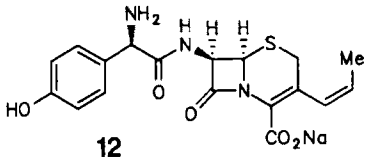
163892, KT-3777) analog of cefaclor indicate a similar antibiotic spectrum (49,50), improved chemical stability and good pharmacokinetics (51-53), but poorer β-lactamase stability than second and third generation agents (54). Reports of synthesis and oral activity for the C-3 methylthio analog 20 of cefedrolor (55), for prodrug esters of cefotiam (56), and for a series of isoxazolyl-oximino substituted cephems were published (57). Laboratory or clinical studies were also reported for cefepime (BMY-28142) (58,59), flomoxef (6315S) (60,61), cefetamet (Ro 15-8074) (62,63), cefpirome (HR-810) (64,65), cefodizime (HR-221) (66-68), cefixime (FK-027) (69), and the C-7 quaternary heterocyclylamino cephalosporins L-642,946 and L-562,813 (70). Additional publications have reported new synthetic routes to O-2-isocephems (71), 7α-methoxy-1-oxacephems (72), 2-(aminothiazolyl)-2-hydrazonoacetamido cephems (73) and Δ^3-1-methylene-1-carbacephems (74).



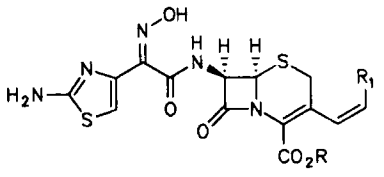
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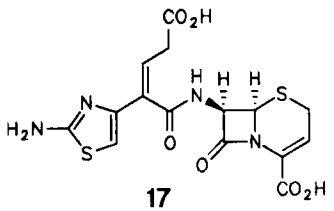
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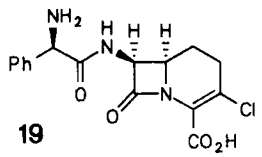
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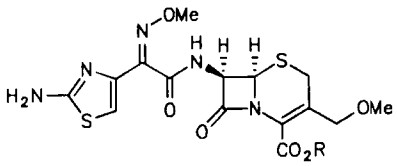
13 R = H R_1 = Me
14 R = $CH(CH_3)OAc$ R_1 = Me
15 R = $CH_2OCOC(CH_3)_3$ R_1 = Me
16 R = H R_1 = H



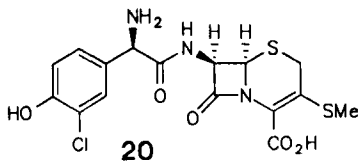
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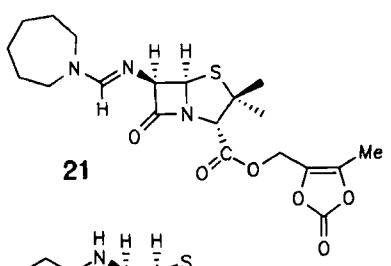
18 R = $CH(CH_3)OCO_2CH(CH_3)_2$



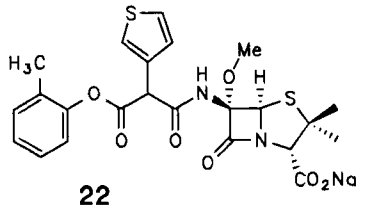
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Penicillins - For several years reports of new penicillins have been less frequent, while development of established agents in combination with β-lactamase inhibitors (*vide infra*) or in prodrug form has been a major emphasis. Clavulanic acid was shown to enhance the efficacy of ticarcillin for treatment of infection due to *P. maltophilia* and this combination achieves therapeutically useful concentrations to inhibit infection in human bone, but was relatively ineffective for the treatment of experimental meningitis in the rabbit (75-77). A new prodrug ester of mecillinam (21) was reported (78), and human bioavailability studies of 22 (BRL-20330) a temocillin prodrug were published (79). Enhanced antibacterial activity for piperacillin derivatives with increased number of

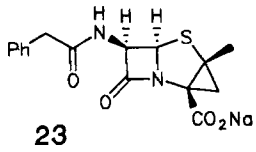
carbon atoms at the N-4 position of the 2,3-dioxopiperazine nucleus was correlated to increased binding affinity for penicillin binding protein 3 (PBP 3) in *E. coli* (80). A series of bactericidal α-(2,3)-methylenepenams (23) bound to PBP 3 in *E. coli*, and to PBP 2 in *S. aureus* at concentrations comparable to the corresponding penams; the corresponding β isomers do not bind to essential PBP's and are only marginally active (81). Potent activity against Gram-negative bacteria including β-lactamase producers was reported for a series of 6-α-formamido penicillin derivatives (82). Serum bactericidal activity (83) and degradation studies (84) of BRL-36650 (24), the lead compound in this series, were reported. The related spirocyclic formamido derivatives **25a** and **25b** were prepared, but were devoid of antibacterial activity (85). Metabolism of the potent Gram negative antibacterial agent AO-1100 by catechol-O-methyl-transferase was decreased by the introduction of chlorine in the catechol moiety (26) affording improved efficacy against *Pseudomonas in vivo* (86). The 2-exomethylene penicillin V system 27 was prepared *via* a decarboxylative Pummerer reaction from a 2R,3R-dicarboxylate system (87). Magnesium enolates derived from anhydro-6,6-dibromopenicillin were shown to react quantitatively with acetaldehyde affording a single aldol product having the 6-R-hydroxyethyl stereochemistry useful for elaboration into penem and carbapenem antibiotics (88).



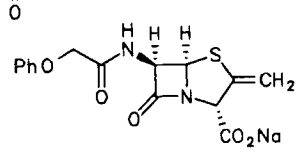
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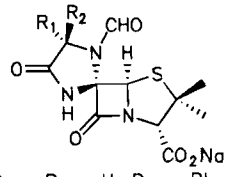
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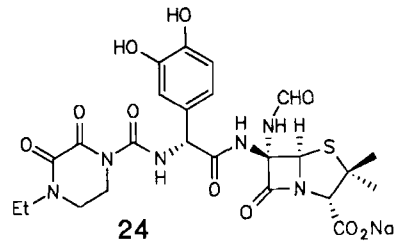
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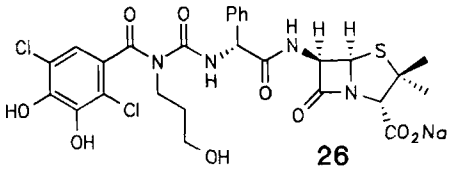
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25a R_1 = H, R_2 = Ph
25b R_1 = Ph, R_2 = H



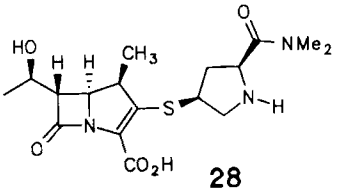
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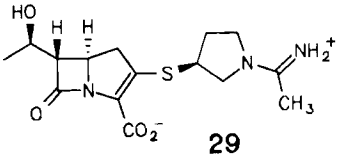
26

Carbapenems - The proceedings of a symposium which presented extensive clinical experience with imipenem-cilastatin has been published and the antibacterial activity and therapeutic efficacy have been reviewed (89,90). SM-7338 (28) a new carbapenem with an antibacterial spectrum similar to imipenem and a high stability to renal dehydropeptidase-I (DHP-I) has been advanced to clinical trials (91,92). The synthesis of RS-533 (29) was reported in 1986 (93), and the antimicrobial spectrum and β-lactamase inhibition by 29 were discussed (94). The synthesis and *in vitro* activity of C-2 quaternary heterocyclic alkylthio carbapenems was published; BMY-25174 (30) displayed a spectrum of activity similar to imipenem and had considerable stability towards DHP-I (95). Several new approaches to the stereocontrolled synthesis of 1-β-methylcarbapenems have been reported (96-98). Synthesis and SAR for a series of 1-β-methyl-2-arylcarbapenems revealed that the presence of the 1-β-methyl group had a net detrimental effect on activity and DHP-I stability relative to the 1-hydrogen series (99). A report on a series of 1-β-alkoxy carbapenems related to 31 (Ro 40-3617) claimed good activity and stability towards DHP-I, but apparently has poorer chemical stability at neutral and higher pH than imipenem (100). Total syntheses were reported for (+)-PS-5 (101,102), and a

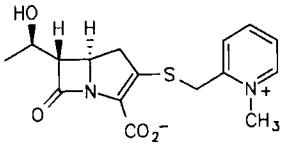
carbapenem with a 6-methyl hydroxyacetate side chain (103). A formal total synthesis and side-chain modified derivatives of thienamycin (104-106) appeared, a stereoselective route to 1-β-methylcarbapenems was reported (107), and a β-lactam annulation to afford the carbapenam skeleton was published (108). The isolation of (3**R**,5**R**)- and (3**S**,5**R**)-carbapenam-3-carboxylic acids from *Serratia* and *Ewinia* species were reported and their possible role in the biosynthesis of more complex carbapenems was discussed (109).



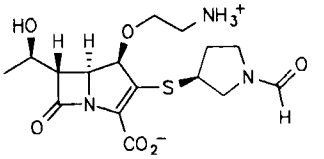
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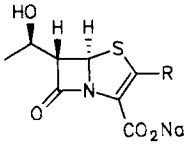


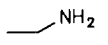
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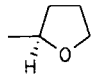


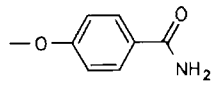
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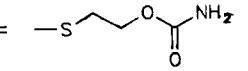
Penems - Comparative *in vitro* antibacterial studies of CGP-31608 (32) have been reported (110,111). This parenteral penem is generally less potent than imipenem except against *P. aeruginosa* and MRSA (112), but is ineffective against *Enterococci* (113). It is stable to plasmid and chromosomal β-lactamases (114), but has a short (0.5 h) half-life *in vivo* (115). Pharmacokinetic analysis of the new orally active penem 33 (SUN-5555) was presented (116,117). SUN-5555 is reported to be well absorbed after oral administration and is highly serum bound (94% in man), but has a relatively short half-life *in vivo* and is highly susceptible to DHP-I mediated hydrolysis. The 2-phenoxy substituted parenteral penem HRE-664 (34) is more active than third generation cephalosporins against Gram positive pathogens including MRSA, but is ineffective against *Pseudomonas* (118-120). Human pharmacokinetics for SCH-34343 (35) were reported, but evidence of further clinical development has not appeared (121). Modest oral bioavailability for prodrug esters of FCE-22101 (36) was demonstrated, and except for having no activity against *Pseudomonas* the antibiotic spectrum of 36 was found to parallel CGP-31608 (122,123); however human pharmacokinetic evaluation indicates a very short half-life after parenteral administration (124). Synthesis of FCE-24362 (37) was reported, and while it has no antipseudomonal activity and a short half-life, 37 is very effective (ED_{50} < 1 mg/kg) for treatment of experimental septicemias (125). The conversion of 2-alkylthiopenems into 2-oxopenam, 2-O-alkylpenem and 2-unsubstituted penems was accomplished by a novel chlorination reaction (126), the synthetic chemistry and SAR for a new series of 2-(1-azolyl) penems has been extended (127), and the stereospecific synthesis of (5**R**,6**S**)-6-(aminomethyl)-2-(ethylthio) penemcarboxylic acid was reported (128).

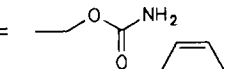


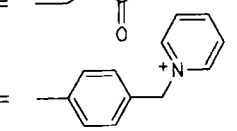
32 R = 

33 R = 

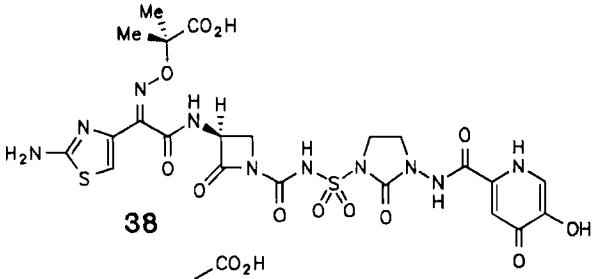
34 R = 

35 R = 

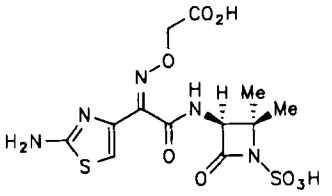
36 R = 

37 R = 

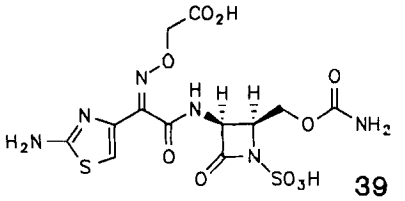
Monocyclic β-Lactams - Synthetic modification of the weakly active naturally occuring monobactams has imparted potent Gram negative activity and improved agents continue to evolve. Pirazmonam (38, SQ-83,360), a parenteral monobactam activated by a novel N-acylaminosulfonyl side-chain, has exceptional activity against *Pseudomonas* (129,130). Mode of action studies indicate that pirazmonam binds to PBP 3 in *E. coli* and *P. aeruginosa* (131), and does not induce β-lactamases (132). Comparative *in vitro* antimicrobial studies of carumonam (39, AMA-1080/Ro 17-2301) another Gram negative parenteral agent undergoing clinical trials appeared (133,134), and renal pharmacokinetics were reported (135). Evaluation of tigemonam (40, SQ-30,213) *in vitro* and orally *in vivo* indicated good bioavailability (85% in dogs) and urinary recovery (75%), with activity superior to other oral β-lactam antibiotics especially when the pathogen was a β-lactamase producer (136,137). The observation that aztreonam is hydrolyzed by an inducible oximinocephalosporinase (CXase) led to an investigation of 4-fluoromethyl derivatives; BO-1165 (41) was more stable to CXases than aztreonam and had a similar antibacterial spectrum (138,139). SAR of C-4 aminooxymethyl monobactam analogs (140), of 4-(2-substituted ethyl)-azetidinone-1-sulfonic acids (141), and for a series bearing an amino acid substituent at the C-4 hydroxymethyl group were reported (142). The structure elucidation of a new class of dehydroasparagine containing monobactams (PB-5266 A,B & C) was published (143). Papers describing synthetic routes to 4-styryl monobactams (144), bis-β-lactams (145), 4-unsubstituted azetidinones (146), and a new nocardicin analog were published (147).



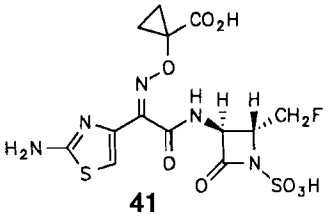
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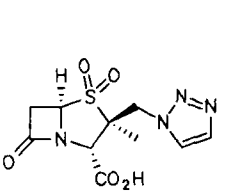


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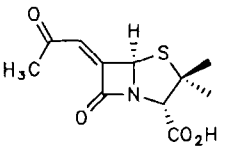


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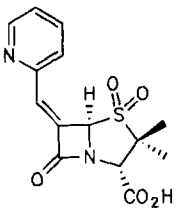
β-Lactamase Inhibitors - SAR of 2-(acylamino)-3-substituted-propenoic acids which led to the discovery of cilastatin, the DHP-I inhibitor employed with imipenem, was reported (148). Analysis of the SAR among the major classes of inhibitors of bacterial β-lactamases was recently published (149). A symposium describing *in vitro* studies, pharmacokinetics and clinical experience with sulbactam/ampicillin appeared as a journal supplement (150), and a drug evaluation for this combination was disclosed (151). The synthesis and β-lactamase inhibition of YTR-830 (42) and a number of related triazolyl sulbactam derivatives was published (152). Comparison with clavulanic acid and sulbactam *in vitro* and *in vivo* indicated that YTR-830 was more potent against a number of chromosomally- and plasmid-mediated enzymes (153), and evidence that YTR-830 is synergistic in lowering the MIC's of cephalosporins and piperacillin against β-lactamase producing clinical isolates was presented (154-156). 6-(E)-acetylmethylene penicillanic acid (43) was shown to inhibit β-lactamases, but was not as potent as Ro 15-1903, the previously descibed 6-(Z)- isomer (157). Synthesis and SAR for a new class of inhibitors based upon 6-(heterocyclyl)-methylene penam sulfones was disclosed; CP-65,372 (44) and CP-68,146 (45) from this series were reported to be equivalent or better than clavulanic acid, 6-β-bromopenicillanic acid, Ro 15-1903 and sulbactam (158). Bactericidal interactions of β-lactamase inhibitors with third generation cephalosporins against aerobic and anaerobic bacteria (159), with ampicillin against *M. tuberculosis* (160), and with ceftazidime in experimental *P. aeruginosa* endocarditis were reported (161).



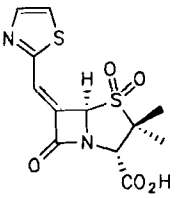
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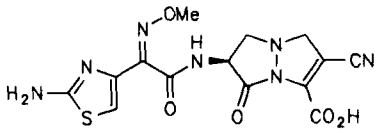


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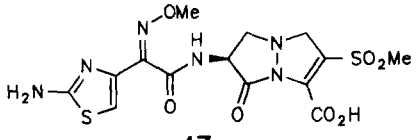


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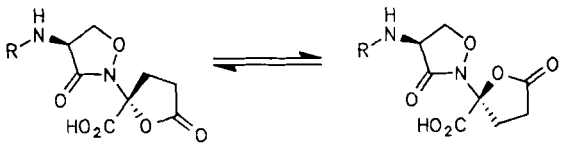
Non-Classical Agents - The synthesis of an interesting new class of bicyclic pyrazolidinone antibacterial agents which was first reported in 1986 has appeared (162-166). Analogs LY-255,262 (46) and LY-193,239 (47) were bactericidal against a variety of G(+) and G(-) bacteria excluding *Staphylococcus* and *Pseudomonas* (167). These γ-lactams were found to bind selectively to PBP-3 of *E. coli* K-12 and to be resistant to hydrolysis by β-lactamases (168). The isolation and structural characterization of lactivicin (48) from culture filtrates of *Empedobacter lactamgenus* and *Lysobacter albus* has been reported (169). Affinity for PBP-1 (1A and 1B) was shown to be the target of lactivicin in *E. coli* (170). The total synthesis of lactivicin was described (171) and the N-acetyl group has been replaced with amide side chains (49) which provide improved bioactivity, however these derivatives are sensitive to β-lactamase and hydrolysis in media at neutral pH (172).



46

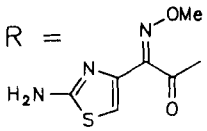


47



48 R = Ac

49 R =



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Chapter 14. Quinolone Antibacterial Agents

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Introduction - In 1987, the research activity in the quinolone area plateaued with fewer new quinolones being reported than in the last four years. This decline is partially related to the success of norfloxacin (NOR), ciprofloxacin (CIP), ofloxacin (OFL) and enoxacin (ENO). These compounds have clinical efficacy rates of approximately 90% and therefore are difficult to improve upon. Significant new findings, however, were reported in the areas of mechanism of killing by quinolones, quinolone resistance, mechanism of adverse effects on the CNS and structure-activity relationships.

Mechanism of Action and Killing - In addition to being the enzyme responsible for DNA supercoiling in bacteria, DNA gyrase has been found to be the principal enzyme responsible for decatenation in *Escherichia coli* (1,2). Tyrosine 122 of the A subunit of *E. coli* DNA gyrase has been identified as the site on the enzyme which binds DNA when the phosphodiester bonds of DNA are broken by the enzyme (3). Using electric dichroism it was determined that a single turn of DNA is wrapped around DNA gyrase with the entry and exit points located close together (4). The average angle between the DNA tails and DNA gyrase is approximately 120 degrees.

An *E. coli* strain containing a *recA*::lac operon fusion was used to determine that *recA* was quantitatively induced by quinolones subsequent to DNA gyrase inhibition and resulting DNA damage (5). *RecA* expression induces the expression of the *sulA* protein which interacts with the *sulB* protein causing inhibition of cell division and filamentation. It is suggested that cell death is caused by the induction of the SOS response which is a DNA repair response to DNA damage and is noted by cessation of cell division and formation of filamentous cells (5).

It has been assumed that the mechanism of quinolone action is the same against all susceptible bacteria. However, DNA gyrase from *Staphylococcus aureus* is not inhibited by the quinolones (6). The 50% inhibitory concentration (IC_{50}) for the quinolones against *E. coli* topoisomerase I was approximately 10 times that for *E. coli* topoisomerase II (7). Although the quinolones can inhibit two different enzymes with opposing activities, DNA gyrase which is responsible for supercoiling and topoisomerase I which is responsible for unwinding DNA, at concentrations achievable *in vivo*, only the inhibition of DNA gyrase is noted. The quinolones were inactive against eukaryotic topoisomerase I (7).

Resistance - The clinical success with the quinolones is clouded by the issue of resistance. The largest number of reports of resistance involve the most potent quinolone, CIP, with *Pseudomonas aeruginosa*, *Serratia marcescens* and *S. aureus* (8). The ratio of the peak serum concentration (C_{max}) to the MIC for the organism may be important in determining the frequency of resistance to the quinolones (9). A mutation in the A sub-

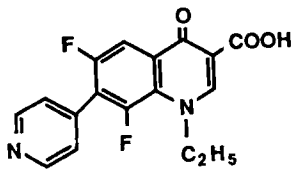
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unit of DNA gyrase and alteration in outer membrane permeability can result in resistance to NOR and altered susceptibility to aminoglycosides and β-lactams in *P. aeruginosa* (10,11). There has also been a report (12) that organisms which become resistant to the new fluoroquinolones are not always resistant to the older quinolones, such as oxolinic acid.

It has been generally believed that plasmid-borne resistance to the quinolones was impossible because the gene for the susceptible DNA gyrase would be dominant over the plasmid borne resistance gene (13). Plasmid mediated resistance to the quinolones was reported from Bangladesh in *Shigella dysenteriae* (14). This resistance gene was found on a large plasmid bearing resistance to multiple antibiotics. Another disturbing report is the cross-resistance between piperacillin and the quinolones which is related to the alteration of porin proteins that prevent the entry of both classes of compounds into bacteria (15).

Strict aerobic mutants of *Salmonella typhimurium* and *E. coli* are resistant to quinolones (16). Using reconstitution experiments, it has been shown that not only altered A subunits, but also altered B subunits can result in DNA gyrase which is less sensitive to the fluoroquinolones. Thus, both the subunits must interact with the DNA binding site of the quinolones (17).

Toxicity - The adverse effects of the fluoroquinolones have been reviewed (18). The binding of quinolones to DNA has raised concern over their potential for mutagenicity. The activities of CIP, NOR, oxolinic acid, nalidixic acid (NAL) and CP-67,015 (1), against mammalian topoisomerase II in an unknotting, catenation and radiolabeled DNA cleavage assay were compared to the activities of anti-tumor agents (19). Only CP-67,015 had significant activity in these tests.

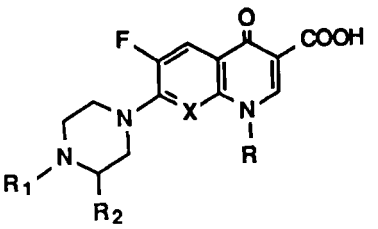


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Additional evidence that quinolones may affect eukaryotic cell function comes from *in vitro* studies on their effect on cells of the immune system. Radiolabeled thymidine uptake into DNA of phytohemagglutinin-stimulated lymphocytes was increased by 1.6 to 6.25 μg/ml by the fluoroquinolones (20). CIP inhibited immunoglobulin secretion by pokeweed mitogen-stimulated human lymphocytes (21) and *de novo* pyrimidine biosynthesis in mitogen-stimulated lymphocytes, resulting in a compensatory increase in pyrimidine precursor uptake (22). IL-2 production by monocytes was suppressed by quinolone treatment (22). NAL has been found to be somewhat effective in treating psoriasis (23), an effect assumed to be related to its activity against topoisomerase II.

The effect of quinolones on the CNS has been further characterized. Some non-steroidal antiinflammatory agents, such as ibuprofen, enhanced the inhibition by quinolones of GABA binding to its receptor in mouse synaptic membranes (24). The problem of why quinolones cause abnormalities at the growing ends of bone has not been resolved. Until then, the use of quinolones will be restricted mainly to the adult population.

Updates on Older Compounds - NOR (2), ENO (3), difloxacin (DIF) (4), pefloxacin (PEF) (5), CIP (6) and OFL (7) were reviewed in detail last year (25). Extensive microbiological, clinical and safety results with NOR have been reviewed (26). The pharmacokinetics and metabolism of the fluoroquinolones have been compared (27,28). The effect of quinolones on the serum concentration of theophylline has been described (29). ENO showed the maximum increase in serum theophylline concentrations. The appropriate clinical situations for using quinolones have been described (30,31).



2 R=C_2H_5, R_1=H, R_2=H, X=CH

3 R=C_2H_5, R_1=H, R_2=H, X=N

4 R=p-FC_6H_4, R_1=CH_3, R_2=H, X=CH

5 R=C_2H_5, R_1=CH_3, R_2=H, X=CH

6 R=c-C_3H_5, R_1=H, R_2=H, X=CH

7 XR=-$COCH_2CH(CH_3)$-, R_1=CH_3, R_2=H

8 XR=-$COCH_2CH_2$-, R_1=CH_3, R_2=H

NOR, OFL and CIP are active *in vitro* against *Campylobacter pylori*, the organism implicated in gastritis and duodenal ulcers (32,33). Resistant organisms were found after therapy with OFL as a single drug (34). However, when OFL was administered with ranitidine, the cure rate with OFL was greater than with ranitidine alone (35).

The proceedings of a symposium on CIP have been published (36). It is now approved in the United States for use in urinary tract infections and a variety of other infections, including upper and lower respiratory tract infections, bacterial diarrheas, gonococcal and non-gonococcal urethritis, septicemia and osteomyelitis. CIP has been used to treat *P. aeruginosa* infection in cystic fibrosis patients (37). CIP, NOR, ENO and OFL have all been found to be effective when administered as a single dose for treating uncomplicated gonococcal infections (31). However, a single dose is not sufficient for treating *Chlamydia trachomatis* co-infection and preventing post-gonococcal urethritis (38). CIP administered at a dose of 750 mg daily for four days eradicated 60% of C. trachomatis infections (38); there was significant variation between individual subjects in the bioavailability of CIP at this dose (38). The bioavailability of CIP upon oral administration was approximately 69% after a 200 and 750 mg dose (39). Food intake, prior to oral administration may decrease the absorption of CIP; antacids definitely decrease absorption. Combinations of CIP-cefotaxime and CIP-clindamycin were synergistic against many anaerobic bacteria *in vitro* (40). The preparation of a series of N-cyclopropyl quinolones including CIP has been published (41).

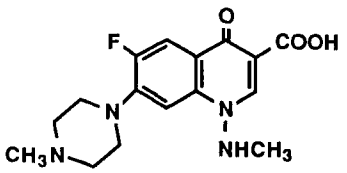
Both NOR and CIP have been found to be useful in preventing infection in immunosuppressed patients (26,42). In a randomized trial in patients with acute leukemia, a CIP-treated group had no infections and no resistant gram-negative bacteria were found, whereas in patients who received trimethoprim-sulfamethoazole, resistant organisms were isolated from 10 out of 28 patients (42). OFL and CIP, the most active quinolones tested against *M. tuberculosis*, *M. bovis*, *M. xenopi*, *M. kansasii* and BCG, were inactive against *M. avium-intracellular* complex at achievable serum concentrations (43).

The clinical experience with OFL has been reviewed (44,45). OFL and PEF have been found to be active against *Mycobacterium leprae* in a murine model (46). A short and efficient synthesis of the two optical antipodes of OFL from (R)- and (S)-alaninol has been published (47). The inhibition of DNA gyrase by the (+) and (-) enantiomers of OFL correlates with the *in vitro* activity (48). The IC_{50}s of the (+) and (-) enantiomers were 7.24 µg/ml and 0.78 µg/ml against *E. coli* DNA gyrase, while the MICs against *E. coli* were 1.56 µg/ml and 0.025 µg/ml, respectively.

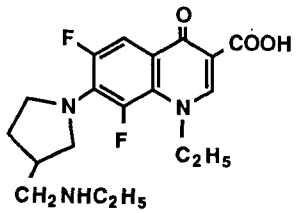
DR-3355, the S-(-) enantiomer of OFL, is ten times more soluble in water (49). The desmethyl derivative (8) has intermediate activity between the the two enantiomers (50). The pharmacokinetic properties of DR-3355 have been described in rats and dogs (51). This compound is distributed to all tissues except the CNS. The C_{max} in the serum after administering 20 mg/kg to rats and dogs was 2.51 and 6.32 µg/ml, and the $t_{\frac{1}{2}}$ was 1.8 and 8.4 hours, respectively.

DIF was as active as nafcillin against experimental *S. aureus* osteomyelitis in rabbits (52).

Amifloxacin (9) (WIN-49,375) - Amifloxacin (AML) was less active than CIP and OFL *in vitro* (53,54). The *in vitro* activity of two metabolites of AML have been described (55). The N-desmethyl metabolite (WIN-49,548) was as active as AML against gram-negative aerobic bacteria, but was 2-8 times less active against *S. aureus* and *Streptococcus pneumoniae*. The N-oxide metabolite (WIN-51,692) was generally 16 times less active than AML against all bacteria.



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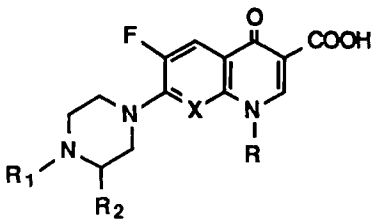


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CI-934 (10) - The *in vitro* activity of CI-934 was compared with CIP, ENO, NOR and vancomycin against a variety of aerobic gram-positive and gram-negative bacteria (56,57). Most *Enterobacteriaceae*, *Aeromonas hydrophila* and *Acinetobacter sp.* were inhibited at concentrations ≤1 µg/ml, while the MIC_{90} of CI-934 for *P. aeruginosa was* ≥8 µg/ml. Gram-positive bacteria, including *Enterococcus faecalis* were inhibited by concentrations ≤2 µg/ml.

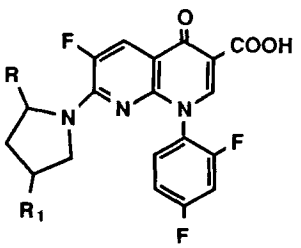
Temafloxacin (11) (A-62254, A-63004 hydrochloride) - *In vitro*, temafloxacin hydrochloride (TEM) was as active as CIP and DIF against *S. aureus* (58, 59). Streptococci were equally susceptible to TEM and CIP and 4 times more susceptible to TEM than DIF. TEM was 2 to 4 times less active than CIP against *Enterobacteriaceae* and *P. aeruginosa* and 2 to 4 times more active than DIF. The potency of TEM was decreased by 2 to 8 times when tested in serum and in urine (58). There was no significant effect of pH on the potency of TEM (58). In mouse protection tests, TEM was as

active as CIP and DIF against enteric bacteria and *P. aeruginosa*, and as active as DIF, and 5 to 10 times more active than CIP against *S. aureus* and *Streptococcus pyogenes* and *Streptococcus pneumoniae* (58). The C_{max} and serum half-life ($t_{1/2}$) of TEM were 13.5 µg/ml and 1.3 hours, respectively, after an oral dose of 100 mg/kg in mice (58).



11 R=o,p-$F_2C_6H_3$, R_1=H, R_2=CH_3, X=CH
14 R=CH_2CH_2F, R_1=CH_3, R_2=H, X=CF
15 R=C_2H_5, R_1=H, R_2=CH_3, X=CF

A-61827 (12) (A-60969, T-3262) - The *in vitro* activity of 12 against aerobic bacteria was similar to that of CIP (60). However, the *in vitro* activity against anaerobic bacteria was better than that of CIP and the MIC_{90} for *Bacteroides fragilis* was 2 µg/ml. To overcome the poor solubility associated with A-61827, a series of 7-[(4S)-4-amino-2-substituted pyrrolidinyl]quinolones and naphthyridines have been prepared (61). While A-65485 (13) exhibits the same level of potency as A-61827, it has significantly improved aqueous solubility.



12 R=H, R_1=NH_2
13 R=α-CH_3, R_1=β-NH_2

Fleroxacin (14) (AM-833, Ro-23-6240) - The *in vitro*, *in vivo* and pharmacokinetic properties have been reviewed (62). The antibacterial activity of this compound was comparable to those of NOR and ENO (63). Most *Enterobacteriaceae* and *S. aureus* were inhibited by the achievable serum concentration of 2 µg/ml (64). The MIC_{90} for *P. aeruginosa* was 8.0 µg/ml (64). Fleroxacin (FLE) was 2 to 8 times less active than CIP against most organisms (65,66). The MIC_{90} against Bacteroides fragilis was 32 µg/ml (67). FLE was effective against intracellular *M. fortuitum* at 2.0 µg/ml but was inactive against *M. chelonei* (68). After administering 400 mg to human volunteers, the C_{max} was 6.1 µg/ml, the $t_{1/2}$ was 11.95 hours and the urinary recovery was 58.6% (69).

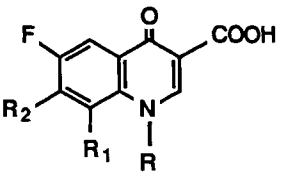
Lomefloxacin (15) (NY-198) - The *in vitro* activity of lomefloxacin (LOM) against aerobic and anaerobic bacteria was compared to those of OFL and NOR (70) and was in general 0.25-1 times as active. In mouse protection tests, LOM is generally 1-2 times as active as NOR if administered as a single dose after infection, reflecting its good pharmacokinetic properties (70).

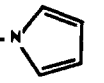
Irloxacin (16) (E-3432) - The *in vitro* activity of irloxacin against enteric bacteria and *P. aeruginosa* was similar to that of NOR (71). The MICs of 16 against *S. aureus* ranged from 0.06-1 µg/ml.

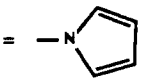
E-3604 (17) - The *in vitro* activity of 17 was in general similar to that of NOR (72). The MIC range of E-3604 for *S. aureus* was 0.03-0.25 μg/ml, more active than NOR and CIP against this organism.

S-25930 (18) and S-25932 (19) - The *in vitro* activity of these compounds against *Enterobacteriaceae* and *P. aeruginosa* was compared with the activities of CIP, ENO and DIF (73). S-25930 was more active than S-25932 and had activity comparable to that of DIF and ENO.

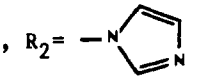
Other Quinolones - An efficient synthetic route to 7-halo quinolones by an intramolecular cyclization accompanied by the elimination of a nitro group has been reported (74). A series of 7-(azole substituted) quinolones has also been reported (75). The 1-imidazolyl derivative (20) was found to possess antibacterial activity slightly better than NOR. A series of 6-fluoro-1,4-dihydro-4-oxo-7-(4-pyridyl)-1,8-naphthyridine-3-carboxylic acids has been described (76). The N-cyclopropyl analog (21) is the most potent member of the series and its *in vivo* efficacy against *S. aureus* is superior to that of ENO. The syntheses and structure-activity relationship of 1-aryl-6,8-difluoroquinolone antibacterial agents have also been published (77).

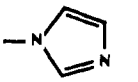


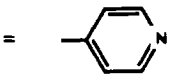
16 $R=C_2H_5$, $R_1=H$, $R_2=$ 

17 $R=CH_2CH_2F$, $R_1=H$, $R_2=$ 

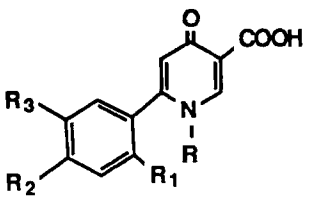
18 $R_1R=-CH_2CH_2CH(CH_3)-$, $R_2=CH_3$

19 $R_1R=-CH_2CH_2CH(CH_3)-$, $R_2=$ 

20 $R=C_2H_5$, $R_1=F$, $R_2=$ 

21 $R=\underline{c}\text{-}C_3H_5$, $CR_1=N$, $R_2=$ 

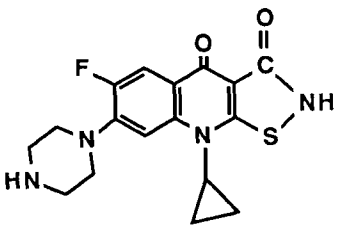
New Structure-Activity Relationships - A computer automated structure evaluation (CASE) program was developed and applied to the quinolones (78). Application of this CASE program may help to evaluate and design potent quinolones. The monocyclic quinolone Ro-13-5478 (22) and a conformationally restricted tricyclic analog, Ro-14-9578 (23), were found to have antibacterial activities equal to NAL (79). They inhibit DNA supercoiling catalyzed by DNA gyrase and produce DNA cleavage. It seems that a bicyclic quinolone is not necessary for DNA gyrase inhibition and antibacterial activity.



22 $R=C_2H_5$, $R_1=H$, $R_2=N(CH_3)_2$, $R_3=H$

23 $R_1R=-CH_2CH(CH_3)-$, $R_3R_2=-OCH_2O-$

Over the years, modification of the 3-carboxylic acid group of quinolones generally produced compounds with a substantial decrease in antibacterial activity. The first successful modification of the 3-carboxylic acid group of quinolones resulting in a series of extremely potent antibacterials was reported (80). The most potent isothiazolo-quinolone, A-62824 (24), in which the 3-carboxylic group of CIP has been replaced by an isothiazolo group, has *in vitro* antibacterial activity 4 to 10 times greater than that of CIP. The isothiazolo system in (24) possess aromatic character and the nitrogen proton is very acidic and can be considered a carboxylic acid mimic. Because of this new finding, research activity in 3-carboxylic acid modification is expected to increase in the future.



24

Conclusion - The focus this year has been on finding the next generation of quinolones, which have lower CNS side-effects, greater water solubility and improved activity against streptococci.

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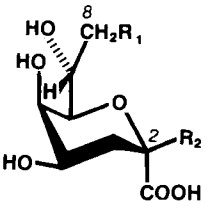
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Chapter 15. Novel Approaches Toward Discovery of Antibacterial Agents

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Introduction — This review attempts to describe some recent work on novel inhibitors. Novelty is defined on the basis of: 1) new mechanism of action (KDO inhibitors); 2) unusual structural features (Gyrase Inhibitors); and/or 3) inhibitory activity aimed at a novel part of a classical pathway (Novel Inhibitors of Protein Synthesis). In addition, suggestions for potentially new targets in the areas of lipopolysaccharide biosynthesis, bacterial cell division, protein secretion and defensins are discussed. Other target areas with novel modes of action have been adequately described in earlier reviews (1,2).

KDO Inhibitors — There have been two reports on a new class of synthetic antimicrobials that inhibit 3-deoxy-*manno*-octulosonate cytidylytransferase (CMP-KDO synthetase; EC 2.7.7.38;(3,4). CMP-KDO synthetase is an essential enzyme in the biosynthetic pathway of lipopolysaccharide, a constituent of the outer membrane of all gram-negative bacteria (3,4). The eight-carbon sugar, KDO (**1**), is unique to gram-negative bacteria (5), providing some security that inhibitors of this enzyme could have selective toxicity. In addition to preventing the synthesis of lipopolysaccharide, CMP-KDO synthetase inhibitors may also inhibit the synthesis of KDO-containing capsular polysaccharides, thereby reducing the virulence of microorganisms that produce this type of capsule (6).



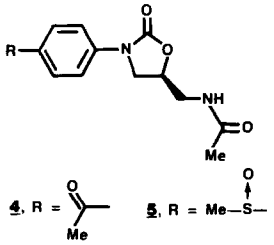
1, R_1 = OH, R_2 = OH
2, R_1 = OH, R_2 = H
3, R_1 = NH_2, R_2 = H

A key discovery was the finding that the β-pyranose form of KDO, the least abundant of the four solution tautomers, was the preferred substrate for CMP-KDO synthetase (7). The 2-deoxy analog of β-KDO-pyranose (dKDO; **2**) inhibits CMP-KDO synthetase with a K_i of 4-10 μM, confirming that the β-pyranose form is the actual substrate (3,4,8). In addition, the potency of dKDO substantiates the proposed nucleophilic displacement mechanism for CMP-KDO formation (9). Further studies revealed that the NH_2-dKDO (**3**) was at least equally active as dKDO (3,4). Neither dKDO nor NH_2-dKDO analogs were active against intact bacterial cells, suggesting that these compounds were not being efficiently transported into these cells. Effective transport of KDO inhibitors was ultimately accomplished by linking amino acid residues by a peptide bond to the 8-amino group of NH_2-dKDO (3,4); this strategy had been used previously to transport other heterologous compounds into bacteria (10,11). This approach, which is dependent upon an intact oligopeptide permease and cytosolic aminopeptidases to release the active NH_2-dKDO (3,4), was successful and reduced the minimal inhibitory concentrations (MIC) 500- to 1000-fold in *E. coli* and *S. typhimurium* (3). Dipeptide derivatives such as alanine-alanine-NH_2-dKDO gave MIC values of 5 to 100 μg/ml depending on the species tested. However, these compounds are not protective in *in vivo* animal models against a variety of gram-negative pathogens due to loss of the peptidyl side chain via the action of non-specific peptidases (12).

Novel Inhibitors of Protein Synthesis — Two antimicrobials of the structurally novel oxazolidinone class have been described (13-21). DuP 721 (**4**) and DuP 105 (**5**) are active

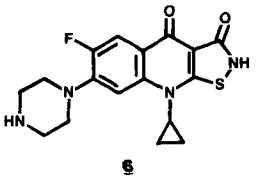
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against gram-positive bacteria and gram-negative anaerobic bacteria. DuP 721 is the more potent of the two with an MIC_{90} of 1-4 μg/ml against staphylococcal isolates (including β-lactamase positive and methicillin-resistant strains), 4 μg/ml against group D streptococci and 4-16 μg/ml against *B. fragilis*. Animal infection models confirmed the activity of the oxazolidinones against the aforementioned pathogens. Pharmacokinetic studies indicate that DuP 721 is well absorbed orally and that peak serum levels, even after single administration of nontoxic doses of compound to rats, dogs, or mice, were in excess of the MIC_{90}s for both staphylococci and streptococci.

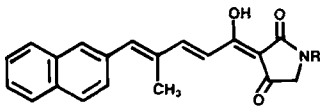


4, R =

5, R =



6



7, R = H
8, R = Me

Although these compounds are bacteriostatic, preliminary *in vitro* studies suggest that resistant mutants are difficult to isolate. It has been suggested that these compounds interfere with interactions between the ribosome and the ribosome recognition sites in the mRNA sequence (19). Since this step precedes the f-met^{tRNA} binding to the ribosome, these compounds may bind to the ribosome during the elongation cycle in a way that still permits elongation and termination to continue, but prevents subsequent initiation from a natural mRNA species.

DNA Gyrase Inhibitors — Possibly the most active current area of antibacterial design involves modifications of the quinolone nucleus (22). Although work on quinolone or naphthyridines has recently focused on C-7, C-8 and N-1 substituents (23), one report suggests that the quinolone SAR story is not a closed issue (24). Specifically, compounds were described in which the C-2 and C-3 positions were bridged by a thiohydroxamic acid chain to form an isothiazolo ring. This carboxylic acid surrogate dramatically improves activity. When the N-1 position is modified with a cyclopropyl substituent (**6**), the resulting compound is 10 times more potent than its parent, ciprofloxacin. When the N-1 position is substituted by a p-fluorophenyl group, the isothiazolo-quinolones are only about 2 times more active than their homologous counterparts. The potency of the isothiazolo-quinolones is reflected in their inherent DNA gyrase inhibitory activities as measured in a supercoiling assay as well as by their superior MIC values. The isothiazolo-quinolones represent the first modification of the carboxylic acid moiety resulting in a series of extremely potent antibacterials.

A second structurally different series with DNA gyrase inhibitory activity was also reported (25). Compounds **7** and **8** are structurally related to streptolydigin and tirandamycin, naturally occurring tetramic acids that inhibit procaryotic RNA polymerase (26). In addition to inhibiting anaerobes like the naturally occurring tetramic acids, the more potent synthetic derivatives also have activity (MICs of 1-3 μg/ml) against *S. aureus* species. These compounds do not have significant activity against purified bacterial RNA polymerase, but were found to have DNA gyrase inhibitory activity in the supercoiling assay; an I_{50} in the 3-10 μg/ml range was observed. Structure **8** was rapidly bactericidal to both *S. aureus* and *B. fragilis* and was active against strains that were either quinolone-resistant, coumermycin-resistant, or tirandamycin-resistant. This result suggests that these compounds may be interacting with different subsites on DNA gyrase and/or DNA, or that they have multiple modes of action.

Inhibitors of Lipopolysaccharide Biosynthesis — Lipopolysaccharide (LPS) provides an attractive target for antibacterial action as it is an essential component of gram-negative cells and contains constituents that are unique to procaryotes (27,28). LPS consists of three domains: lipid A, core oligosaccharide and O-antigen side chain. Lipid A is the most highly

conserved structural domain of LPS and forms the lipid monolayer of the outer leaflet of the outer membrane. The core sugar domain is attached to lipid A via KDO. The O-antigen side chains are glycosidically attached to the the distal core sugar and extend outward from the cell surface.

Perhaps the most tractable target in the LPS biosynthetic pathway is one or more of the enzymes involved in the early stages, *i.e.*, in the formation of the lipid A disaccharide (29-33). The key to the elucidation of the lipid A biosynthetic pathway was the identification of lipid X or 2,3-diacylglucosamine-1-phosphate (**11**) in a double mutant that was selected as a temperature-conditional mutant for phosphatidylglycerophosphate synthesis (27). This finding stimulated research that has provided us with the current concept of lipid A biosynthesis (Figure 1; from reference 34 with permission of the author and publisher).

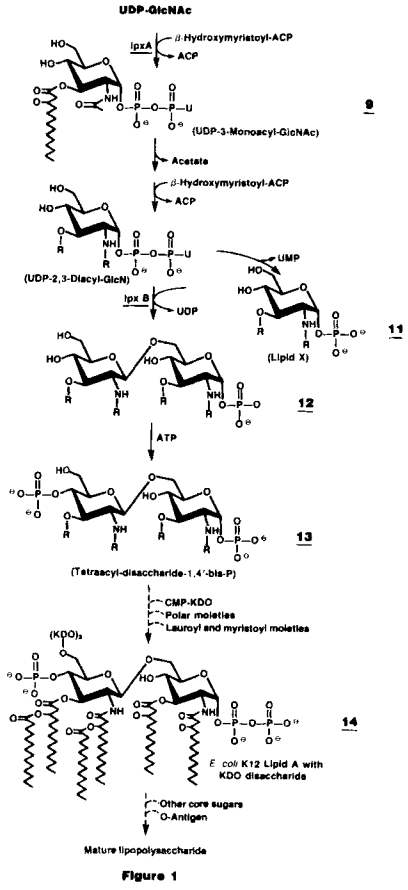


Figure 1

Since β-hydroxymyristate is unique to gram-negative LPS, inhibitors of the UDP-*N*-acetylglucosamine acyltransferase or lipid A disaccharide synthase are attractive. UDP-*N*-acetylglucosamine acyltransferase, the product of the *lpxA* gene, is responsible for the transfer and esterification of a β-hydroxymyristoyl moiety from its acyl carrier protein derivative to the 3 position of the glucosamine ring of UDP-*N*-acetylglucosamine, resulting in the formation of UDP-3-monoacyl-*N*-acetylglucosamine (**9**) (30,34). The lipid A disaccharide synthase, the product of the *lpxB* gene, forms tetraacyl-disaccharide-1-phosphate (**12**) from UDP-2,3-diacylglucosamine (**10**) and 2,3-diacylglucosamine-1-phosphate (**11**) (29,32,34). The product of this reaction is phosphorylated (**13**) and further modified (**14**) to form the mature LPS species that can be antigenically distinguished primarily based on differences in sugar moieties.

A program based on the identification of mutants in LPS synthesis or translocation could be used to identify inhibitors of these processes. This strategy would take advantage of monitoring a late stage in LPS maturation and could use a radiolabel or antibody to core sugars or O-antigen side chain (in strains other than *E. coli* K-12) to monitor LPS synthesis. Inhibitors of lipid A biosynthesis, of carrier lipid biosynthesis and/or function, and inhibitors of O-antigen transferases would potentially be detected in this assay. Secondary *in vitro* biochemical assays would help pinpoint the enzyme(s) that is the primary target of the compound.

LPS is a biologically important molecule in gram-negative cells because it is responsible for maintaining the selective permeability of the outer membrane (27). In addition, LPS is also an important biological molecule to man as it has been shown to modulate immune reactions (27,28,35,36). Lipid X, or 2,3-diacylglucosamine-1-P, retains many functions of lipid A, including mitogenicity in the presence of serum (27,28,37). It has been shown to be devoid of the toxicity associated with disaccharide forms of lipid A (endotoxin; 38,39) and protective against the effects of bacteremia and endotoxemia in mice (40). Thus, struc-

tural analogs to early precursor forms of lipid A might be immunostimulatory or protective against septic shock as well as antibacterial.

Targeting Bacterial Cell Division — Many of the more potent and successful antibiotics to date have had as their target enzymes in the peptidoglycan biosynthetic pathway. Enzymes in this pathway are good targets not only because peptidoglycan is an essential component of all bacteria but also because this macromolecule is unique to procaryotes, thereby theoretically ensuring selective toxicity.

It has long been felt that there is more than one system or basic set of enzymes for murein (peptidoglycan) synthesis in bacteria (41). This view has been substantiated by the finding that certain β-lactam antibiotics have greater affinity for one penicillin-binding protein over another (42). In addition, there are mutants which are blocked only in lateral wall formation (*rodA, pbpA*) and mutants blocked only in septum formation (many of the *fts* alleles; 41). Double mutants constructed by combining either *rodA* or *pbpA* with either *ftsA, ftsI* (*pbpB* or *sep*), *ftsQ,* or *ftsZ* have implicated three systems involved in the morphogenesis of rod-shaped cells (43). The first system, in the absence of other modifying systems, is capable of forming a complete murein sacculus around a growing cell. When only this basic system is allowed to function in rod-shaped organisms, spherical cells are produced that enlarge without any division. Modifying reactions for lateral wall elongation result in a rod-shaped organism and are specified by a second set of enzymes known to include *rodA* and *pbpA* (PBP2). The third system appears to be responsible for cell division or septum formation and includes a large number of the *fts* gene products. It is thought that the second and third systems compete, the result ultimately determining cell shape and cell division.

Antibiotics whose modes of action involve inhibition of membrane-bound transpeptidases, carboxypeptidases and/or transglycosylases have already been identified (41). Many of these antibiotics belong to the β-lactam structural family. The inactivation of some of these compounds by β-lactamases or by the presence of new or altered target enzyme (changes from PBP2 to PBP2′, for example; 44) has led to more sophisticated derivatives or the need for a second inhibitor to prevent inactivation of the primary antimicrobial (42,45-47). Discovery of clinically useful inhibitors of cytosolic enzymes involved in peptidoglycan synthesis has been more difficult due to troubles with delivery (10,11) or lack of selectivity (common pathways for procaryotes and eucaryotes).

A possible novel target of antimicrobial action exists within the morphogenetic system that determines septum formation. A large number of gene products (some 15-17) are thought to participate in septum initiation and formation (41). The ease with which these essential genes, or *fts* alleles, can be identified within this system suggests that septum formation is easily perturbed and that there are multiple targets. As a broad-spectrum agent is highly desirable, it is clear that steps common to septum formation in both gram-positive and gram-negative bacteria are of interest. At present, the molecular functions of many of those *fts* proteins are largely unknown and only limited literature exists to ensure that these compounds are found in both gram-positive and gram-negative bacteria.

The cluster of *fts* genes that are located at minute 2 on the *E. coli* chromosomal map are perhaps the best characterized of the *fts* genes (41,48-50). Any one of these essential genes that are involved in cross-wall formation could be a potential target for an antimicrobial agent. FtsA protein is a 45,000 dalton protein thought to be involved in an event late in septum formation (41,50). It appears to interact with PBP3 (51), the product of the *ftsI* (*sep* or *pbpB*) gene, that codes for transpeptidase and transglycosylase activities (41). The function of FtsA in septum formation is not entirely understood, but it is reputed to be either the termination protein that links chromosome replication with cell division and/or a structural part of the septum (52). The synthesis of this protein is required for each cell division, but

only for a short period immediately prior to septum initiation (41). The product of the *ftsQ* gene, a 31,500 dalton protein, is involved in an intermediate stage of septum formation, acting at a stage earlier than the *ftsA* protein (41,43,50). The FtsZ protein is thought to initiate septum formation (41,43). It is probably not fortuitous that these genes are closely clustered and potentially translationally coupled (48). Interaction among FtsQ, FtsA, and FtsZ proteins can be surmised in certain double mutant strain constructions (53).

In terms of an antibacterial target, perhaps the most interesting and best described protein of those involved in cross-wall formation (above) is the *ftsZ* (*sulB, sfiB*) gene product. There is good evidence that this 40,000 dalton protein controls a rate determining early step in septum formation (43). Studies with *ftsZ-lacZ* operon fusions have shown that the level of expression of *ftsZ* is in proportion to the number of cross-walls formed irrespective of growth rate or cell size (41). Thus, this gene seems to be regulated with septum formation. In addition, FtsZ protein appears to be ubiquitous to many bacterial species as demonstrated by the presence of cross-reacting genomic DNA sequences to an *ftsZ* gene probe and/or the presence of a major immunoreactive band (54). Therefore, this protein target has potential as a broad-spectrum antibacterial agent.

The importance of the FtsZ protein in cell division is also substantiated by its well-recognized interaction with the SulA (SfiA) protein (55-57). SulA protein is induced in response to DNA damage and interacts with FtsZ protein, rendering it immediately nonfunctional in its role as a septum initiator (55-57). Lateral wall formation, specified by a separate set of enzymes (above), is not inhibited; the cells elongate as filaments. If this filamentous growth continues unchecked, it is lethal. Therefore, cells have evolved a system to regulate their collective response to DNA damage (SOS system). The SulA protein is part of this system.

In order to understand the importance of the *sulA/ftsZ* interaction and to highlight another potential target for antimicrobial action (*sulA* gene synthesis, see later), it is necessary to describe the SOS system. SulA synthesis, like other genes of the SOS regulon, is under negative control by the *lexA* gene product (57,58). When DNA is damaged, an inducing signal of unknown nature is produced. In the presence of this inducing signal, RecA protein is activated to become a protease specific for the LexA receptor. Other genes that are part of the SOS regulon are also induced (including *recA* and *lexA*) as a result of LexA cleavage and many of them function to repair the DNA that is damaged. As the DNA damage is repaired, the signal that activates RecA protein dimishes, and LexA protein accumulates to once again negatively repress synthesis of the SOS genes. The inhibition of cell division via the *sulA/ftsZ* interaction is reversed by another protease that has also been induced. This protease, encoded by the *lon* gene, cleaves preexisting SulA protein, releasing FtsZ in an active form that functions to initiate cross-wall formation (57,59).

In *lon* (protease$^-$) mutants in which SulA protein is stabilized, the release from SOS-induced cell division inhibition cannot be controlled, leading to filamentous death upon *sulA* induction (60). An over-production of FtsZ can help relieve the lethality associated with the *lon* phenotype (61). Thus, if a strain that is protease deficient (*lon*) and capable of overproducing *ftsZ* is induced for *sulA* synthesis, growth is relatively normal because FtsZ protein is in excess of SulA protein. If the function or synthesis of the FtsZ protein is impaired, the strain will die upon *sulA* induction because there is no excess FtsZ protein to initiate cross-wall formation. Therefore, by way of strain construction, a system can be built to allow the detection of any mutation or compound that interferes with FtsZ function (61). If the goal is to detect only compounds that cause death due to inerference with *ftsZ* function and not *ftsZ* synthesis (rule out general inhibitors of protein synthesis), the synthesis of *ftsZ* could be monitored using operon fusion technology. Strains containing a *ftsZ-lacZ* operon fusion have the operator region of the *ftsZ* gene fused to a functional sequence encoding β-galactosidase. The β-galactosidase assay provides a convenient spectrophotometric measure of

ftsZ synthesis. Therefore, compounds that cause death in a *lon*$^-$ *sulA*$^+$ *ftsZ*$^{++}$ strain without inhibiting *ftsZ* synthesis would presumably do so by interfering with the role of the FtsZ protein in cell division.

Another interesting target would be SulA protein. Although this gene is nonessential, it appears to have been conserved throughout the *Enterobacteriaceae* family (54). Its primary role is to inhibit cell division only during the SOS response by interacting with FtsZ protein. Since SulA synthesis is not constitutive, one would need to identify compounds that induced the synthesis of *sulA*. Such an antibiotic, if possible to find, would represent a first in a type of antibiotic that would have regulatory properties at a transcriptional level. Thus, a chemical that induced the synthesis of *sulA* would result in bacteria committing suicide (62).

Induction of *sulA* could be monitored by using strains that have *sulA-lacZ* operon fusions (57). If induction of *sulA* in the absence of induction of other SOS genes is desired, a strain containing a fusion to another SOS gene (*recA*, for example) could be monitored for comparison (57). Proof that induction of *sulA* in the absence of general SOS induction is lethal to cells is seen in studies with strains that have *sulA* under the control of an inducible *lac* operon (62). In these strains, when the synthesis of *sulA* is induced, there is a rapid arrest of cell division, growth of cells as filaments and loss of cell viability.

Inhibitors of Protein Secretion — One of the most extensively researched areas in cell biology today is the study of protein export or secretion. Investigations include genetic and biochemical studies to define how a cell distinguishes the synthesis of cytosolic proteins from proteins destined to be secreted (63-71). Many proteins destined for export are initially made as precursors with amino-terminal extensions called signal sequences. The overall role of the signal sequence is to direct the nascent polypeptide/ribosome complex to the endoplasmic reticulum (ER) in eucaryotes and the cytoplasmic membrane in procaryotes (Figure 2; from reference 71 with permission of the author and publisher). As the signal sequence emerges from the ribosome, it interacts with an important component of the secretion machinery, the signal recognition particle (SRP). SRP is an 11S nucleoprotein that is composed of six heterologous proteins and one molecule of 7SL RNA. SRP can exist in

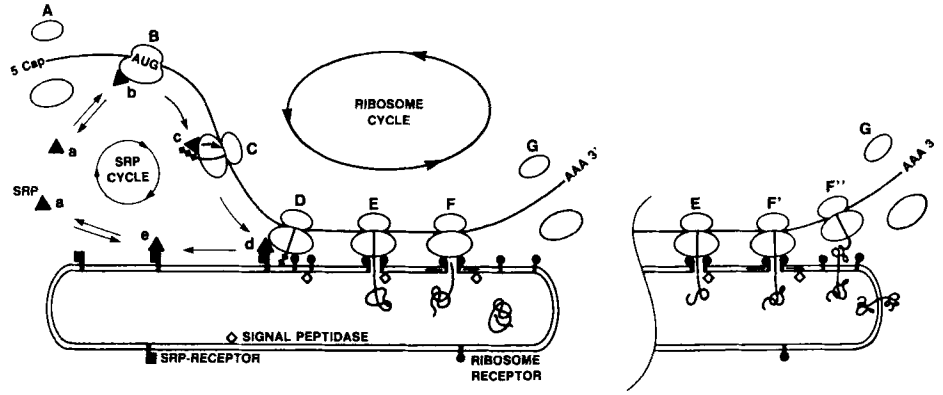


Figure 2

a soluble form in the cytosol (a), bound to ribosomes (b) and bound to SRP receptor (c). SRP binds more tightly to a ribosome translating a mRNA encoding a secretory protein (steps A-C); this binding often results in a detectable translational arrest. This elongation arrest is relieved when the SRP complex interacts with its specific receptor in the ER membrane, SRP receptor (step D). Once the nascent chain/ribosome/SRP complex has been correctly targeted to the ER membrane, SRP and SRP receptor dissociate. As protein synthesis continues, compatible sequences are translocated into (steps E,F',F'',G) or across (steps

E-G) the ER membrane with the aid of at least one protein, the translocon, and ATP. During the translation of the polypeptide or shortly after its synthesis, the signal peptide is cleaved by an endoprotease, signal peptidase, that faces the lumenal side of the ER. The translocation complex disengages and is recycled as the synthesis and translocation of the polypeptide is completed.

The above model for protein translocation is largely based upon the result of biochemical efforts in eucaryotic systems. Genetic support for the involvement of proteinaceous factors in protein secretion in procaryotes has come from the isolation of mutants in *E. coli* that are defective in protein translocation (63,67,68). Biochemical efforts have proceeded differently, with the best described components of the procaryotic secretion machinery being the signal peptidases (72-74). Signal peptidase I has been purified from *E. coli*, its primary sequence determined and its activity reconstituted *in vitro* with fidelity (64,72,75). Two different genetic approaches have shown that this enzyme is the major endoprotease that cleaves non-lipoprotein presecretory proteins and that its activity is essential for bacterial viability (75,76). Its location in the inner membrane with the majority of the polypeptide chain situated in the periplasm makes it an attractive target theoretically because inhibitors would not have to cross the cytoplasmic membrane (75). Procaryotic signal peptidase I is a single polypeptide of 37,000 daltons (75) whereas the homologous eucaryotic enzyme appears to consist of a protein complex located on the lumenal side of the ER (77,78). Although its substrates, the amino termini of presecretory proteins, are quite diverse at the level of the primary sequence, there are some secondary features that are preferred by the enzyme (79). The minimal inhibitory peptide based on the signal sequence of procoat has been described (80). Since virtually all bacteria translocate essential proteins outside their cytosol, selective inhibitors of signal peptidase I would theoretically have broad-spectrum antimicrobial activity.

Signal peptidase II and its specific inhibitor, globomycin have been reviewed previously (2,72). This enzyme cleaves the signal sequence from lipoprotein-like presecretory proteins. Although globomycin is not useful clinically, it has provided a valuable tool to isolate mutants in the biosynthetic pathway of lipoprotein synthesis and maturation. The isolation of a third signal peptidase from *E. coli* has been described (72), but its role in the secretory pathway remains to be determined.

Other less well characterized genes that are thought to be involved in the secretory pathway include the *secA, secB, prlA (secY), prlC, prlD* and *prlE (secD)* genes (63,67,68,81-84). These genes were identified by one of several genetic strategies (63,67,68). Primarily the phenotypic properties of the *lamB* and *malE* genes of the *E. coli* maltose operon have been exploited in this mutant hunt. The LamB protein is an outer membrane porin protein that participates with MalE protein, a periplasmic binding protein, in the uptake of maltose and maltodextrins. LamB is also the receptor for the bacteriophage λ. Cells containing the complete maltose operon grow normally when provided with maltodextrin as the sole carbon source (Dex$^+$). Strains containing the lactose operon can also utilize lactose as a carbon and energy source (Lac$^+$). Strains encoding hybrid proteins containing the N-terminus of *lamB* or *malE* fused to *lacZ*, a gene of the lactose operon specifying the cytoplasmic enzyme, β-galactosidase, have been constructed (63,67,68). If the hybrid protein localizes to either the inner or outer membrane, β-galactosidase cannot form a functional tetramer and the cells will be Lac$^-$. In these strains, a selection for Lac$^+$ provides a simple way to enrich for mutations in the secretion pathway without simultaneously detecting mutations that affect the synthesis of the selected polypeptide. Mutations in the secretory pathway would be expected to localize the hybrid protein to the cytosol where β-galactosidase is functional and the cells are phenotypically Lac$^+$. This type of selection was used to identify the *secA* and *secB* genes.

SecA protein is an essential peripheral inner membrane protein of 92,000 daltons whose synthesis appears to be regulated in response to the cell's secretion needs (85). It has been suggested that this protein may be the membrane receptor for presecretory proteins, *i.e.*, SRP receptor (67). The *secB* gene is nonessential and encodes a 12,000 dalton protein that is required for the normal secretion of only a subset of exported proteins (81).

A second strategy to identify components of the secretory pathway is to look for mutants that rescue or suppress the secretion of proteins that contain a defective signal sequence (63,67,68). For example, certain alterations in the *lamB* signal sequence prevent the proper interaction of the signal sequence with the secretion machinery, resulting in the aberrant precursor form accumulating in the cytosol. Since LamB protein cannot function as either a porin or phage receptor when it resides in the cytosol, this strain will have a $Dex^- \lambda^-$ phenotype. A selection for growth on maltodextrin and/or sensitivity to λ can be used to identify suppressor mutations that facilitate the proper translocation of the aberrant LamB protein. The *prlA* gene was identified by this approach. It is an essential integral membrane protein that appears to interact with the signal sequence to effect translocation (86). PrlA protein has also been shown to be absolutely required for translocation of proteins in an *in vitro* biochemical assay (87) and has been proposed to be part of the translocon (69). Certain *prlA* alleles suppress the *secA*ts mutation, suggesting that these two proteins interact in the secretory pathway (88).

Although it is not clear what the rate-limiting component is in the secretion pathway, it is clear that there are multiple targets within this pathway, many of which are essential to normal growth. Compounds that interfere with the secretion pathway at different steps could be detected in screens based on either of the above genetic strategies. There are many mutants available (63,67,68) and biochemical assays (64,65,72,75,87) to devise appropriate secondary screens to help localize the step in the pathway that is being inhibited.

Defensins — The term, defensins, refers to a family of microbiocidal and cytotoxic peptides that have been found in human, rabbit and guinea pig polymorphonuclear leukocytes (PMN) and in rabbit alveolar macrophages (89-93). These cationic peptides are composed of 29 to 34 amino acids and are rich in cystine, arginine and aromatic residues (94,95). The primary structures of the three human defensins differ in sequence only at their amino termini (95). The remarkable homology between species, especially the conservation of all six cysteinyl residues, suggests that these peptides are important to the host defense mechanism of vertebrates (95). Defensins are primarily found associated with azurophil granules and are thought to play a significant role in intraphagosomal killing (96). Defensins can constitute as much as 10% of the total protein in PMN granules (96). Any extracellular release of defensins would most likely occur in confining spaces such as intercellular clefts between PMNs and their targets or in PMN-rich exudates (96).

The *in vitro* spectra of the peptides include several gram-positive and gram-negative bacteria (89,93), fungi (89,98-100) and selected enveloped viruses including Herpes virus (89,92,101). Even if a specific defensin does not have inherent activity against a pathogen, it may act synergistically with another family member as has been demonstrated for NP-5, a component of the rabbit defensin family (102). Presently, the mode of action of these peptides is unknown. However, the primary sequences of defensins provide a starting point for chemical modification to ensure stability in serum and/or increase potency. The cloning of the gene(s) for defensins could facilitate a complete analysis of the effects of amino acid substitutions. In addition, this cloning effort could facilitate the production of clinically efficacious defensins as recombinant products.

Recently, two peptides with broad-spectrum antimicrobial activity were isolated from the skin of the African frog *Xenopus laevis* (103,104). Many clinically relevant species exhibit

MICs in the 5-50 μg/ml range upon treatment with these peptides. These water-soluble peptides called magainins also have activity against fungi and induce osmostic swelling of protozoa although they do not appear to be simple membrane disrupters as evidenced by their lack of hemolytic activity against human erythrocytes (up to 150 μg/ml). Both peptides are 23 amino acids long and have an amino acid sequence consistent with a strongly amphiphilic nature. The peptides are derived from a common precursor that codes for one copy of magainin 1 and two copies of magainin 2. Studies with synthetic magainin 2 analogs demonstrate that three amino-terminal residues can be deleted without appreciable loss of antibacterial or antiprotozoan activity (104).

Although there is no structural similarity to defensins, the magainins are functionally similar in that they are part of their host's defense system. Structurally they resemble the cecropins, a family of amphiphilic, nonhemolytic small peptides that constitute the antibacterial defense system of insects (105,106). The development of any of these peptides to be clinically effective would be a challenge that could have multiple indications.

Conclusions: Areas in procaryotic cell biology with potential for an antimicrobial target have been suggested. Strategies to enrich for compounds with novel mechanisms using genetic and biochemical approaches that have proven successful in the identification of mutants or protein components in the respective areas have been reviewed. Additionally, molecules designed as part of naturally-occurring host defense systems were discussed in light of their antibacterial activities and potential as chemical leads.

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Chapter 16. Chemical Modification of Antibodies for Cancer Chemotherapy

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Introduction- Monoclonal antibodies (MoAb's) which can discriminate between normal tissues and their cancerous counterparts offer a vector by which drugs, toxins, immunomodulators and radioisotopes can be targeted to tumors selectively, avoiding the damage inflicted upon normal tissues with classical administration of such agents. No truly tumor-specific MoAb's have as yet been developed. Nevertheless, impressive results have been achieved in some instances with MoAb-based therapies in spite of normal organ cross-reactivities. This chapter updates the chemistry behind conjugation of agents to MoAb's (1-3). Recent reviews dealing with other aspects of MoAb-based therapy have appeared, covering subjects such as the generation of hybridomas, the various tumor-associated antigens, problems inherent in MoAb-based approaches to tumor therapy, and clinical experiences with MoAb's and their conjugates (4-9).

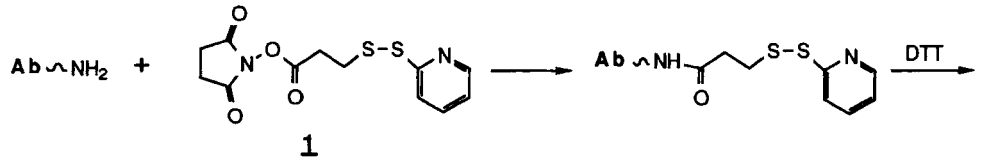
Chemical Considerations- Most attempts at conjugation have dealt with the IgG class of antibodies because their smaller size (~150,000 MW) makes them better candidates for penetration into solid tumors than the pentameric IgM isotype (10). Smaller functional fragments, derived from enzymatic digestion of antibodies, have also been used. The minimal dimeric units are designated as $F(ab')_2$, and monomeric fragments as Fab or Fab'. Covalent bond formation between side-chains of the amino acid backbone and drugs has relied predominantly on the nucleophilicity of lysine ε-amino groups. Modifications at more specific sites have involved cysteine residues or carbohydrate chains. Several recent reviews have dealt with the restrictions placed on useful conjugation reactions due to the nature of MoAb's (8,11). Briefly, all methods of activating agents for conjugation must yield intermediates which are stable to, but reactive, under aqueous conditions. All reactions must preserve the immunoreactivity of the original MoAb by not involving functionalization in the antigen recognition sites. Although maximum loadings achieved by a particular method are mentioned in the following discussion, such loadings differ from one MoAb to the next, and often reflect a point at which immunoreactivity was lost upon further loading.

Covalent Non-specific Conjugations

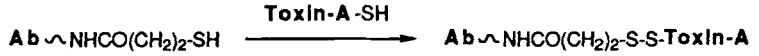
Immunotoxins- Plant toxins such as abrin, ricin and diphtheria toxin are unique proteins so potent that one molecule is sufficient to shut down protein synthesis completely and therefore kill a cell (12-14). They generally consist of an enzymatically active subunit, referred to as the A-chain, which is nontoxic by itself unless it is covalently linked by a disulfide bond to a targeting subunit, called the B-chain. The B-chain binds to cell surface galactose receptors indiscriminately prompting endocytosis and delivery to ribosomes. The strategy in developing an immunotoxin (IT) is therefore to have a MoAb serve as a surrogate B-chain and direct the toxin to target cells, sparing normal tissues.

The literature in the field of IT construction and testing is extensive (15). The most commonly used conjugation method for IT's is the formation of a reducible disulfide linkage between the antibody and the A-chain of the toxin. A sulfhydryl group is introduced into the antibody by reacting, for example, the heterobifunctional agent SPDP (1) with a primary amine of the MoAb, followed by dithiothreitol (DTT) cleavage. A sulfhydryl group is introduced into the toxin by reacting it with SPDP, 2-iminothiolane, or by cleaving the disulfide bond between the A- and B-chains with DTT. The conjugation is completed as shown:

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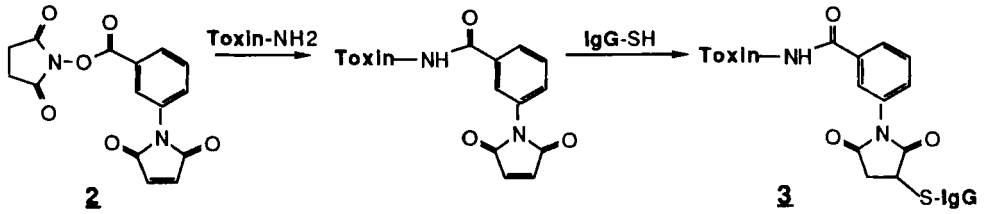


1



Ricin A-chain (16-19), diptheria toxin A-chain (20), abrin A-chain (21), gelonin (22), antiviral protein from *Phytolocca americana* (23-26), *Pseudomas* exotoxin (27) and *Saponaria offinallis* toxin (28) have all been conjugated to antibodies with the SPDP method. Efficacy of disulfide linked conjugates has been inconsistent. In some tests IT's showing *in vitro* protein synthesis inhibition had low activity *in vivo* (29). Potentiation of A-chain conjugates has been observed if they are coadministered with either free B-chain (30) or a B-chain conjugate targeted to the A-chain complex (31). To prevent lysosomal digestion of conjugates by target cells, lysosomotropic amines such as chloroquine, monensin and ammonium chloride have been used in conjugation with A-chain conjugates (29,32). Modification of A-chain carbohydrate by sodium periodate and cyanoborohydride treatment at pH 3.5 has been found to decrease liver uptake of ricin A-chain conjugates and dramatically increase circulatory half-life (33,34). Modifications of the antibody carbohydrate and attachment of polyethylene glycol or dextran also enhanced the circulating half lives of IT's (35). Attempts to increase serum stability of disulfide-linked IT's has prompted the design of new linkers; 3-(2-pyridyldithio)butyryl N-hydroxysuccinimid and 4-succinimidyloxycarbonyl-α-methyl-α-(e-pyridyldithio)toluene (SMPT) both produced conjugates with hindered disulfide links and markedly increased serum stability (14,36).

If either the toxin or MoAb are modified with MBS (2), non-reducible thioether bonds (3) are formed to thiol-modified conjugation partners as shown below (37). This technique has been used primarily to prepare whole ricin conjugates (38). Water soluble sulfo-MBS and 4-(p-maleimidophenyl)butyryl sulfo-N-hydroxysuccinimid (SMPB) have been developed to improve yields and solubilities (39). 4-(Iodoacetylamino)benzoyl N-hydroxysuccinimid (SIAB), and the maleimido-6-aminocaproyl ester of 4-hydroxy-3-nitrobenzenesulfonic acid have also been used as cross-linkers in this approach (36,40).

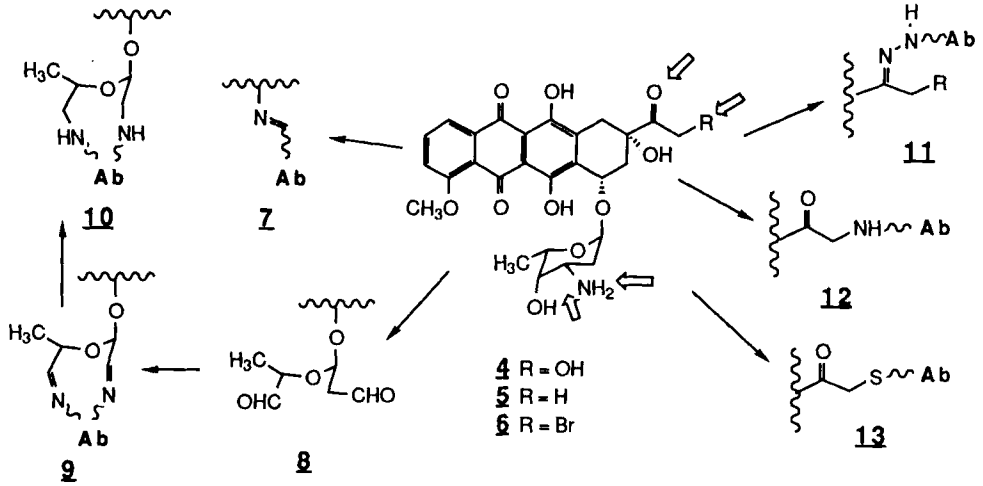


2 3

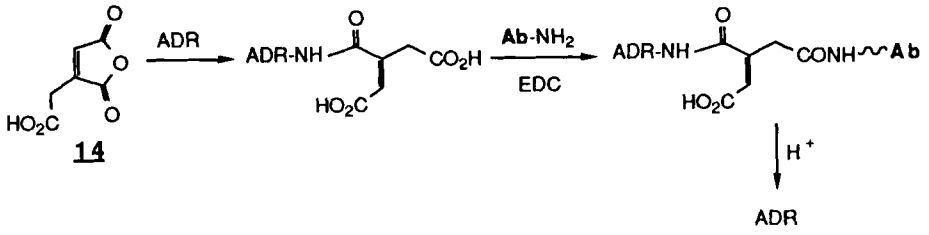
Binding of the B-chain to galactose residues of non-target cells interferes with the selectivity of whole toxin IT's. Co-administration of galactose or lactose to block this binding confers selectivity *in vitro* (13). Photoaffinity labels based on galactose have been prepared and reacted with ricin to block this receptor, but the technique is limited because the optimum uv wavelength for activating the photolabel caused degradation of ricin itself (41). Alternatively, by selecting those IT constructs in which the galactose-binding sites of the B-chain have been sterically blocked by the MoAb, increased selectivity of whole ricin conjugates has been observed (42). Genetic engineering may provide the ideal toxins for IT construction. The genes for diphtheria and ricin toxin have been cloned and the site-specific mutagenesis of residues responsible for galactose binding may produce toxins which no longer exhibit non-specific binding yet transport the toxin from the lysosomal compartment to the ribosomes (31).

Anthracyclines- Conjugates of both adriamycin (ADR, 4) and daunomycin (DNR, 5) have been constructed by modifications at one of the four sites shown below. Glutaraldehyde has been used to cross-link amines of the MoAb lysine groups with the 3'-amino group of the sugar residue (7) to

produce loadings of up to 5.7 drugs per antibody (43). Oxidation of the daunosamine sugar with sodium periodate cleaves the 3',4'-carbon bond to generate a dialdehyde (8) which likewise reacts with MoAb amines to form Schiff's base linkages 9 (44,45). In some studies, the Schiff's base linkages have been reduced with $NaBH_4$ (to 10) to increase the stability of the conjugates (44). Not all functionalizations through the sugar have yielded active immunoconjugates. Cross-linking ADR with MoAb carboxyl groups in the presence of a carbodiimide generated inactive materials, as did incorporation of a succinic acid spacer between the amines of ADR and a MoAb (44,46). The 14-bromo derivative of DNR (6) has been used as a conjugation partner. Displacement of the bromine by MoAb lysines (to give 12) has produced loadings of up to 8.5 DNR (46). Sulfhydryl-modified MoAb's have likewise been used to produce (13) with up to 4 DNR per antibody (46).

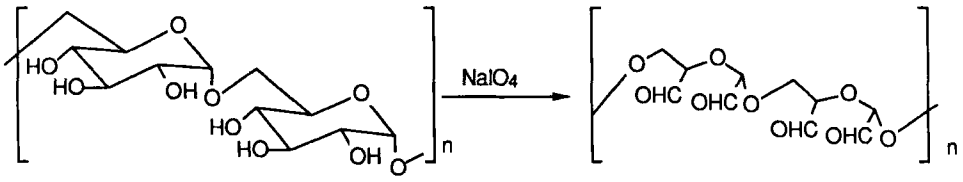


Several attempts have been made to prepare true pro-drug forms of the anthracyclines by incorporating spacers which could be cleaved subsequent to targeting. Cis-aconitic anhydride (14) has been reacted with the amine of ADR, then coupled to MoAb's using the EDCI method discussed later (46-48). These conjugates, which contained up to 10 ADR per MoAb, were stable at physiological pH, but released free drug in the acidic environment of lysosomal compartments after endocytosis by target cells (49). Similarly, since both succinyl-Ala-Leu-Ala-Leu-DNR and -ADR were processed by lysosomal enzymes to liberate the parent drugs, MoAb conjugates with these spacers have also been prepared. No more than three anthracyclines could be loaded per MoAb, presumably due to poor solubility conferred to the conjugate by the hydrophobic nature of the spacer (50).



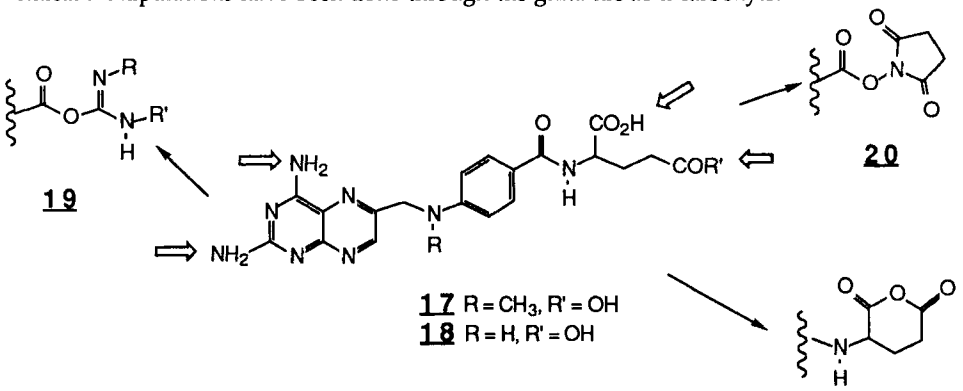
Poly-(L-glutamyl-γ-hydrazide) has been used as an intermediate carrier for the anthracyclines in an effort to increase molar loadings by reaction with the 13-keto group of DNR (to form 11) followed by crosslinking to MoAb with glutaraldehyde (51). Alternatively, the N-terminus of poly-(L-Glu) has been reacted with SPDP in DMF to substitute a single 3-(2-pyridyldithio)-propinamido group on the polymer (52,53). Reaction of the polymer with DNR in the presence of EDCI introduced 7 to 10 DNR onto a 21,000 MW unit. Conjugates were assembled by reacting the sulfhydryl of the polymer with a m-maleimidobenzoate or 4-(N-maleimidobutyrate)-modified MoAb.

Dextran derivatives have been used extensively as intermediate carriers. Oxidation of dextran (15) with $NaIO_4$, if done exhaustively, generates two aldehyde groups per glucose unit yielding polyaldehyde dextran (PAD, 16). The aldehydes react with the amino group of the anthracyclines to form Schiff's base linkages, and attachment to a MoAb is achieved through a residual aldehyde using the same reaction. The enamine bonds are generally reduced to di-alkylamines with $NaCNBH_3$ or $NaBH_4$ to stabilize the materials (44,51,54). High loadings of anthracyclines, up to 50 molecules per IgG, have been achieved using this method. In fact, the highest reported loading of 500 molecules of DNR per MoAb used this method with an IgM (55).



15 16

Methotrexate- Numerous non-specific conjugates of this anti-metabolite have been described. Because of the unreactive nature of the methotrexate (MTX, 17) amino groups, nearly all of the chemical manipulations have been done through the glutamic acid carboxyls.



19 20

17 R = CH_3, R' = OH
18 R = H, R' = OH

21

Activation of the carboxyl groups has been accomplished with N,N'-dicyclohexylcarbodiimide (DCC) in DMF to give 19, R, R' = cyclohexyl (56,57), by treatment of MTX with N-hydroxysuccinimide and DCC yielding 20 (58-60) or by formation of the glutamyl anhydride (21) with acetic anhydride (56). Alternatively, MTX has been activated *in situ* in the presence of MoAb using the water-soluble carbodiimide 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, variously know as EDCI, EDC or EDAC (56,61). All of these procedures produce amide bonds between MTX and the MoAb. The first method is claimed to produce conjugates with the best balance between high loading and the least loss of immunoreactivity (56). Loadings as high as 15 molecules of MTX per MoAb have been reported with only a 25% sacrifice of immunoreactivity using EDCI (61), compared with 12 molecules using the OSu ester 20 (58). $F(ab')_2$ fragments have been likewise reported as MTX carriers, though loadings have been correspondingly lower, as have been the amounts of drug localized in the target tumors (62,63). Even though the 2- and 4-amino groups of the diaminopteridine nucleus of MTX are generally considered to be non-nucleophilic, a carrier-based conjugate of MTX with PAD (16), presumably linked through the amines, has been described (64).

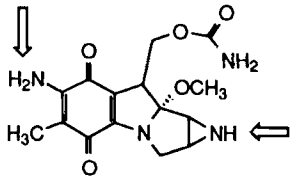
Since direct conjugation of MTX to some MoAb's leads to precipitation of the product after as little as two or three molecules of MTX have been conjugated, human serum albumin (HSA) has been used extensively as an intermediate carrier for MTX-MoAb conjugates (59,65). HSA contains numerous amino groups for amide formation and solubilization, is relatively small (~65,000 MW), and contains a cysteine at residue 34 which makes the complex amenable to later attachment to a MoAb. Both ethylcarbodiimide (59,66) and EDCI (67) have been described for use in a "single pot" procedure and MTX-OSu has been used for a two-step reaction sequence (68).

Better molar loadings of MTX onto HSA (up to 38:1) have been achieved with ethylcarbodiimide at a pH of 6.5 (68). Attachment of the MTX-HSA complexes to MoAb's has been achieved in several ways. Reduction of the cysteine with DTT generates a sulfhydryl group (24,69). Alternatively, a sulfhydryl group is introduced into HSA with the SPDP procedure prior to reaction with MTX (70). The thiol-containing complex is reacted with MoAb's which have been modified with iodoacetyl or γ-maleimidopropionate groups (59,65-70). Either single or multiple units of the HSA carrier complex have been conjugated to MoAb's depending on the extent they have been functionalized with the sulfhydryl-reactive groups.

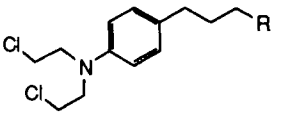
Considerable biological and pharmacological information comparing free and conjugated MTX in animals has been accumulated. In general, most of the conjugates have shown greater specificity toward target tumors and lower systemic toxicity than the parent compound, though real tumor-destroying capabilities of the conjugates have been variable. Biodistribution of MTX conjugates approximates that of unmodified MoAb (71). Sub-populations of target cells resistant to MTX remain sensitive to MTX conjugates (72), implying differences in modes of entry into cells by the two entities. Processing of conjugates by lysosomal enzymes appears necessary for cytotoxicity, since agents such as ammonium chloride, chloroquine, and suppressors of thiol proteinases significantly reduce the toxicity of conjugates, but not of free MTX (60,73,74). Inhibition of the enzyme dihydrofolate reductase is diminished, though not eliminated, by conjugation (75). However, tight binding to the enzyme requires a free α-carboxyl group on the glutamyl residue. None of the above methods described for activating MTX is specific for one carboxyl group over the other (76).

Mitomycin C- Relatively few reports covering conjugation of mitomycin C (MMC, **22**) to MoAb's have appeared. Acylation of MMC with succinic or glutaric anhydride through its aziridine nitrogen not only stabilizes the aziridine, but also provides a carboxyl group for MoAb conjugation. Conversion to an N-hydroxysuccinimide ester followed by reaction with an anti-AFP (alphafetoprotein) MoAb produced molar loadings of 6 to 8 (77). Cyanogen bromide-mediated coupling produced a loading of only 2 (78).

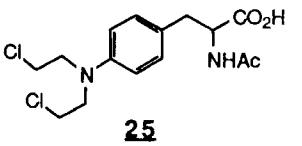
PAD (**16**), when first functionalized with MoAb, then reacted with MMC presumably through its primary amino group followed by $NaCNBH_3$ reduction produced loadings of 87 MMC's per MoAb (79). Bovine serum albumin (BSA) has likewise been used as carrier for MMC (80). The aziridine group of MMC was reacted as above and this was treated with 1-pyridyldithio-activated BSA. Conjugation was achieved to the backbone of two IgM's by partially reducing the MoAb's with cysteine to expose sulfhydryl bonds, then reacting this with the BSA complex to produce a loading of 32 MMC's per IgM.



22



23 R = NCO
24 R = CO_2H

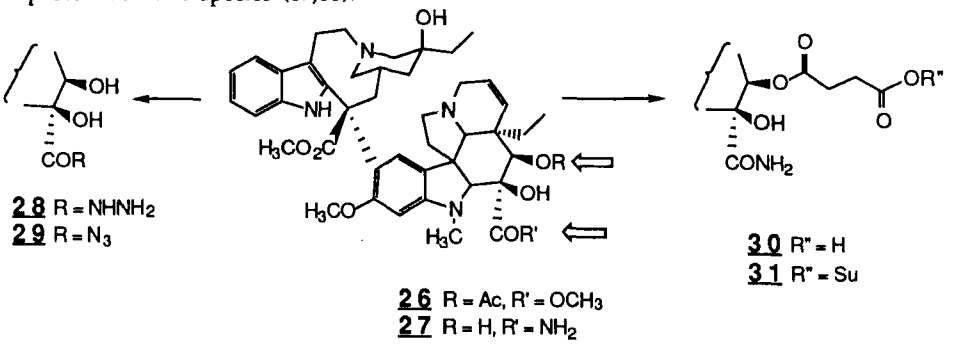


25

Chlorambucil/Melphalan- The isocyanate **23**, produced from chlorambucil (**24**) using a modified Hoffman reaction, has been conjugated directly to the surface of an anti-AFP and anti-CEA (carcinoembryonic antigen) MoAb to produce, in the latter case, material containing 37 chlorambucil molecules per MoAb (81,82). The products were from 3 to 30 times more active than the parent drug against target cells. Similarly, N-acetylmelphalan (**25**) was activated as its N-hydroxysuccinimide ester and conjugated to both an intact MoAb or its corresponding $F(ab')_2$ to generate molar loadings of 30 and 25 molecules, respectively (83,84).

Vinca alkaloids- The experience with non-specific vinca conjugates has been reviewed to 1985 (85). Reaction of vinblastine (**26**) with hydrazine produced 4-desacetylvinblastine hydrazide (**28**), which upon treatment with nitrous acid generates the lysine-reactive acyl azide **29**. Up to 10 vinblastines per MoAb have been conjugated using this procedure (86). Alternatively, when vindesine (**27**) was reacted with succinic anhydride, functionalization occurred at the secondary

C-4 hydroxyl group. Conversion of **30** to the corresponding hydroxysuccinimide ester **31** produced the protein-reactive species (87,88).

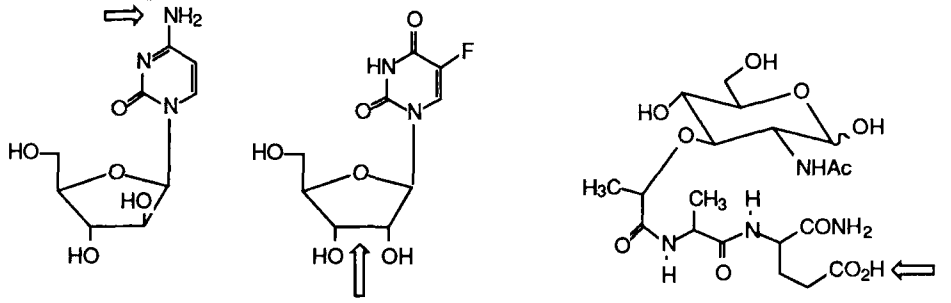


28 R = $NHNH_2$
29 R = N_3

26 R = Ac, R' = OCH_3
27 R = H, R' = NH_2

30 R" = H
31 R" = Su

Neocarzinostatin- This agent belongs to a group of DNA-reactive substances which contain a chromophoric unit noncovalently surrounded by a protective protein coat. For neocarzinostatin (NCS), the coat is a 12,000 MW protein which contains only three side-chain amino groups. Two procedures have been described for conjugation of NCS to MoAb's. In the first of these, the chromophore was removed and only the protein coat was functionalized using the SPDP procedure to form a disulfide bond to a MoAb. The chromophore was reinserted into the protein subsequent to conjugation (89). More recent procedures have employed the same coupling strategy, but used intact NCS with its chromophore unit in place (90,91). MoAb conjugates have been prepared which contain up to 4 NCS units, exhibiting activity as high as 74 times that of unmodified NCS toward target cells.



32 **33** **34**

Miscellaneous cytotoxic conjugates- Bleomycin has been conjugated to a MoAb through PAD (**16**) using the sequence previously described (92). The resultant conjugate, containing 57.6 bleomycin molecules per MoAb, was 15 times more toxic to target cells than free bleomycin. The antimetabolites cytosine arabinoside (Ara-C, **32**) and 5-fluorouridine (FUR, **33**) have likewise been conjugated to MoAb's through dextran bridges (93,94). Ara-C was bound directly through its amine to oxidized dextran, FUR was first oxidized to generate a dialdehyde from its carbohydrate moiety, then reacted with dextran hydrazide. These methods produced loadings of 25 to 60 molecules of Ara-C per IgG, 80-130 molecules of Ara-C per IgM, and 7 to 24 molecules of FUR per IgG. Cis-platinum containing conjugates have been prepared by reacting MoAb's directly with potassium tetrachloroplatinate to yield materials containing up to 20 molecules of platinum (95). The amino group of misonidazole, a radiosensitizer, was reacted with succinic anhydride to introduce a carboxylic group into the molecule, then conjugated to a MoAb as a mixed anhydride. Although a maximum of 70 misonidazoles could be attached, only loadings of 10 or less preserved the immunoreactivity of the MoAb (96).

A somewhat different tact for antitumor therapy has been taken in an attempt to deliver carboxypeptidase G_2 to tumor cells. Since the enzyme cleaves glutamic acid from folates, localization of carboxypeptidase G_2 in the vicinity of a tumor might "starve" the cells of folates. The enzyme was functionalized with bromoacetyl or MBS groups, then reacted with SPDP-modified MoAb (97). Loadings of 0.66 to 5 molecules per MoAb were achieved, with only a 20% loss in enzyme activity.

Immunomodulators- Several agents which exhibit no cytotoxicity themselves but which are capable of activating components of the immune system have been conjugated to MoAb's for delivery to tumors. The macrophage activator muramyldipeptide (**34**) has been cross-linked through its γ-glutamyl carboxyl group to an IgM with carbodiimide to produce loadings of 45 molecules per MoAb (98). Formyl-methionyl-leucyl-phenylalanine (fMLP), a mediator of macrophage chemotaxis, has been conjugated to a series of both polyclonal and monoclonal antibodies using the water soluble 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (99). The same carbodiimide, when used on polyclonal antibodies, produced loadings of 3 to 5 molecules of fMLP per Ab, but when applied to MoAb's, loadings were decreased by two orders of magnitude (100). Nevertheless, even conjugates with low loadings induced macrophage accumulation in tumors. Several examples of interferon (INF) conjugates have been reported, all employing the SPDP procedure to form disulfide bonds. It is interesting to note that loadings of up to 6 INF's were achieved using naturally-derived INF-α, yet only 10^{-4} to $5x10^{-2}$ molecules could be conjugated if recombinant INF-α was used (101,102).

Cobra venom factor (CVF), a 140,000 MW functional analog of complement C_3/C_5 convertase has been attached to several MoAb's in an effort to achieve cytotoxicity by complement fixation. A number of hetero-bifunctional cross-linking reagents were evaluated for their potential as spacers between CVF and MoAb's including 4-(N-maleimidomethyl)cyclohexane-carboxyl N-hydroxysuccinimide, γ-(N-maleimidobutyryl) N-hydroxysuccinimide, MBS, SPDP and iodoacetyl N-hydroxysuccinimide (103). Only SPDP did not alter the hemolytic activity of CVF; the remainder caused 50 to 95% loss in activity. Moreover, SPDP-linked materials yielded conjugates with circulating half-lives of about 25 hr, whereas the MBS-conjugated material exhibited a $T_{1/2}$ of 3.5 hr. SPDP-linked conjugates have shown good complement-fixing activity when assessed *in vitro* (104).

Site-Specific Conjugates

Random covalent modifications of MoAb amino acids as described above always involves the risk of reacting at residues in or near the antigen binding sites. This can alter or eliminate the targeting ability of a MoAb. Cystine residues, particularly those which hold the heavy chains together, offer potential for modification in areas of the MoAb remote from the antigen-binding sites. Unfortunately, access to these cysteines requires the breaking of disulfide bonds and thus distorting the tertiary structure of the antibody. The carbohydrate residues, since they are attached to asparagine or serine residues in the Fc region of IgG molecules and thus distant from antigen binding domains, offer sites for drug conjugation which are accessible without disruption of the MoAb backbone (105). Oxidation of *vic*-diols with galactose oxidase, or more commonly with periodate, generates aldehydes suitable for covalent bond formation (106-108).

Conjugates of 4-desacetylvinblastine-3-carboxyhydrazide (**28**), joined through oxidized carbohydrate residues, have been made with several different antibodies (109,110). Constructs containing 4-6 moles of drug per mole of antibody retained 100% of their immunoreactivity and contained less than 10% aggregate. The hydrazide-linked vinca conjugates were more potent than their non-specifically linked counterparts (109,111). Conjugates of methotrexate-γ-hydrazide (**17**, R' = $NHNH_2$) and aminopterin-γ-hydrazide (**18**, R' = $NHNH_2$) likewise had greater potency than their lysyl-attached versions (112).

Techniques combining site-specific linkage with polymeric carriers have been employed to overcome the limitations of low drug loading. As many as 91 moles of methotrexate have been loaded on a MoAb through carbohydrate linkages using aminodextran (113). The OSu ester of methotrexate (**20**) has been coupled to dextran T-40 to load 25-50 methotrexate molecules. Two dextran units were then linked to each MoAb to produce a conjugate which contained up to 100 moles of methotrexate yet still retained its immunoreactivity (114). Chlorin-e_6 monoamide was first reacted with poly-(L-Glu), then attached as a unit to an oxidized antibody (115). This latter material, when irradiated, exhibited laser-induced phototoxicity for target cells.

Non-covalent Delivery of Drugs Using MoAb's

The simplest approach toward drug targeting might be to take advantage of strong non-covalent interactions between certain drugs and proteins, applying the concept to MoAb's. Considering the number of proteins such complexes would encounter in serum before targeting is achieved *a priori* makes the approach dubious, yet several successes have been noted. Mixtures of chlorambucil **24** (116) with MoAb's have shown higher antitumor activity than their individual components. In one reported case, an ADR (**4**) and MoAb mixture even outperformed a covalently-bound counterpart (117).

A more rational approach has been to tailor the non-covalent interaction between the MoAb and delivered agent. This has been achieved in one of two ways: conjugation of drug to a hapten recognized by the MoAb, or selection of MoAb's specific toward the agent of interest. Derivatives of both bleomycin and mitoxantrone containing an L-benzyl-EDTA unit have been reported (118). Although co-administration of these agents with a MoAb which recognizes L-benzyl-EDTA involved an attempt at altering the biodistribution of the drugs rather than true tumor targeting, the delivery of bleomycin to a tumor increased from 0.3 to 15% of the injected dose.

Several different approaches have been reported for the use of dual-specificity MoAb's for localizing drugs or toxins in tumors. All of these approaches rely on the ability of the construct to recognize both the tumor antigens and the agent to be delivered. By administering the antibody first and allowing it to localize, receptors for the agent are created on tumor cells for subsequent administration of the agent. A tetrameric hybrid has been constructed by linking an intact anti-ricin A-chain MoAb with a targeting MoAb (16). Reassociation of the heavy-light chain half of an anti-ricin A-chain antibody to the heavy-light chain half of an anti-prostate MoAb has been reported (119). The production of and screening for a hybridoma which secretes hybrid MoAb 28.19.8 with specificity for both vinblastine (**26**) and CEA has also been described (120). This material, when allowed to localize to CEA-expressing tumors for seven days before administration of vinblastine increased the concentrations of vinblastine by at least ten-fold over untargeted levels (121,122).

Conclusion

An impressive variety of drugs and toxins have been reacted with MoAb's to confer target specificity to the agents. Early efforts at forming covalent bonds to MoAb's relied on relatively crude crosslinking techniques. As the field has matured, more attention has been paid to the chemistry of MoAb modifications. Issues such as retention of full immunoreactivity, delivery of large amounts of an agent, and release of the active agent after targeting occurs have been addressed and to a degree solved individually. However, in spite of many demonstrations of activity in rodents, the utility of MoAb-based delivery systems can be established only after human trials with a variety of these constructs have been completed.

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Chapter 17. Antiviral Agents

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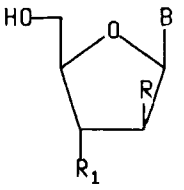
Introduction - A number of reviews have appeared over the last year (1-8) with several concentrating on antiviral therapy against AIDS caused by human immunodeficiency virus (HIV) infection (5-8). Rimantadine was recommended for approval for the treatment of influenza (9), while AZT was approved for AIDS (10).

Retroviruses - The transmission (11), pathogenesis (12) and epidemiology (13) of HIV have all recently been reviewed. Several papers have appeared which highlight the neurological manifestations of AIDS (14,15). Many different therapies are under investigation for HIV infections with several nucleoside analogues being evaluated (16-18). Those nucleoside analogues prepared as anti-HIV agents are targeted against the unique virus enzyme, reverse transcriptase (RT). The suggested mode of action of 3'-azidothymidine (AZT, **1**) requires that it be metabolized sequentially via the monophosphate (AZT-MP) to the triphosphate (AZT-TP); the triphosphate then inhibits RT and terminates the growing DNA chain (19,20). In clinical studies, AZT decreased mortality and the frequency of opportunistic infections and to some extent reverses neurological symptoms (21,22). AZT produces bone marrow toxicity leading to anemia (23-24); this toxicity can now be evaluated *in vitro* (25). A proposed explanation for this toxicity involves a build up of thymidine monophosphate (dT-MP) by competitive inhibition of thymidylate kinase by AZT-MP. The resultant depletion in thymidine triphosphate pools in host cells starves host DNA synthesis (20,26). AZT-TP has only a weak effect on human ribonucleotide reductase (27). AZT also shows activity against certain bacteria capable of phosphorylating it (28). The major metabolite of AZT appears to be the 5'-glucuronide, which is not an RT inhibitor (29,30). AZT has shown synergy with dextran sulphate, a number of biological response modifiers and acyclovir, potentially allowing for dose reduction of AZT (31-35). The related analogues 3'-azidouridine (AZU) and 3'-azidoguanosine (AZG) also show antiretroviral activity (36,37).

Although results from *in vitro* assay systems are highly variable, 2',3'-dideoxycytidine (ddC, **2**) is generally the most potent of the dideoxynucleosides. In a phase I trial with ddC, a peripheral neuropathy which appears to be dependant on the cumulative dose, developed in all those patients who completed the trial (38). Neutropenia was also seen in a number of patients in this trial. This is consistent with the finding that ddC showed bone marrow toxicity *in vitro* (39). A rash, which subsided with continued therapy, was also seen. AZT and ddC, given alternately a week at a time, were well tolerated for up to 28 weeks (38). DdC was not significantly deaminated nor was it a substrate for pyrimidine nucleoside phosphorylase (40,41). In addition to ddC mono-, di-, and triphosphates, an unexpected metabolite, ddC diphosphate choline was formed. High urinary recovery was seen with ddC and the oral bioavailability was good (42).

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3'-Fluorothymidine (FT, **3**) is a very active pyrimidine nucleoside analogue (43-45); however, the greater toxicity of FT highlights a common problem that increased potency is often accompanied by higher toxicity (46). The unsaturated analogues 2',3'-dideoxy-2',3'-didehydrocytidine (d4C, **4**) and 2',3'-dideoxy-2',3'-didehydrothymidine (d4T, **5**) are both very potent anti-HIV agents (47-53).



1	R = H	$R_1 = N_3$	B = Thymine
2	R = H	$R_1 = H$	B = Cytosine
3	R = H	$R_1 = F$	B = Thymine
4	2',3'-olefin		B = Cytosine
5	2',3'-olefin		B = Thymine
6	R = H	$R_1 = H$	B = Adenine
7	R = H	$R_1 = H$	B = Inosine
8	R = F	$R_1 = H$	B = Adenine

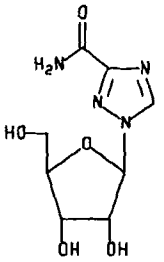
Of the purine analogues, 2',3'-dideoxyadenosine (ddA, **6**) has limited bone marrow and cell toxicity (39). DdA is readily deaminated to 2',3'-dideoxyinosine (ddI, **7**) by adenosine deaminase (ADA). While both ddA and ddI independently exhibit an antiviral effect, they do so through the same triphosphate metabolite, ddA-TP. DdA can, by the usual pathway, be taken through to ddA-TP (54). More likely, ddA is deaminated to ddI which upon monophosphorylation becomes ddI-MP. The ddI-MP can be converted to ddA-MP by the adenylosuccinate synthetase enzyme. DdA-MP is then further phosphorylated to ddA-TP (55). The 2'-fluoro analogue of ddA (**8**) is less susceptible to glycosidic bond cleavage and is not readily deaminated (56,57). 2,6-Diaminopurine-2',3'-dideoxyriboside is also resistant to ADA, has good anti-HIV activity and is a strong inhibitor of the deamination of ddA and araA which may be useful for combination therapy (58,59). The dideoxynucleosides AZT, ddC, and ddA all fail to inhibit the infection or replication of HIV in macrophages, a reservoir for the virus; this is probably due to reduced phosphorylation within these cells (60).

In an uncontrolled clinical trial, ribavirin (**9**), administered orally, gave a transient clinical improvement associated with HIV suppression (61). Also oral ribavirin treatment at escalating doses for at least 1 month inhibited RT activity with acceptable toxicity, and appeared to inhibit HIV replication (62). Ribavirin antagonises the effect of pyrimidine nucleosides but enhances the inhibitory effects of purine nucleosides (63,64).

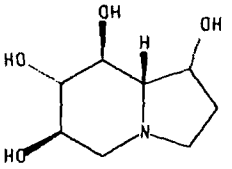
Avenues for the treatment of HIV infection other than RT inhibition have become evident over the last year. Plant alkaloids which modify the glycosylation of gp120 inhibit the infectivity of HIV (65,66). Castanospermine (**10**) is believed to exert its antiviral effect by reduction in virus infectivity and reduction in cell-to-cell spread of the virus by inhibition of cell fusion events (66).

A number of pyrophosphate analogues, fusidic acid, phosphonoformic acid (PFA), oxetanocin, and dextran sulphate were reported to be active against HIV (67-70). The ability of Peptide T to act as a ligand for the CD4 receptor, resulting in the inhibition of HIV infectivity, remains controversial (71-72).

In vitro mutagenesis of the murine leukemia virus (MuLV) genome produces a protease-defective MuLV which lacks infectivity (73), providing evidence that the HIV aspartyl protease may be a good target enzyme for antiviral drugs (74-76). Another possible target is the cell fusion peptide of HIV which is believed to be part of the gp41 transmembrane peptide and may be responsible for the infection of cells and the cytopathic effects of fusion (77).



9



10

Biological response modifiers have also been used for the treatment of AIDS. Recombinant GM-CSF (granulocyte-macrophage colony stimulating factor) administered to AIDS patients with leukopenia has been shown to be well tolerated, and to give an increase in the number of circulating leukocytes (78). The use of ampligen as an anti-HIV agent has recently been reviewed (79). Five reports have appeared on the preparation and isolation of a recombinant soluble CD4 and shown it to inhibit in vitro HIV replication and syncytia formation by binding to gp120 (80-84).

RNA Viruses - Reports on the ability of amantadine to stop the spread of influenza when given prophylactically continue to appear (85). Even when taken within 48 hr of the onset of symptoms, its therapeutic use will shorten the illness and reduce fever (86). Recent data suggest that at 100 mg (half the normal dose), amantadine is also effective (87). Two studies, the first in children and the other a double blind clinical study, showed that the related compound rimantadine (α-methyl-1-adamantanemethylamine hydrochloride) is well tolerated with fewer side effects than amantadine and improved efficacy against influenza A/H_3N_2 (88,89).

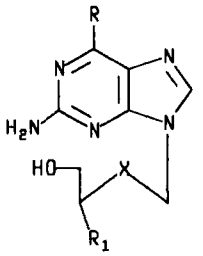
The experimental compound 1,3,4-thiadiazole-2-ylcyanamide (LY253963) has activity against both influenza A and B type viruses (90,91). LY253963 provides protection over a wide range of doses and treatment can be delayed for up to 68 hr post-infection and still provide a high degree of protection when given to mice by gavage. Ribavirin when given as an aerosol is effective in treating respiratory syncitial (RSV) infections, especially in children (92-94). This aerosol treatment appears to have no deleterious effect on non-infected individuals (95). In vivo experiments with higher doses of the drug for shorter times indicate that shorter treatment schedules may be possible without loss of efficacy or increased toxicity (95). A loading dose and larger maintenance doses of oral ribavirin were given to patients with Influenza A and B in a phase I clinical trial (96). The drug showed no significant toxicity and appeared to give minor beneficial results.

The known activity of neplanocin A, an inhibitor of RNA methylation, against influenza results from inhibition of the 2'-O-methylation of nucleosides within mRNA cap structures. The resultant reduction of

influenza specific mRNA leads to an inefficient cap recognition by the influenza polymerase (97).

DNA Viruses - Those nucleoside analogues designed for activity against herpes simplex virus types 1 and 2 (HSV 1 and 2) require activation to the monophosphates by viral-specified thymidine kinase (TK). It is the triphosphate, derived from the monophosphate, which inhibits the virus DNA polymerase. The phosphorylation of ganciclovir (DHPG) to the monophosphate has been studied and shown to be stereospecific. The triphosphate (S)-DHPG-TP has the same stereochemistry as dGTP and is a good inhibitor of the virus DNA polymerase, whereas (R)-DHPG-TP has little or no inhibitory effect against the enzyme (98).

Acyclovir (ACV, **11**) is still the only drug proven prophylactically effective against HSV infection (99-104). Oral ACV suspension has been given to young children and found to be well tolerated with no adverse side effects (105). Oral ACV was effective in treating herpes labialis in a double-blind, placebo-controlled study (106). ACV, when taken for up to 21 months, showed no side effects or decreased potency (107,108). Resistance to ACV still does not appear to be a significant clinical problem (1,109). An additional indication for ACV may be its prophylactic use against HSV in transplant patients (110). ACV suppresses persistent varicella zoster virus (VZV) in Vero cells (111). The poor oral absorption of ACV requires that high or repeated doses be used when treating a less sensitive virus such as VZV, where higher serum levels are required (112-114). Desciclovir (DCV, **12**), which is converted to ACV by xanthine oxidase, has better absorption than ACV resulting in higher plasma levels than can be achieved with ACV. However oral ACV at a higher dose can be used with positive results against VZV in either immunocompromised patients or in children with cancer (115-119). DCV showed no serious or adverse effects when given to healthy volunteers (120). Oral DCV was beneficial at 125 mg/d/tid in an open trial of 20 patients with herpes zoster and 7 patients with oral hairy leukoplakia (121,122).

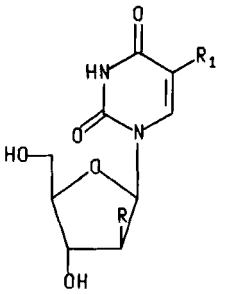


11	R = OH	R_1 = H		X = O
12	R = H	R_1 = H		X = O
13	R = OH	R_1 = CH_2OH		X = O
14	R = OH	R_1 = CH_2OH		X = C
15	R = OH	R_1 = OH		X = C

DHPG (**13**) is the first drug to be shown to bring consistent therapeutic relief to patients with cytomegalovirus (CMV) including CMV retinitis (123-127). Since CMV may be one of the earlier manifestations of AIDS (128), DHPG has been administered to many AIDS patients. Although DHPG is beneficial in these cases, neutropenia can require discontinuation of the drug (129-133). DHPG gives positive results when taken by patients who develop CMV complications during transplant therapy (134). The pharmacokinetics of oral DHPG show that while it has limited bioavailability, it is sufficient for oral administration (135). Cerebrospinal fluid (CSF) levels indicated penetration of 24-67% (136). A number of esters of DHPG have been prepared in order to improve the oral absorption of the drug (137,138). The carba analogue, BRL 39123

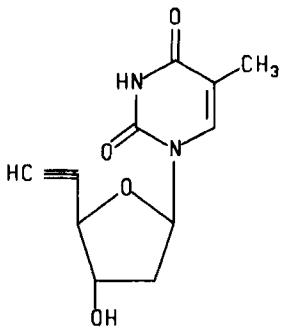
(**14**), has been found to reduce virus replication more efficiently than ACV, and this replication remains depressed for long periods after drug discontinuation (139-140). DHPC, the cytosine base analogue of DHPG, is inactive against HSV but is as active as DHPG for CMV and EBV (141). While the structures and *in vitro* biological data for a number of compounds related to buciclovir (BCV, **15**) appear similar, their *in vivo* biological activity differs widely. Biochemical and pharmacokinetic parameters which may account for this discrepancy have been examined (142-144).

5-Ethyl-2'-deoxyuridine (EDU, **16**), currently being pursued for topical HSV 1 and HSV 2 treatment (145), derives its antiviral activity and selectivity from greater phosphorylation in HSV-infected cells (146). The fluoroarabinoside FEAU (**17**) achieves its impressive selectivity against HSV 2 by similar selective phosphorylation by the viral enzymes (147,148). FEAU also shows good in vivo activity against hepatitis B (149). A number of 5-haloalkyl substituted analogues of the fluoro-arabinoside series have been prepared; none are as effective *in vivo* as CEDU (**18**), the lead compound in this 5-haloalkyl series (150,151). While 5-bromovinyl-2'-deoxyuridine (BVDU, **19**) is susceptible to phosphorolysis by thymidine phosphorylase to give BVU in HSV 1 infected cells, the related compound BVaraU (**20**) is not. The electron withdrawing OH group in the arabino configuration at the 2'-position strengthens the glycosidic bond. BVaraU showed good antiherpes activity (152). 5'-Ethynylthymidine (**21**) has been reported to be a very potent inhibitor of TK (153). TK is believed to play a role in the reactivation of the virus from neural cells (154,155). A TK inhibitor may, therefore, have utility in inhibiting reactivation.

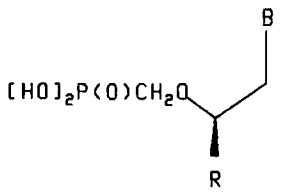


16	R	=	H	R_1	=	Et
17	R	=	F	R_1	=	Et
18	R	=	H	R_1	=	CH_2CH_2Cl
19	R	=	H	R_1	=	CH=CHBr
20	R	=	OH	R_1	=	CH=CHBr

The phosphonate (S)-HPMPA (**22**) is a nucleotide analogue which bypasses the need for TK activation as it is a monophosphate equivalent. The triphosphate analogue [(S)-HPMPApp] has been synthesised and shown to be incorporated to a very low extent in both virus and mock infected cells. The compound exerts the antiviral effect by inhibiting virus DNA synthesis at a concentration much lower than that required to inhibit cellular DNA synthesis (156). The cytosine analogue, (S)-HPMPC (**23**), has higher potency against CMV than DHPG, while PMEA (**24**) is active against HIV (157). These phosphonates and a number of other nucleotide analogues show high potency against a broad spectrum of viruses and offer the possibility of treating CMV and VZV.



21



22 B = Adenine R = CH_2OH
23 B = Cytosine R = CH_2OH
24 B = Adenine R = H

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Section IV – Endocrinology, Immunology and Metabolic Disorders

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Chapter 18 . Immunomodulatory Approaches to the Treatment of Inflammation

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Introduction – Inflammation is a complex and multifaceted process which allows blood proteins and cells to enter tissues in response to injury or infection and which initiates tissue repair and host defense. The propensity of the inflammatory process to cross over from protection to pathology, as exhibited in autoimmune diseases such as rheumatoid arthritis, glomerulonephritis, systemic lupus erythematosus, insulin-dependent diabetes, thyroiditis, and multiple sclerosis, makes inflammation a focus of medicinal research (1-3). Control of these immunopathological diseases might be achieved by modulation of the signals controlling the activation and proliferation of the cells of chronic inflammation. Many such lymphokine (i.e., produced by one lymphocyte population with activities on another) and monokine (i.e., produced by macrophages and related cells) growth and differentiation factors are known, some of which have been described only phenomenologically. Recent advances toward definition of these factors at the molecular level have allowed recognition that many reported disparate activities reside in a relatively few molecules. Among those best characterized are the interleukins 1-6, tissue necrosis factor (TNF), and the interferons (particularly gamma-interferon). Recent general reviews have appeared on lymphokines and monokines (4,5). This review focuses on their possible roles in inflammation and to the possible rational strategies available for modulation of their actions.

The Role of Lymphokines and Monokines in Inflammation

Interleukin-1 (IL-1) – IL-1 ("Endogenous Pyrogen", "Lymphocyte-Activating Factor", "B-cell Activating Factor", "Leukocyte Endogenous Mediator", "Catabolin", "Osteoclast Activating Factor", "Mononuclear Cell Factor", "Fibroblast Proliferation Factor", "Hematopoetin 1", and others) is both a hormone and a local mediator, produced classically by activated cells of the macrophage line, with a remarkably broad spectrum of actions, as suggested by its many early appellations (6-8). There are two families of cloned and sequenced IL-1 molecules, known as IL1-α and IL-β (8). Despite only a 25% sequence homology, both of these species bind indistinguishably to a single receptor on T-cells and on connective tissue (9,10). A number of non-monocytes, including dendritic cells, kidney mesangial cells, endothelial cells, vascular smooth muscle cells, epithelial cells, fibroblasts, brain astrocytes and glial cells, and keratinocytes, have been reported to produce interleukin 1-like molecules, the precise chemical identity of which is uncertain (11). A recent report describes the ability of human PMN to produce two IL-1 species, indistinguishable from IL1-α and IL-β by Northern blotting, upon stimulation with granulocyte/macrophage colony-stimulating factor, GM-CSF (12). IL-1 is a primary enhancer of lymphokine release (notably interleukin 2) from T-cells (8), and is active in the differentiation and proliferation of B-cells (5,13). It has a wide variety of pro-inflammatory actions on other cells, including growth and stimulation of fibroblasts, stimulation of synovial cells toward release of collagenase and prostaglandins, stimulation of adhesion, prostaglandin release, superoxide release, and platelet activating factor release in endothelial cells, secretion of acute phase proteins by hepatocytes, stimulation of osteoclasts toward bone resorption, and stimulation of chondrocytes for cartilage breakdown (6,11,14-16).

With this myriad of activities, it is not surprising that IL-1 has been suggested as a possible causative or exacerbating factor in a number of diseases. Purified IL-1 has been shown to be capable of extremely potent mediation of both acute dermal inflammation and of the classic local Schwartzman reaction, where it can both prime the site and provoke a

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necrotic inflammatory lesion upon subsequent challenge injection (17).

A strong link has been proposed between IL-1 and rheumatoid arthritis (RA), with high levels of IL-1 found in synovial joint fluid (18). Monocytes from RA patients with recent onset or exacerbation of disease had enhanced spontaneous IL-1 secretion, whereas patients with stable disease had normal levels, suggesting that enhanced IL-1 activity might be a primary factor in the early stages of the disease (19). While in vitro stimulated peripheral leukocytes isolated from RA patients have been reported to produce less IL-1 than those from normal controls, the relevance of these peripheral measurements to the immunological state at the site of inflammation is unclear (20). Injection of recombinant IL-1 into rabbit knee joints produced, in just 24 hours, PMN infiltration and cartilage degradation which were similar to that seen in antigen-induced arthritis models after one week (21).

Provocative recent data has suggested that IL-1β may be responsible for the islet cell destruction which precipitates type I diabetes. Cultured rat and human pancreatic islet of Langerhans cells were shown to be exquisitely sensitive to IL-1, which reduced insulin release in culture, and caused microscopically-defined degeneration of the cells (22,23). Neither TNF,gamma interferon, nor IL-1α had any effect. No effect was noted on human islet alpha cells, indicating a great deal of cell specificity.

Several other inflammatory diseases have been linked to elevated IL-1 activity, including psoriasis, gingivitis, systemic lupus erythematosus (SLE), chronic inflammatory bowel disease, and allergic encephalitis (6,24-27). Increased levels of IL-1 have been shown in crystal arthritis (gout), and enhanced release has been demonstrated of IL-1 from synovial fluid mononuclear cells stimulated with urate crystals (28). Reports that rat glomerulonephritic kidneys contain two- to three-fold more IL-1 mRNA than normals and that IL-1 stimulates mesangial cells to release gelatinase suggests an important role for IL-1 in glomerulonephritis (29,30).

Tumor Necrosis Factor (TNF) – TNF-alpha (TNFα, "cachectin", "TNF") and TNF-beta (TNFβ, "lymphotoxin", "alpha lymphotoxin") are broad spectrum cytokines with many activities similar to IL-1, including pyrogenicity, acute phase responses, and local inflammatory actions, and TNF is a direct cytotoxin for some tumor cells (14,31,32). Although the two TNF sequences show only modest homology, the molecules appear capable of binding to the same receptor, reminiscent of the IL-1's (33). TNF is thought to be the major mediator of endotoxin shock, and monoclonal antibodies to TNFα have been shown to block the lethal effects of toxic shock in baboon (34).

Recombinant TNFα stimulates resorption and inhibits synthesis of proteoglycan in explants of cartilage, and stimulates the mitogenesis of fibroblasts, mimicking the action of IL-1 (35,36). TNFα appears to be a ten to one hundred-fold more potent than TNFβ as a pro-inflammatory substance, with more potency in chemotactic and chemokinetic stimulation of PMN's and in increasing human endothelial-dependent neutrophil adherence (37,38). Both TNF species, like IL-1, have been reported to stimulate fibroblasts in vitro to produce various hemopoietic factors, including GM-CSF (39).

TNF has been suggested as an important mediator in a variety of inflammatory diseases. Measurements by radioimmunoassay of TNF in the serum of RA patients showed elevated levels in three of fourteen patients which were higher than those seen in any of forty normal controls (40). An unexpected and seemingly paradoxical protective effect of TNFα has been reported in a mouse model of spontaneous lupus-like nephritis, where decreased production of TNFα has been linked to a defective TNFα gene, and the development of lupus-like disease can be significantly delayed by supplementation with recombinant TNFα (41).

Interleukin 2 (IL-2) – IL-2 ("T-Cell Derived Growth Factor", T-cell Maturation/Stimulating Factor", "Killer Helper Factor") is the primary growth factor produced by T-cells which stimulates the growth of activated T-cells, is directly mitogenic for lymphokine-activated killer (LAK) cells, is a major growth factor for the maturation of antigen-stimulated cytotoxic T lymphocytes (CTL), stimulates the release of IL-2 and other T-cell cytokines, and is an important growth and differentiation factor for B-cells (42,43). The majority of T-cells of the helper line and a minority of suppressor/cytotoxic T-cells produce IL-2 (42). IL-2 provides an amplifying signal, following antigen recognition, for expression of its own receptor, which is present at undetectable levels on non-activated T-cells (42). Unlike IL-1 and TNF, IL-2 appears to be a single gene product (44).

Although IL-2 clearly plays a central role in T-cell mediated processes, its direct involvement in inflammatory processes has been somewhat difficult to assess, due in part to

the fact that IL-2 is only transiently produced and is bound rapidly by its high-affinity receptor. There have been many conflicting reports about the presence of free IL-2 in RA synovial fluid (45). Failure to detect IL-2 in several studies has been ascribed to in vivo absorption of of IL-2 by its receptors, a capacity which appears to be increased in RA patients (45). In a clonal analysis of IL-2 dependent T-cells from RA patients, a shift toward phenotypic suppressor/cytotoxic cells was noted in synovial fluid, compared with the predominance of helper clones obtained from peripheral blood, suggesting that an imbalance of T-cell populations may be a factor in the disease accounting for the low responsiveness seen in synovial lymphocytes (46).

Reduced responsiveness to IL-2 and reduced stimulated production of IL-2 has been reported in SLE, which is characterized by B-cell hyperreactivity and T cell deficiency (47). In a murine model of SLE, activated B-cells show a hyper-responsiveness to IL-2 as compared to cells from normal control mice, suggesting that while IL-2 production may be low, it might still be driving a hyper-responsive B-cell population (48). Studies of the hypo-responsiveness to IL-2 of cultured lymphocytes from SLE patients suggest that the observed hypo-responsive state could be due to "helper cell exhaustion", possibly as a result of chronic overstimulation (49).

Conflicting reports have noted either normal or decreased stimulated IL-2 production from isolated peripheral lymphocytes of diabetes mellitus patients (50,51). IL-2 treatment strongly enhances and accelerates the development of spontaneous diabetes in a rat model (52). In intraocular fluids of uveitis patients significantly increased T-cell activation, measured by the expression of IL-2 receptor, has been noted, which correlates with the clinical severity of disease (53). Lymphocytes freshly isolated from Hashimoto's thyroiditis patients were found to be highly activated, with a predominance of cytolytic T cells, suggesting IL-2 activation (54).

Interleukin 3 (IL-3) – IL-3 ("Panspecific Hemopoietin", "Multiple Colony Stimulating Factor", "Megakaryocyte Colony Stimulating Factor", "Hemopoietic Growth Factor", "Erythroid Colony Stimulating Factor", "Mast Cell Growth Factor", "P-Cell Stimulating Factor", "Burst-Promoting Activity", "Eosinophil Colony Stimulating Factor", "Mixed Colony Stimulating Factor") is an activated T-cell-derived local hormone which supports the growth of multi-potential stem cells and may be an important local mast cell growth factor (55-57). Although there is no evidence that IL-3 participates in maintenance hematopoiesis, it may be an important link between local T-cell activity and local hematopoiesis, especially that directed toward mast cell generation (55). Recombinant IL-3 has been shown to stimulate eosinophil function (58). Keratinocytes have been shown to produce an IL-3-like factor, which can stimulate production of mast cells, implicating IL-3 in inflammatory processes in the skin, especially inflammatory fibrotic processes such as scleroderma, where mast cell infiltrates have been identified (59). IL-3-like factors have been characterized in astrocytes and may play a role in cytopoiesis within inflammatory brain lesions (60). Spontaneous release of IL-3 has been measured in supernatants from spleen cells in a SLE model in mice (61). These spleen cells seem to have an impaired synthesis of and response to IL-2, suggesting that the IL-3 release may be causally linked to the B cell hyperactivity which characterizes this disease (62).

Interleukin 4 (IL-4) – IL-4 ("B Cell Stimulatory Factor 1", "B Cell Growth Factor", "B Cell Stimulatory Factor p1", "IgG_1 Inducing Factor", "Macrophage Fusion Factor", "T Cell Growth Factor-2", "Mast Cell Growth Factor-2") is a T-cell-derived growth and differentiation factor with its primary effect on B cell lines, first characterized by its ability to co-stimulate resting B cells in conjunction with anti-Ig or with antigens (63-65). It can powerfully stimulate Class II major histocompatibility molecules (Class II MHC) on resting B cells, which enhances the ability of B cells to present antigen to T cells, and which should allow the immune system to respond to lower antigen levels (66,67). IL-4 promotes the expression of IgG_1 and IgE from activated B cells (63). The importance of the latter effect has been demonstrated in vivo, where the normal 100-fold increase in IgE production in mice infected with parasites can be significantly blunted by administration of anti-IL-4 antibodies (68). The role of IL-4 in stimulating IgE production in allergic inflammatory responses is unknown, but IL-4 has been reported as required for the IL-3 stimulation of murine connective tissue mast cells (69). Receptors for IL-4 have been reported on a wide variety of non-hemopoietic cells (70). IL-4 alone has recently been shown to be a macrophage activating factor, capable of increasing Class II MHC expression and increasing tumorcidal activity of elicited murine peritoneal macrophages (71). The abundance of IL-4 mRNA in the developing thymus has suggested an important role for IL-4 in early T-cell

differentiation (72). IL-4 has been reported to be more effective than IL-2 in stimulating development of CTL from primed small resting murine precursors (73).

Murine IL-4 is produced by a particular subset of helper T-cells ("Type 2", T_H2) which do not produce either IL-2 or gamma interferon (74). The growth of this IL-4 producing subset of T helper cells appears to be dependent on or is enhanced by IL-1. In contrast, IL-2 and gamma interferon-producing helper T cells ("Type 1", T_H1) are insensitive to IL-1 (63,75). IL-4 also can act directly on activated human and murine T-cells as a growth factor independent of IL-2 (63,75,76). In this scheme, IL-4 producing helper cells stimulated by IL-4 and macrophage-produced IL-1 respond optimally to B cell-presented antigens to drive humoral immunity, while macrophage presentation of antigens to Type 1 helper cells produces IL-2 driven cellular immunity (63,75). Thus, this IL-4 circuit could explain the observed B-cell hyper-responsiveness in the presence of low IL-2 levels and IL-2 hypo-responsiveness which is seen in humoral autoimmune diseases such as SLE. Whether this division of helper cell populations applies to human cells is unknown.

Interleukin 5 (IL-5) – IL-5 ("Eosinophil Differentiation Factor", "Eosinophil Colony Stimulating Factor", "B-Cell Growth Factor II", "IgA-Enhancing Factor", "T-Cell Replacing Factor", "Killer Helper Factor", "IL-4") is a T-cell derived differentiation factor for eosinophils, acid-staining granulocytes which are especially prevalent in the inflammatory response to parasites and in certain allergic conditions (77,78). IL-5 (briefly called "interleukin 4") also has been shown to be a B-cell growth factor, acting with antigen on isolated B cells to promote both growth and antibody formation (79-81). In the absence of antigen, IL-5 has little or no effect on B cells (79). Using anti-IL-5 serum, it has been shown recently that IL-5 is required along with IL-4 for antigen-specific stimulation of small B cells as well as for polyclonal B cell responses of large B cells isolated from spleen (82).

IL-5, but not IL-1, IL-2, IL-3, or IL-4, has been shown to induce the expression of IL-2 receptors on activated B cells isolated from spleen, and that these IL-5-stimulated B cells then respond to IL-2 by proliferation, suggesting a role for IL-5 not only in IL-4 driven B cell differentiation and growth but in IL-2 driven growth as well (83). Effects on T cells have been seen also, including enhancement of IL-2 receptor expression on some T cell lines and participation, along with IL-2, in the generation of CTL (84,85). It is unknown whether IL-5 plays a role in the eosinophilia of inflammatory diseases such as eosinophilic pneumonia or autoimmune eosinophilic fasciitis, but B cells isolated from SLE patients have been found to exhibit a markedly enhanced proliferative response to an IL-5-like factor, while responding in a normal fashion to IL-2 or IL-4 (86). T cells from Peyer's Patches have been shown to produce both IL-4 and IL-5, possibly accounting for the high frequency of IgA-committed B cells produced there, suggesting that IL-5 might be important in mucosal inflammatory responses (81,87).

Interleukin 6 (IL-6) – IL-6 ("B-Cell Stimulatory Factor-2", "B-Cell Differentiation Factor", "B-Cell Stimulatory Factor p2", "Interleukin HP-1", "Plasmacytoma Growth Factor", "26kDa Protein", "$IFN\beta_2$", "Fibroblast-Derived Hybridoma Growth Factor") is produced by T cells, fibroblasts, monocytes, macrophages, and various cell lines upon stimulation by IL-1 or $TNF\alpha$ (88-90). Activities include both stimulation of B cell differentiation and growth, the latter activity sometimes observable only when the target B cells are cultured at low cell densities, where the overriding effects of other growth factors produced in culture conditions are lessened (91-93). IL-6 has also been reported to act synergistically with IL-3 on the proliferation of multipotential B cell progenitors in culture (94).

Several reports have appeared describing the inability of IL-1 alone to activate purified T-cells, stimulated with either Con A or anti-T-cell-receptor antibodies, for the production of IL-2 (95). Using purified peripheral murine T cells, cloned human IL-6, but not IL-1α or β, has been shown to be an effective second signal for IL-2 production by Con A or anti-T-cell-receptor stimulated mature T cells (95). Since IL-1 is a potent inducer of IL-6 production in fibroblasts, the previously claimed ability of IL-1 alone to stimulate IL-2 production in T cells could be due to small numbers of contaminating fibroblasts or other antigen presenting cells (95). IL-6 appears to regulate the full acute phase protein response from hepatocytes. Serum levels of IL-6 have been shown to correlate very well with the rise in body temperature which accompanies the typical severe inflammatory reaction in non-infected burn patients, preceding and predicting the subsequent release of acute phase proteins (96,97). T cells in initial culture from SLE patients were found to have enhanced IL-6-like activity on B cells, along with normal or reduced IL-4 activity, as compared to T cells obtained from normals (98).

Gamma Interferon (INFγ) – INFγ ("Interferon Gamma", "Macrophage Activating Factor", "Type II Interferon", "Immune Interferon") is a primary macrophage activating factor produced by T cells and natural killer cells in response to IL-1 or TNF. It stimulates the phagocytic capability of macrophages and drives the expression of major histocompatibility complex class II antigens (MHC class II) on macrophages,other monocytes, and histiocytes, allowing them to present antigens to the T cell receptor (99,100). INFγ shares some antiproliferative and antiviral effects with the structurally unrelated alpha and beta interferons, and stimulates MHC class I expression in virus-infected cells (99,100). The antiviral and macrophage-activating properties of INFγ may reside on separate parts of the molecule, since monoclonal antibodies have been prepared which differentially effect these two activities (99). INFγ in the mouse is predominantly a product of the Type 1 subset of T_H cells, and markedly inhibits IL-4 production in murine Type 2 T_H cells, and thus could inhibit B cell responses or shift B cell activity away from IgG_1, IgA, and IgE production (101-103). INFγ has been shown in mice to enhance selectively IgG_{2a} secretion, and IgG_{2a} antibodies are known to be more effective than the IgG_1 subtype at activating complement and interacting with macrophages (104).

INFγ can also induce MHC class II expression on epithelial, endodermal, and parenchymal cells, allowing these cells in some cases to function as antigen-presenting cells (105,106). This ability to induce antigen presentation in cells which normally do not express MHC class II has implicated aberrant INFγ production in a number of inflammatory and autoimmune diseases (107-110). Thyroid epithelial cells (thyrocytes) can be induced by INFγ to express MHC class II, and T cell clones generated from infiltrates from thyroiditis patients showed a higher frequency of INFγ-producing clones, which was correlated with higher than normal natural killer cell and CTL activity (111,112). In the inflammatory progression toward diabetes mellitus, the target islet beta cells are known to express MHC class II. While normal islet beta cells cannot be induced to express MHC class II with INFγ alone, INFγ and TNFβ can synergize to induce this expression (112). Tissue-specific expression of INFγ in the pancreatic β cells of transgenic mice leads to an inflammatory destruction of the islets and development of diabetes, but other effects of INFγ in addition to inappropriate MHC class II expression appear to be involved (113). In an autoimmune encephalomyelitis model, astrocytes from a susceptible rat strain showed much higher levels of MHC class II expression induced by INFγ than a resistant strain (114). The proximity of mononuclear leukocytes and keratinocytes is a hallmark of a number of lymphocyte-mediated inflammatory skin diseases, including SLE and erythema multiforme, and INFγ treatment of cultured keratinocytes has been shown to enhance their binding to peripheral blood leukocytes (115).

Although increased expression of MHC class II antigens is a central feature of the local lesions of RA, INFγ levels are low in blood, in synovial fluid and in synovial tissue explants from rheumatoid patients (116). However, a quantitative study in RA patients of MHC class II expression in short-term cultures of adherent synovial lining cells showed increased MHC class II expression compared with normals, which rapidly disappeared in culture. Recombinant INFγ added to the cultures induced the re-expression of MHC class II antigens (117). In SLE patients, decreased levels of induced INFγ are seen from peripheral mononuclear cells, but added INFγ substantially increases the secretion of polyclonal immunoglobulins and anti-DNA autoantibodies characteristic of the disease (118). In a mouse model of spontaneous SLE, treatment with monoclonal antibodies to INFγ significantly delayed onset of disease and increased survival time, while administration of INFγ itself accelerated development of disease (119).

Clinical Use of Interleukins and Gamma Interferon as Immunomodulators – IL-1 has not been studied in humans as a potential immunomodulator, although is is being considered to promote wound healing and to stimulate myelopoeisis. TNF has been considered for cancer therapy, but early results are unpromising (120). In mice bearing cells expressing low levels of TNFα severe weight loss is seen reminiscent of cancer cachexia (121).

IL-2, administered intravenously, has been investigated as a possible anti-cancer agent (42). At doses necessary to see modest anti-tumor effects, a massive increase in total lymphoid and IL-2 receptor positive cells was seen, sometimes more than ten-fold, and there was a profound eosinophilia. Gamma interferon levels rose. Systemic side effects included fever and chills, controllable with prostaglandin inhibitors, and a profound capillary leak syndrome. On the basis of animal experiments, IL-2 treatment might be expected to exacerbate some inflammatory and autoimmune conditions (52,122).

INFγ has been employed in several clinical studies, both in the cancer area and for

immune dysfunction diseases (100,123). INFγ administration intravenously leads to fever, hypotension, and flu-like symptoms, which appear to be manageable, but only modest anti-tumor effects have been observed (100,123). Results in inflammatory diseases have been generally modest or negative. Some favorable responses in short term experiments with low doses of recombinant INFγ have been reported in RA, but longer term benefits were viewed as doubtful (124,125). It may seem difficult to understand how INFγ might be expected to benefit RA patients, but inhibition by INFγ of IL-4 induced B cell response could be involved (126). Treatment of multiple sclerosis patients with recombinant INFγ led to significant exacerbations of disease in seven of eighteen cases, with increases measured in MHC class II expression on monocytes and in leukocyte proliferation (127).

Strategies for Therapeutic Intervention

Transcriptional and Post-Transcriptional Control – The pathways from any particular biological stimulus to any specific gene promoter or repressor and the mechanisms by which mRNA levels are regulated are not well understood at the molecular level, and specific targets for medicinal intervention are not presently available (128,129). Initial studies have begun toward identification of transcriptional and post-transcriptional control points for the interleukins and interferons based upon the gene cloning of these immunoregulants (130-137). In the absence of precise information on molecularly-defined targets for specific transcriptional control, it is notable that the potent immunosuppressive cyclic peptide, cyclosporin A, appears in vitro to inhibit primarily the transcription of IL-2, which may account for the clinical utility of cyclosporin A in suppression of transplant rejection and its reported broader utility in autoimmune diseases (138). The recent report of a structurally unrelated macrolide, FK-506, which may function as an immunosuppressive agent by a related transcriptional control mechanism, underscores the possible opportunities for intervention at the transcriptional level to control lymphokine and monokine action (139).

Protein Processing Inhibitors – Both IL-1α and IL-β appear to be produced as 31-33 kDa precursors, which are proteolytically processed to the mature 17.5 kDa species detected as the released monokines (7,140,141). IL-1 is not produced with a recognizable N-terminal signal sequence to direct secretion. Analysis of precursor sequences and study of intracellular location of IL-1 precursors suggests that IL-1 is not secreted via the endoplasmic reticulum, the Golgi apparatus, or the membrane (142). While most intracellular IL-1 appears to be present in the cytosol in the precursor form, some reports have described also a plasma membrane-bound species (141,142). Recently it has been found that the 30 kDa IL-1α precursor binds to the IL-1 receptor, while the IL-1β precursor does not, suggesting the possibility that the membrane-bound form of IL-1 could be higher molecular weight IL-1α, an active species (143). Thus it is possible that the IL-1α species, as a precursor, functions in direct macrophage-T-cell contact, while the processed IL-1β species is the major soluble monokine. The enzymes responsible for IL-1 processing have not been identified, but processing of the human IL-1β precursor to the mature species would require an unusual cleavage at an Asp-Ala site, suggesting involvement of specific processing enzymes (140,141). TNFα is synthesized also as a larger precursor form, which is processed to the mature species by removal of 76 amino acids at the N-terminus (144).

IL-2, IL-3, IL-4, IL-5, IL-6 and INFγ are produced containing typical N-terminal signal sequences, and so their proteolytic processing may not be subject to specific intervention (44,63,78,145-147). A membrane-associated form of IL-2, distinct from receptor-bound IL-2, has been reported, although its precise nature and function are unclear (148).

Receptor Antagonists – Antibodies targeted for the IL-2 receptor have been shown to be effective in prevention of human kidney transplant rejection and in suppressing diabetic insulitis and lupus nephritis in animal models, demonstrating the validity of the receptor antagonist approach (149,150). The development of small molecule antagonists for the interleukins and for INFγ is the goal of intense research, but no potent receptor antagonists have been reported to date.

Several fragment studies of IL-1 suggest that nearly the full mature sequence, 115-270, is required for full biological activity, although a "core" sequence of 62 amino acids, 136-197, has been proposed as the active site area (151,152). However, a synthetic fragment, 237-269, of the C-terminus of IL1β has been reported to bind at the IL-1 receptor with K_d = 2-40 nM as an antagonist (153). A large (> 18 kDa) protein inhibitor of IL-1 which can block binding of IL-1 to the IL-1 receptor has been reported in the serum and urine of febrile patients and those with systemic juvenile chronic arthritis (154). Anti-peptide antisera against fragments of IL-3 have implicated sections in the middle and at both termini in

biological activity (155). Antibodies to small peptides at the N- and C-termini of IFNγ have implicated these regions in both anti-viral and immunoregulatory activities (156,157). A synthetic peptide representing fragment 1-39 of mouse INFγ was 1/1000 as potent in a competitive binding assay as recombinant mouse INFγ (157).

A rational approach to receptor antagonist design could begin with three-dimensional structural knowledge of the agonist molecules. X-ray investigations of crystalline IL-1, Il-2, and INFγ are underway, and the structures of IL1-β and of IL-2 have been reported at 3Å resolution (158-160). Prior to the availability of the X-ray data on IL-2, structure activity studies using monoclonal antibodies and site mutations had suggested that regions 3-17 and 36-54 might contain the residues responsible for direct receptor contact (161-163). These conclusions are consistent with the IL-2 X-ray data, but the particular residues implicated by the point mutation studies do not define a binding site on the X-ray structure, demonstrating the difficulty in translating these studies into an atomic description of the binding site (159).

Inhibition of Post-Receptor Events – The signal transduction pathways for lymphokines and monokines are not well understood, although the usual cast of second messengers has been implicated, including the protein kinase C and the phosphatidyl inositol pathways (164-169). Internalization of the ligand-receptor complexes for both IL-1 and IL-2 has been reported as an additional possible transduction pathway (164,170). Information is not available presently to determine whether specific intervention is feasible at these levels. Examples of more distal approaches are suggested by the identification of the early IL–2 induced expression of c-myb, a DNA-binding protein, and the induction by IL-1 of the proto-oncogenes c-fos and c-myc (171,172).

Inhibition of Receptor Synthesis or Expression – The structure and regulation of the receptors for IL-1 and IL-2 have been reviewed recently (164,173). The IL-1 receptor common for both IL-1α and IL-1β appears by crosslinking studies to be a 80 kDa protein, although two other proteins of 42 kDa and 200 kDa have been reported to copurify with this binding unit (164). The kinetics of expression of IL-1 receptor on murine splenic T cells have been found to parallel those for the IL-2 receptor, suggesting that these two receptors may be regulated coordinately (164).

The IL-2 receptor is now known to consist of two non-covalently-associated proteins, IL-2Rα ("Tac") and IL-2Rβ ("75 kDa"), which can account for reports of low affinity ($K_d = 10^{-8}$ M), moderate affinity ($K_d = 10^{-9}$ M), and high affinity ($K_d = 10^{-11}$ M) binding sites (173). Recent work has demonstrated that the IL-2Rβ protein is both necessary and sufficient for IL-2 recognition and signal transduction, although affinity for IL-2Rβ alone is moderate (173-175). IL-2Rβ is present in excess on resting T cells, while IL-2Rα is absent until the early stages of T cell activation (173). Large granular lymphocytes, which include both natural killer cells and LAK cells, initially express only IL-2Rβ, which may account for the reported high levels of IL-2 necessary for their augmentation and activation. In the early stages of activation, when only IL-2Rβ may be present, IL-2 stimulation of IL-2Rα expression, and subsequent formation of high affinity IL-2 receptors, can be driven through IL-2Rβ alone (174,175). Internalization of receptors may involve only the high affinity receptor form (170).

IL-2Rα is a highly N- and 0-glycosylated protein produced initially with a 21-amino acid signal sequence. It appears to be sulfated and phosphorylated, but the functional significance of any of these post-translational modifications has not been established (176). The molecular events involved in stimulation of IL-2Rα expression are under study, with both calcium mobilization and activation of protein kinase C implicated in receptor induction stimulated by interactions at the T-cell antigen receptor (176).

Conclusions

The certainty of continued discovery and characterization of new cytokines should increase the urgency of clarifying the roles of the major cytokines reviewed here. There is abundant evidence suggesting that inappropriate production of lymphokines and monokines plays a significant role in various inflammatory diseases, but definitive proof awaits the development of potent and specific medicinal agents applicable to clinical situations. The most straightforward strategy for intervention would appear to be the development of specific receptor antagonists. Attractive possibilities exist for specific intervention either before or after the ligand-receptor step, but the molecular description of these pathways is sketchy. Due to the complexity and interconnection of the network of natural immunomodulatory signals, it may be necessary to attempt intervention at several points in a particular system in order to locate the most clinically favorable molecular target.

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Chapter 19. Modulation of Arachidonic Acid Metabolism in the Treatment of Rheumatoid Arthritis

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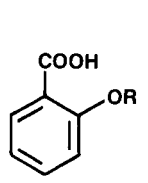
Introduction

The classical analgesic and antiedemic action of non-steroidal antiinflammatory drugs (NSAIDs) is related to the inhibition of the metabolism of arachidonic acid (AA) to prostaglandins (PG) via the cyclooxygenase (CO) pathway (1). Recently, however, further understanding of the underlying pathology has led to interest in the 5-lipoxygenase (5-LO) pathway of AA metabolism and hence, cell chemoattractants such as the leukotrienes (LT) as mediators of inflammatory diseases (2). The purpose of this review is to survey the NSAID literature from late 1985 to the present (for previous reviews, see refs. 3, 4) and to discuss the treatment of inflammatory diseases from the standpoint of biochemical interventions in the AA cascade.

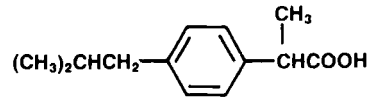
Selective CO Inhibitors

Carboxylic acid derivatives — Numerous clinical studies continue to demonstrate the efficacy of this class against pain and inflammation. Studies have also shown that some carboxylic acid NSAIDs possess mechanisms of action beyond their classical CO pathway inhibition. Salicylate, **1**, the major metabolite of aspirin, **2** (5), has been observed to be effective in blocking rat paw edema induced by platelet activating factor (PAF), an activity not shared by other NSAIDs (6).

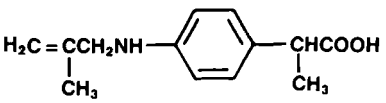
New studies have revealed that intravenous injection of ibuprofen, **3**, inhibits the neutrophil dependent edema response of rabbit skin to $C5a_{des\text{-}arg}$ plus PGE_2, an observation which suggests a non-CO action of this agent (7). Alminoprofen **4** has demonstrated antiinflammatory activity in a clinical trial, as well as inhibition of platelet aggregation (8,9). Although it is an effective analgesic (10), pirprofen, **5**, has been associated with acute benign hepatitis (11), which has been related to the drug's ability to inhibit mitochondrial β-oxidation of fatty acids in mice (12).



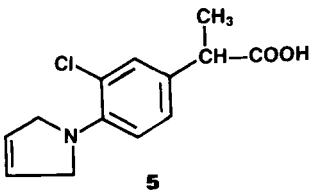
1 R = H
2 R = $OCCH_3$



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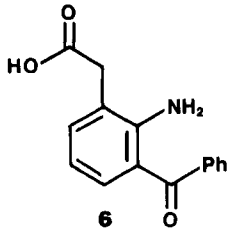


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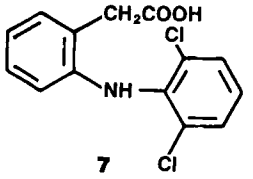
At a single oral dose of 100 mg, amfenac (AHR-5850, **6**), is reported to produce more rapid and effective analgesia than aspirin at 600 mg (13). Albeit at a higher concentration than that required for CO pathway blockade, diclofenac, **7**, appears to inhibit 5-LO pathway product formation. This activity has been explained by **7**'s stimulation of the reuptake of deacylated AA into membrane triglycerides (14). Recently, tolfenamic acid, **8**, has been reported to have a protein synthesis inhibitory component to its activity based on its blockade of collagen and proteoglycan synthesis in skin and cartilage tissue of weanling rats (15). Further, at concentrations in the 10^{-5}M range, both **7** and **8** prevent superoxide production by N-formyl-methionyl-leucyl-phenylalanine-stimulated human neutrophils; the action of **8** appears to be exerted at the receptor level while **7**'s does not (16). Fentiazac (WY-21894, **9**) has demonstrated symptom reduction (according to subjective evaluation) in an acute gouty arthritis

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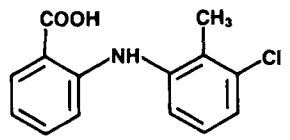
trial (17), and has shown a significant analgesic effect in patients with tendonitis and bursitis (18). Lonazolac, **10**, is reported to be a submicromolar inhibitor of thromboxane B_2 production by AA stimulated platelet rich plasma (19). In a trial where **10** was administered at a 3X200 mg dose, a marked decrease of joint pain and inflammation was observed, although reports on toleration of **10** by man vary (20,21). Initial results on the once-a-day (1200 mg) NSAID, oxaprozin, **11**, show efficacy in symptom remission of painful rheumatic and antiinflammatory conditions (22). The toleration profile in rats is favorable compared to that of indomethacin and aspirin (23). In a study involving patients with rheumatoid arthritis (RA), administration of single daily doses of pirazolac, **12**, for 6 days resulted in a ca. 50% decrease in PGE levels in the synovial fluid (24) where the "non-protein bound" fraction of drug is higher than that in the plasma (25). Another trial in patients with RA has shown by evaluation of pain and joint mobility tests that tiaprofenic acid (RU-15060, **13**) is equally efficacious either as a 2X300 mg/day tablet or a 1X600 mg/day sustained release capsule (26).



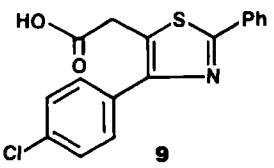
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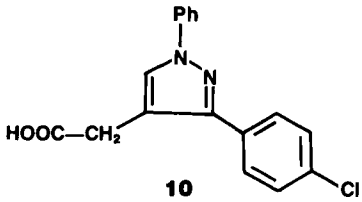
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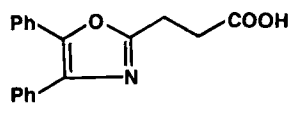
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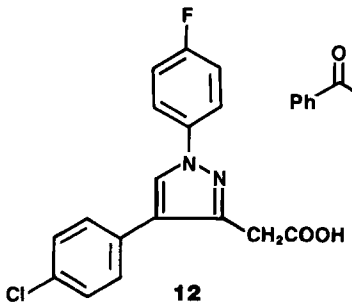
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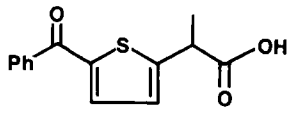
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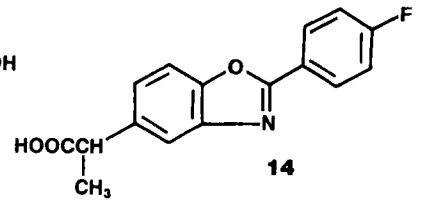
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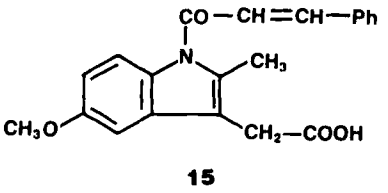


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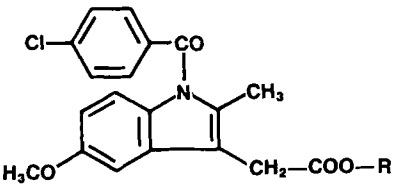
Flunoxaprofen, one of the enantiomers of **14**, decreases the amount of thromboxane B_2 and PGE_2, but not LTB_4, present in the inflammatory exudate provoked by carrageenin-soaked sponges implanted in rats. It also reduces the number of infiltrating inflammatory cells (27). In a small trial with osteoarthritic patients it was effective in reducing pain and improving joint motility, while displaying minimal side-effects (28). Cinmetacin, **15**, an indomethacin analog, shows improved toleration (29), possibly related to the fact that it binds to albumin more tightly than does indomethacin (30). Two indomethacin prodrugs have been prepared: acemetacin, **16**, and proglumetacin, **17**. Administration of **16** (60 mg t.i.d.) and **17** (150 mg t.i.d.) to human volunteers for 5 days caused significantly fewer gastroduodenal lesions than did indomethacin (50 mg t.i.d.) (31).

Carprofen (Ro 20-5720, **18**), an example of the fused tricyclic class of acidic NSAIDs, has a low incidence of gastrointestinal side effects in man (32). The indolecarboxylic acid, etodolac (Ay-24236, **19**), has displayed greater potency in the adjuvant arthritis assay relative to its activity in the rat foot edema (RFE) assay (33). Administration of **19** for 28 days to rats with adjuvant arthritis resulted in a significant abrogation of joint pathology (34). **19** displays a high degree of enantioselectivity in its action on CO (35), and is more effective on synoviocyte and chrondrocyte than on macrophage CO activity (33). Gastric and duodenal endoscopic examinations of normal volunteers have shown that

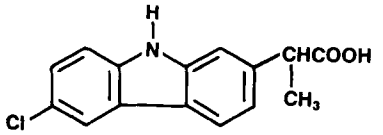
19 is significantly better tolerated than indomethacin, ibuprofen, or naproxen (36); in a smaller study, its effects on renal function were only transient (37). It has recently been launched in a number of countries.



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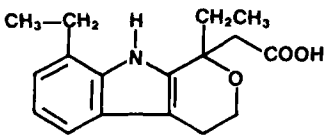


16 R = $-CH_2-COOH$

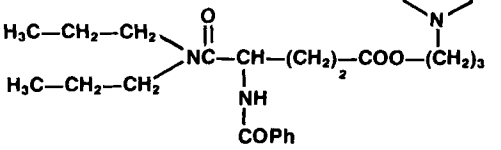


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17 R = $-CH_2-CH_2$

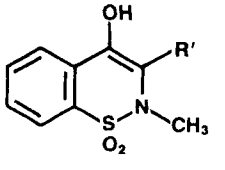


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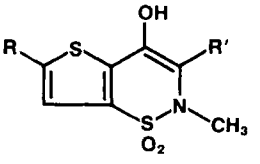


Oxicams — Several compounds derived from the oxicam class (analogs of piroxicam, **20**), have recently been introduced or are in various stages of clinical development. Displaying a 70 hour half-life in man (38), tenoxicam, **21**, at a daily dose of 20 mg has been shown to be efficacious and well tolerated in clinical trials for rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, gout, and non-articular rheumatism (39). **21** has been launched in several countries. By contrast, chlortenoxicam, **22**, has a shorter half-life of 7.5 hours in man (40). **22** is 20-fold more potent than **20** in rat adjuvant arthritis, although it is unexpectedly weak in the RFE test (ED_{50} = 50 mg/kg), in comparison with other oxicams (40,41). Isoxicam, **23**, was recently withdrawn worldwide due to skin reactions and is no longer available. Currently in Phase II trials, meloxicam, **24**, displays a half-life of 25 hours in humans (41), and is claimed to be superior to other oxicam analogs (42-45). An aza analog of **20**, TY-10,4744, **25**, has greater analgesic potency than piroxicam, indomethacin, diclofenac, or naproxen in animal models (46,47); however, with an ED_{50} of ca. 9 mg/kg, it is 4-fold weaker than **20** in the RFE test (48).

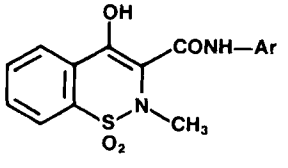
Several prodrugs of **20** are currently undergoing clinical evaluation. Droxicam, **26**, shows potency equivalent to **20** in animal models (49,50), but with delayed activity onset (51,52). Administered at a 20 mg daily dose in humans, **26** shows efficacy and toleration similar to that of **20** (53,54). Examined in patients with mono- or polyarticular arthritis, the pivalate prodrug, pivaloxicam **27** (CHF 10/21) has an acceptable efficacy and toleration without any onset delay, suggesting that the compound undergoes rapid hydrolysis to **20** *in vivo* (55).

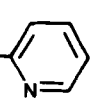


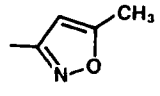
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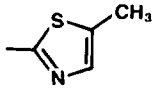


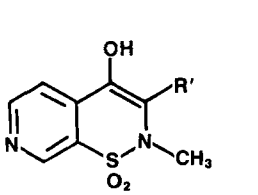
21 R′ = H
22 R′ = Cl



R′ = $-CONH-$ 

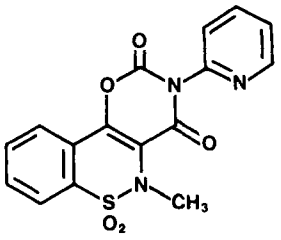
23 Ar = 

24 Ar = 

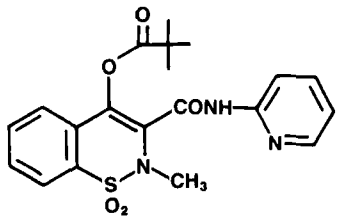


25

R' = —CONH—



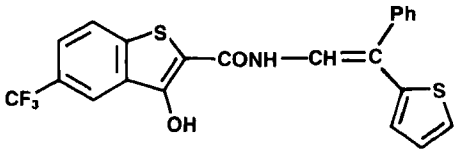
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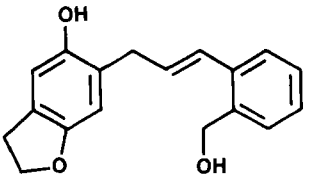
27

Dual 5-LO/CO Pathway Inhibitors

A variety of structurally diverse compounds continue to be reported as combined 5-LO/CO pathway inhibitors. L-652,343, **28**, demonstrates *in vitro* half-maximal inhibition of LT and PG biosynthesis in the micromolar range (56). Consistent with its potent CO inhibitory activity, this acidic compound (pKa = 4. 0) is orally effective in the RFE assay (ED_{50} = 18 mg/kg), adjuvant arthritis (ED_{50} = 10 mg/kg) and phenylbenzoquinone-induced abdominal stretching models (ED_{50} = 1.1 mg/kg) (57,58). Surprisingly however, no corresponding *in vivo* inhibition of 5-LO pathway product formation has yet been demonstrated (59). A recent patent claiming dual 5-LO/CO inhibitory activity for positional isomers of this compound has appeared (60). A potent PGE_2 inhibitor in cell culture, L-651,896, **29**, is also an effective topical inhibitor of LT biosynthesis associated with AA induced mouse ear inflammation (61), acting potentially as a free radical scavenger.

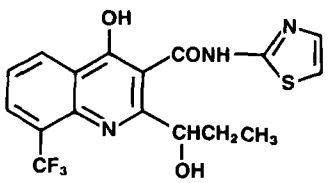


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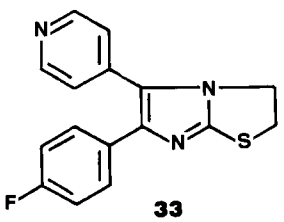


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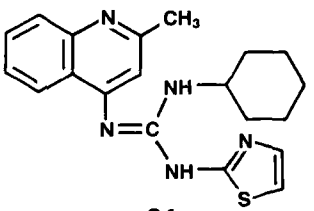
RU-41,819, **30**, has combined 5-LO/CO activity *in vitro* as well as antiedema and analgesic properties in animal models (62). Resolution of this compound has shown that the (-)-enantiomer (RU-45,021, **31**) is 4000X more potent a CO inhibitor than the (+)-enantiomer (RU-45,020, **32**), while little enantioselectivity can be found for inhibition of the 5-LO pathway. Most of the *in vivo* antiinflammatory activity (RFE) also resides in the (-)-enantiomer. SKF-86,002, **33**, a levamisole-like 5-LO/CO blocking agent, inhibits the peritoneal infiltration of neutrophils stimulated by carrageenin injection, an activity not shared by other NSAIDs which were selective CO inhibitors (63-65). Effective activity of **33** also extends to the AA induced ear and paw edema in the rat where conventional NSAIDs are inactive. Another AA metabolism inhibitor with 5-LO/CO blocking action is CP-66,248; this agent is discussed in the New Horizons section.



30 (±)
31 (−)
32 (+)



33



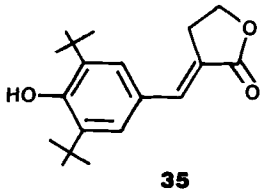
34

Timegadine (SR-1368, **34**), one of the earliest reported dual 5-LO/CO inhibitors (66,67) is apparently still undergoing clinical evaluation. In humans, **34** has been recently reported to undergo extensive first pass metabolism, providing three different compounds, two of which can be measured in serum (68). Current studies of both **33** and **34** will perhaps define the utility of dual LO/CO inhibitors in various

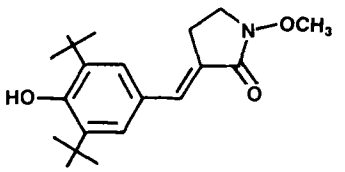
inflammatory diseases. Lastly, as an adjunct to drug mediated blockade of arachidonate metabolism, a dietary approach involving the use of fish oils (rich in 22:6 and 20:5 fatty acids) to exchange for membrane arachidonate shows encouraging results in animal models of arthritis (69,70), as well as in arthritic studies in man (71,72).

Antioxidants

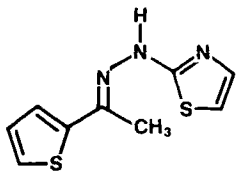
Several 2,6-di-*t*-butylphenol derivatives exhibit combined 5-LO/CO inhibitory activity, attractive antiinflammatory profiles in standard models, and reduced gastrointestinal side effects. These antioxidants have emerged as a new class of NSAIDs (73,74) and numerous compounds have been reported (75). A typical activity profile of these agents is best exemplified by KME-4, **35**, currently in Phase II clinical trials. With its equipotent 5-LO/CO pathway inhibition, **35** is orally active in blocking edema and granuloma formation, and inhibiting leukocyte migration in animal models, while provoking only a fraction of the gastric lesions caused by indomethacin and naproxen. Its analgesic potency is equivalent to that of naproxen in the acetic acid abdominal stretching test (74,76-79).



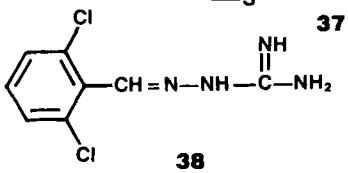
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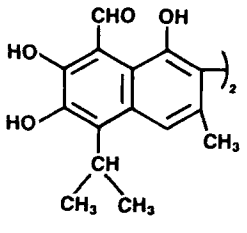
38

A recent addition to this class of compounds is E-5110, **36**. In addition to its excellent antiinflammatory/analgesic activity, **36** also inhibits LTB_4, superoxide anion formation, and interleukin-1 production by isolated human neutrophils (80-83). **36** is in Phase I of clinical development.

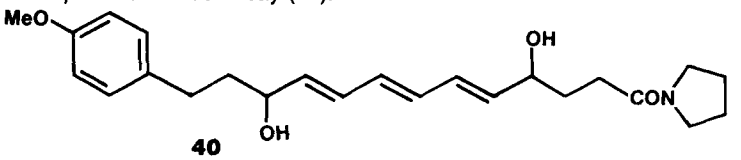
CBS-1108, **37** (84), and the α_2-agonist guanabenz, **38** (85), are among several hydrazine derivatives which are reported as antiinflammatory agents with combined 5-LO/CO blockade. In rats, **37** inhibits the infiltration of leukocytes in inflammatory sites in a dose dependent fashion (84). *In vitro*, **38** inhibits LTC_4 and PGE_2 formation by mouse macrophages ($IC_{50} = 16$ and 28 μM, respectively) as well as LTB_4, PGE_2, and TXB_2 by rat PMNs. It is not clear whether these inhibitory effects are due to 5-LO/CO inhibition or to a free radical scavenging activity. Recently, the antiinflammatory activity of several other α_2-adrenoreceptor agonists has been reported (86).

LTB_4 Antagonists

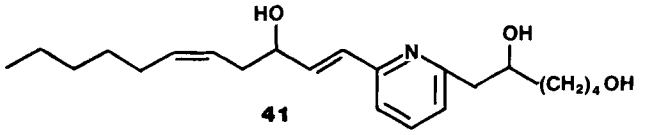
Studies indicate that LTB_4 is a potent mediator of immune responses (87), although it has not been conclusively shown that an antagonist of such a mediator will be efficacious in the treatment of inflammation. Initial studies have identified two natural products, gossypol, **39**, (88) and ichthammol (ammonium bituminosulfonate) (89,90), and two synthetic compounds, SM-9064, **40**, and U-75,302, **41**, as antagonists of LTB_4. **40** suppresses the Arthus reaction in mice at low doses, although it fails to act against carrageenin-induced edema (91). **41** is also claimed to have LTB_4 antagonist activity and to be active in the reverse passive Arthus assay (92).



39



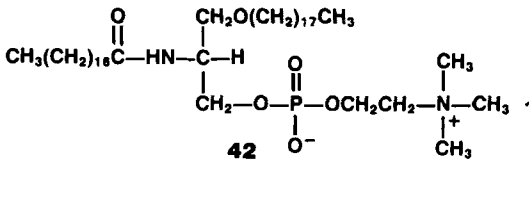
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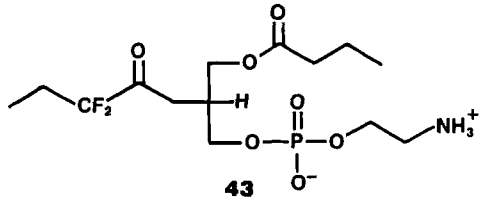
41

PLA_2 Inhibitors

There continues to be much interest in phospholipase A_2 (PLA_2) inhibitors (93-97), especially since human lipocortin I can now be produced in large quantities by recombinant DNA technology (98). Further studies have identified a ca. 80 amino acid region within this 38.5 kD protein which appears to be important for activity (99). Recent studies indicate that PLA_2 inhibition occurs via interaction between lipocortin and the phospholipid substrate in a Ca^{++} dependent fashion, thereby preventing substrate binding to enzyme and consequent liberation of arachidonic acid (100,101). This Ca^{++} dependent activation was also observed in other experiments which determined that calmodulin antagonists also inhibit PLA_2; this inhibition is thought to be caused by their interaction with acidic phospholipids and not with calmodulin (102).



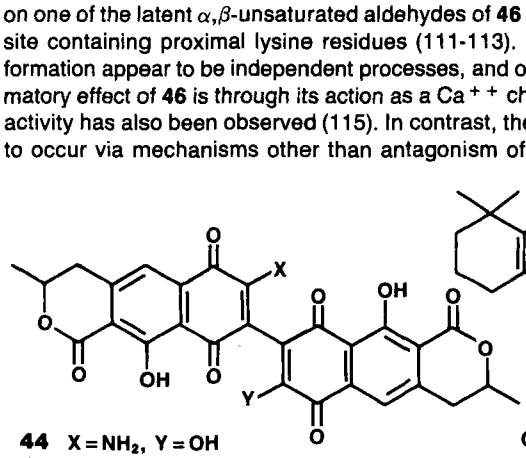
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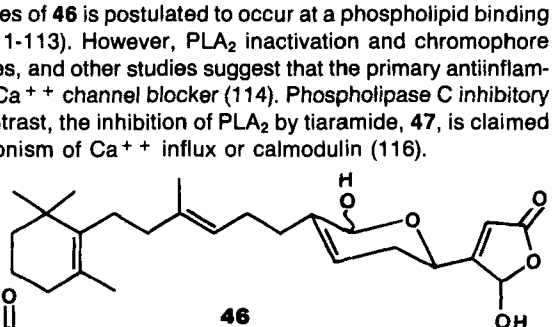
43

Several other PLA_2 inhibitors have been described recently, such as **42**, a synthetic analog of a phospholipid (103). The enhanced binding was attributed to a more electron rich carbonyl oxygen binding to the active site electrophile (possibly a Ca^{++} ion) in addition to enhanced hydrophobic interactions by the ether linkage. **43** was recently synthesized and exhibits micromolar binding activity ($K_i = 50\ \mu M$) to PLA_2 (104). This compound was designed to act as a transition state analog. Two natural products, plastatin, **44**, and luteosporin, **45**, isolated from the filamentous fungus, *Penicillium chermesium*, have been shown to be inhibitors of porcine pancreatic PLA_2, although they do not exhibit significant activity towards other PLA_2 preparations (105). In other programs designed to find inhibitors of porcine pancreatic PLA_2, a family of acylated decapeptides, the plipastatins, has been isolated (106). Vitamin E has also been shown to be an endogenous inhibitor of PLA_2 (107).

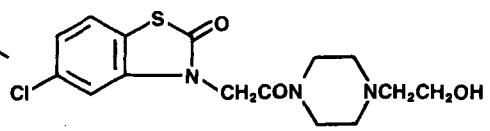
A potent inhibitor of purified bee venom (108) and cobra venom PLA_2 (109), manoalide, **46**, has been the subject of intense study in the last few years. As compared with its *in vitro* binding to PLA_2, the *in vivo* potency of **46** is greatly reduced, although the compound does demonstrate antiinflammatory activity (mouse ear edema) (110). Based on a variety of data, an irreversible nucleophilic attack on one of the latent α,β-unsaturated aldehydes of **46** is postulated to occur at a phospholipid binding site containing proximal lysine residues (111-113). However, PLA_2 inactivation and chromophore formation appear to be independent processes, and other studies suggest that the primary antiinflammatory effect of **46** is through its action as a Ca^{++} channel blocker (114). Phospholipase C inhibitory activity has also been observed (115). In contrast, the inhibition of PLA_2 by tiaramide, **47**, is claimed to occur via mechanisms other than antagonism of Ca^{++} influx or calmodulin (116).



44 X = NH_2, Y = OH
45 X = Y = OH



46



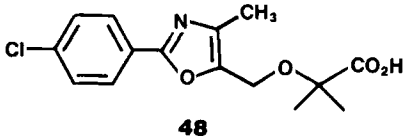
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New Horizons In NSAID Therapy

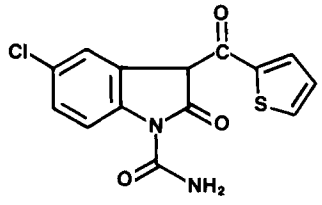
New and intriguing leads for prospective antiinflammatory agents with expanded profiles of activity are emerging. Two of these are compounds which have the potential for regulating interleukin-1 (IL-1) production and/or action. Recent reports have implicated the monokine IL-1 as a mediator associated with the pathobiology of joint deterioration in rheumatoid arthritis (117-120). Ro 31-3948, **48**, currently

in Phase II trials, reverses the change in concentration of plasma acute phase proteins (ACPs) in rat adjuvant arthritis and blocks the IL-1 induced rise in serum amyloid P (an ACP) in mice (121). Evidence suggests that the synthesis of hepatic ACP is stimulated at least in part by IL-1 (122); hence, drug induced reduction in the concentration of these proteins implies the potential for lowering IL-1 concentration (123). In addition to CO inhibitory activity (124), the enolic oxindole, CP-66,248, **49**, also in Phase II trials, possesses properties which distinguish it from conventional NSAIDs. In particular, **49** blocks IL-1 biosynthesis by lipopolysaccharide-stimulated murine macrophages as well as by human monocytes in cell culture (125,126); it also inhibits 5-LO inhibitory activity (124). Early clinical results from patients with rheumatoid arthritis have revealed that this agent reduces the IL-1 levels in synovial fluid (126).

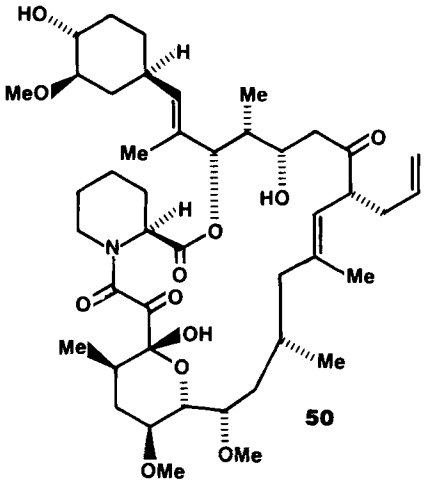
Other agents with novel pharmacological properties possessing potential utility in rheumatoid arthritis are appearing. A natural product FK-506, **50**, which is thought to suppress the production of a number of mediators such as IL-2, has recently been described (127-129). This compound is reported to be considerably more active than cyclosporin A in a number of assays, including mouse and human mixed lymphocyte reaction tests. CGP-28237, **51**, displays a unique profile in that it inhibits prostaglandin synthesis, yet is not a CO inhibitor;it is nonulcerogenic in rats under both acute and chronic test conditions (131). Lastly, as an seleno-organic compound (130) with glutathione-peroxidase activity and the capacity to inactivate LTB_4, ebselen, **52**, represents an interesting approach to inflammation via inhibition of peroxide formation (131-133).



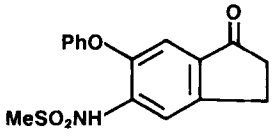
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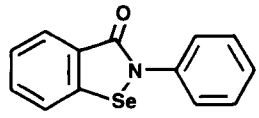
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Conclusion

The approach to treating inflammatory diseases via manipulation of the arachidonic acid cascade has expanded markedly in the last few years, from selective CO inhibition to a whole host of other agents, such as phospholipase A_2, combined 5-LO/CO, or selective 5-LO inhibitors. Since the action of these PLA_2 and 5-LO/CO inhibitors more closely mimics that of steroids on AA metabolism, these agents should in theory offer improved therapy over conventional NSAIDs. Furthermore, research into the role of other mediators, such as IL-1, has led to their identification as potential targets for drug intervention. This increased understanding of the underlying pharmacology, as well as the vigorous pursuit of novel structural classes of agents, is giving rise to an exciting new era in the management of inflammatory disease.

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Chapter 20. The Pharmacological Treatment Of Obesity

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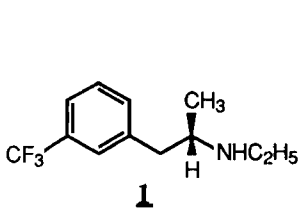
Introduction - Obesity is a disease characterized by multiple physiologic and metabolic abnormalities, anyone of which could be targeted for pharmacologic intervention. The treatment of obesity has, however, proven difficult because of the redundancy built into the body weight regulatory mechanism. Consequently when one system is perturbed, there is often a compensatory reaction which minimizes or negates the initial effect. An example of such a response is the observed increased food intake occurring in normal animals treated with thermogenic agents (1,2). The net effect when two such counterproductive actions are energetically equal is to leave body weight unaltered. Thus it may be necessary to treat obese patients with agents which act by more than one mechanism, or with a combination of therapeutics which decrease energy intake and increase energy expenditure. Since the last review here (3), there has been considerable progress toward the development of successful therapeutics for the treatment of obesity. Reviews and monographs describing more comprehensively some of the topics discussed below have appeared (4-8).

ANORECTIC AGENTS

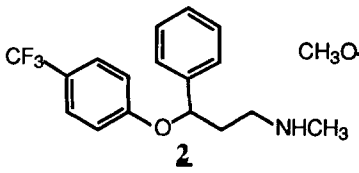
Anorectics Acting Through Serotonergic Mechanisms - Serotonergic agonists acting at 5-HT_1 receptors continue to receive attention as potential anorectic agents (9). The role of these drugs in the treatment of obesity secondary to mild depression has been reviewed (10, 11). In animal studies, it has become apparent that the anorectic response to drug treatment is dependent on the nature of the feeding stimulus, and that while some stimuli act through serotonergic pathways, others act via dopaminergic or adrenergic mechanisms (12-14).

In addition to the serotonergic mechanism of action of fenfluramine and dexfenfluramine (**1**) evidence has been found for a thermogenic component of activity (15,16). Dexfenfluramine, the S enantiomer of fenfluramine, is presently undergoing clinical trials for the treatment of obesity. It affects both the uptake and release of serotonin and binds to 5-HT_1 receptors, although its regional distribution of binding to rat brain differs from that of serotonin. Its anorectic activity is blocked by the non-selective serotonin antagonist metergoline, but not by the 5-HT_2 receptor antagonist ritanserin (17).

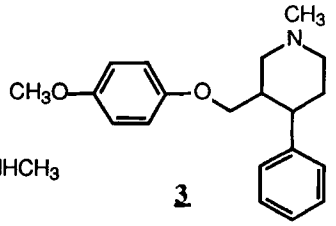
Fluoxetine (**2**) is a serotonin uptake inhibitor which has been approved for use as an antidepressant and is also being studied as an anorectic and for the treatment of bulimia (18). The results of phase I studies summarizing pharamacokinetic and safety data have appeared (19, 20). With the exception of rash in approximately 2% of obese patients, no significant adverse effects were reported in these trials. In two double blind clinical trials in non-depressed obese patients receiving minimal dietary advice, **2** was significantly better than placebo and comparable to benzphetamine in promoting weight loss after 8 weeks (21, 22).



1



2

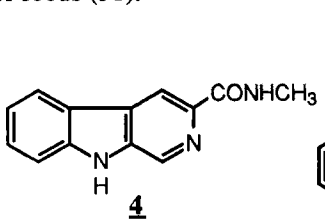


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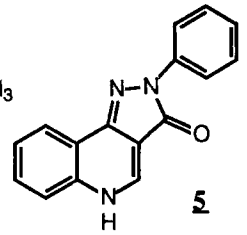
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Femoxetine (3) is another serotonin uptake inhibitor which was compared with placebo in a double blind study in patients who were >20% above their ideal weight. After 16 weeks no difference in weight loss was seen between placebo and the drug treated groups (23).

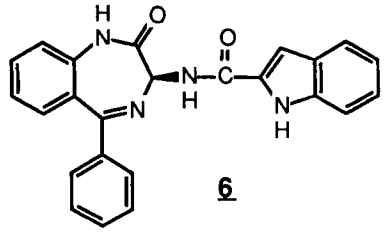
Agents Acting Through The Benzodiazepine Receptor - The stimulatory effect of benzodiazepines on food intake in animals has been known for some time, but this effect has not been demonstrated in humans. Moreover, benzodiazepine receptor antagonists do not suppress food intake (24). Two partial inverse agonists, FG 7142 (4) and CGS 8216 (5), reduced food intake. In rats, 4 produced a dose dependent decrease in consumption of a palatable diet, reversed the hyperphagia caused by clonazepam treatment and reduced the duration and rate of eating (25,26). At lower doses than used in the preceding studies, 4 increased food intake. Thus it was suggested that the decreased intake at higher doses might be due to locomotor depression (27). Decreased intake of palatable foods or a saccharin-glucose solution (28-30), but not a powdered diet (27) were seen with 5. It also reversed the chlordiazepoxide-induced enhancement of palatability, suggesting that the effects of these partial benzodiazepine inverse agonists on food intake behavior are related to the palatability of foods (31).



4



5



6

Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2

7

Anorectic Peptides - CCK-8 (7), the C-terminal octapeptide of cholecystokinin, has been shown to suppress food intake in lean and obese patients by reducing meal size without producing notable side effects (32,33). The satiety effect of 7 has been established in a variety of animal models following either peripheral or central administration (34). Its site of action is thought to be peripheral, based on the observed presence of 7 receptors on the pyloric sphincter (35), the induction of pyloric contraction *in vitro* (36), the correlation between inhibition of gastric emptying and suppression of food intake induced by 7 (37), the involvement of vagal afferents in the transmission of a peripherally generated CCK signal (38) and the reversal of its effects by L-364,718 (6), a benzodiazepine CCK-8 receptor antagonist, which also independently stimulates food intake.(39,40,41).

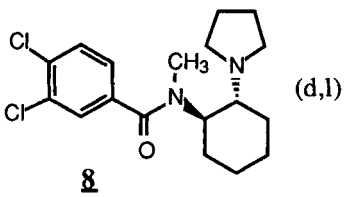
A suppression of meal size in response to administration of 7 to rats at the end of each free feeding meal was maintained over a five day period of treatment (42), but an adjustment toward increased meal number resulted in no net change in 24 hour intake. Consequently, efforts for improving on 7 have focused on the design and synthesis of biologically stable analogs. The nona analog of 7 was shown to suppress food intake and stimulate pancreatic amylase release in patients (43). U-67827, the bis Nle analog of Ac-CCK-8, was shown to be 10 times more potent than the native peptide as a satiety agent in 19-hour food deprived rats (44). Among several analogs found to be more effective than 7 in reducing food intake in 3-hour meal fed rats, the most notable were those which contained a methyl-Phe substitution at the C-terminal end of the molecule (45). Other analogs of 7 for which satiety activity has not been measured have been shown to be equipotent or more potent than 7 in a number of *in vitro* and *in vivo* assays (46-50).

Bombesin, like 7, decreases food intake in humans (51) although its effect on food intake in rats appears to be nonspecific (52,53). Glucagon decreases and glucagon antisera increases food intake in rats (54). Lean rats were more sensitive to the inhibitory effect of glucagon than obese rats, but it was speculated that glucagon's satiating activity was indirect. Centrally administered corticotropin releasing factor (CRF) decreases food intake in rats (55). The CRF related peptides, sauvagine and urotensin I, peptides isolated from frog skin and

fish, respectively, also decrease food intake in rats following central or peripheral administration (55). Histidyl-proline diketopiperazine (56) suppresses food intake and reduces weight gain in rats treated for 13 days by continuous intracerebral ventricular (icv) infusion. Crude mouse tumor necrosis factor (TNF-α) causes a dose dependent decrease in food intake in mice (57). Human recombinant TNF-α and murine recombinant interleukin-1 (IL-1α) also suppress food intake in mice and rats after single or multiple intraperitoneally administered doses, but not after icv administration (58,59). Human recombinant IL-1α and IL-1ß were less effective than murine recombinant IL-1α, while IL-2 was without effect in mice (58).

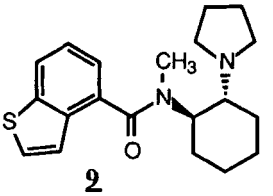
Peptides that stimulate food intake have been recently described suggesting a complex role in food intake regulation for peptidic neurotransmitters. Their value lies in the possibility that specific antagonists may prove to be therapeutically useful. Centrally administered peptide YY (PYY) or neuropeptide Y (NPY) stimulate food intake and induce weight gain in rats in a dose dependent fashion (60,61). Injection of galanin into the medial hypothalamus also stimulates feeding (62). The icv injection of growth hormone releasing factor (both rhGRF and hpGRF) produces small, dose independent increased food intake in rats (63).

Opioid Anorectics - The role of opioids in feeding and drinking behavior has been reviewed (64,65). Although opioid agonists increase and opioid antagonists decrease food intake in animals, there is no evidence of food intake suppression in moderately obese men treated with the long acting opioid antagonist, naltrexone (66). Food intake is not decreased by either naloxone or naltrexone treatment of obese Prader-Willi patients in whom ß-endorphin levels were abnormally elevated (67-69). Naloxone, given by intravenous infusion, decreases caloric intake and the duration of binge eating episodes in bulimic patients, suggesting a role for opioids in this eating disorder (70). Although several opioid receptor subtypes have been implicated in the regulation of feeding behavior, it has been suggested that kappa receptors play a more specific role. However, U-50,488H (**8**) and PD117302 (**9**), which are specific kappa agonists, decrease 24-hour food intake in rats following a transient increase in intake after a single subcutaneous injection (71,72). The role of opioid receptors in animal feeding behavior and their value in treating human obesity remain unclear.



(d,l)

8



(d,l)

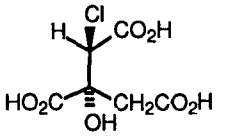
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Anorectic Analogs of Endogenous Carbohydrates - (-)-Threo-chlorocitric acid (**10**), an appetite suppressant which inhibited gastric emptying in rats (3), when tested in obese men, produced a weight change compared to placebo (-1.6 kg/wk). This was accomplished without a significant decrease in food intake, hunger ratings or gastric emptying (73).

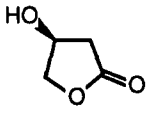
A large number of glucose analogs, among which D-glucosamine was the most potent, were shown to increase food intake by increasing meal size and duration in rats following central administration (74). 1-Deoxy-glucosamine, however inhibits food intake for as long as three days following an initial transient stimulation of intake (75). It was suggested that interference with glucose metabolism was responsible for these effects.

Two endogenous carbohydrate acids isolated from fasted rat serum were described as facilitating or inhibiting food intake (76). 3,4-Dihydroxybutanoic acid γ-lactone (**11**) injected into the cerebral ventricle inhibits food intake and suppresses single-neuron activity in the lateral hypothalamic area, an area known to contain glucose sensitive neurons. 2,4,5-trihydroxypentanoic acid γ-lactone (**12**) stimulates food intake and single-neuron activity under the same experimental conditions. Intravenous injection of **12** stimulates food intake in a dose dependent fashion, but **11** has no effect.

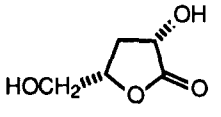
Other Anorectics - AO 124 (**13**) decreases food intake in lean and obese rats and beagle dogs (77). A dose dependent decrease in both food and water intake is seen in normal rats. Serotonin receptor blockade with methysergide does not reverse its anorectic effect; it does not suppress norepinephrine or muscimol-induced feeding, suggesting it does not act through dopamine, serotonin, or GABA pathways. **13** delays gastric emptying and decreases glucose transport, which probably explains the decreased insulin secretion following an oral, but not an intravenous glucose tolerance test. The S but not the R enantiomer of 3-((benzyloxy)methyl)morpholine hydrochloride (**14**) suppresses food intake in a dose dependent manner in dogs in the absence of cardiovascular or CNS side effects (78).



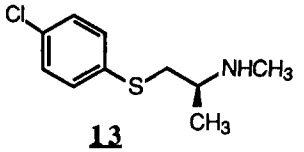
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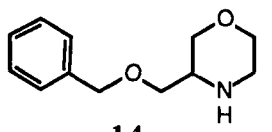
11



12



13



14

AGENTS AFFECTING LIPID AND CARBOHYDRATE METABOLISM

Lipolysis Inhibitors - Lipolysis can be inhibited by α_2 adrenergic agonists through suppression of adenylate cyclase (79). Thus, α_2-adrenoreceptor antagonists would be expected to promote lipolysis and, since a rise in plasma free fatty acid (FFA) levels are associated with an increase in metabolic rate, enhance weight loss (80,81). Indeed, chronic administration of the α_2-antagonist yohimbine decreases body weight and fat content of rodents with hypothalamic, genetic and dietary obesity but, with the exception of obese Zucker rats, has little affect on body protein (82). Furthermore, yohimbine increases energy expenditure in these same animals, particularly in hypothalamically obese mice and genetically obese rats, without markedly altering food intake. In a recent clinical study acute administration of yohimbine increased plasma FFA and glycerol levels and potentiated the exercise-induced increase in energy expenditure of both lean and obese subjects (83). Yohimbine also accelerated weight loss in a group of obese women ingesting an energy restricted diet without apparent side effects (83).

Lipid Absorption Inhibitors - Reducing the absorption of dietary lipid should lead to an energy deficit and weight loss. Tetrahydrolipstatin (THL, **15**) an analog of a microbially derived agent, is a potent, selective and irreversible inhibitor of pancreatic lipase *in vitro* (84,85). A dose dependent decrease in intestinal fat absorption is observed in rats and monkeys following a single administration (85). The effect appears due to inhibition of pancreatic lipase and not to post-lipolytic events,since **15** does not impair the absorption of fatty acids from the intestine. In animals receiving a moderately high fat diet, chronic treatment with **15** decreases body weight gain of mice and causes a loss of body weight and a decrease in fat of diet-induced obese rats even though energy intake increases during treatment (85,86). The antiobesity activity of **15** is attributed to the marked reduction in dietary fat absorption which remains unchanged throughout treatment.

Another approach to decreasing lipid absorption is by the incorporation of a nonabsorbable fat substitute into the diet. Sucrose polyester (SPE) is a nonhydrolizable synthetic compound consisting of octa-esters of sucrose and long chain fatty acid which when used as a replacement for dietary fat to reduce intake of adsorbable energy, causes weight loss in obese subjects (87,88). Its utility as a cholesterol lowering agent has been demonstrated in both normal and hypercholesterolemic subjects receiving SPE as a substitute for dietary fat (87-91). The cholesterol lowering activity of SPE is due to its ability to

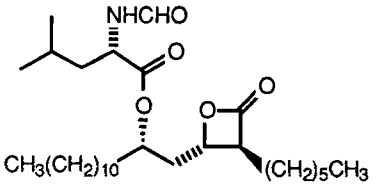
decrease cholesterol absorption from both dietary and enterohepatic sources (92) by creating a persistent lipophillic phase in the intestine which interferes with the dissolution of cholesterol. Another nonabsorbable fat substitute that may have potential as an antiobesity agent is phenylmethylpolysiloxane (PS), which is a linear polymeric derivative of silica (93). Administration of PS, as a dietary admixture to genetically obese rats, decreases body weight, carcass fat and retroperitoneal fat cell size while conserving lean body mass. The antiobesity activity of PS is likely related to both fat malabsoption and a decrease in energy intake resulting from the noncompensated energy dilution of the diet.

Preventing the absorption of lipid may also be achieved by sequestering lipids in the intestine. Diethylaminoethyl-dextran (DEAE-D), a nonabsorable polycationic derivative of dextran (94,95), promotes excretion of neutral sterols, fatty acids and bile acids in patients with and hyperlipoproteinemias (96). This effect is attributed to the binding of DEAE-D to anionic amphipathic components such as bile acids, phospholipids and fatty acids and the subsequent inhibition of micellar formation (97). Long term administration of DEAE-D leads to marked reductions in serum cholesterol and triglyceride levels in hyperlipidemic patients (96,97). In addition, treatment with DEAE-D produces body weight loss in individuals with refractory obesity (98) and in obese subjects with hyperlipoproteinemias (97).

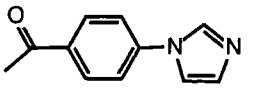
Carbohydrate Absorption Inhibitors -Three carbohydrate absorption inhibitors have recently undergone limited clinical testing. The α-glucosidase inhibitor, acarbose, the most advanced of these, reduced weight regain following weight loss in obese subjects but is more effective in non-insulin dependent diabetics than in obese patients (99,100). Trestatin, a pancreatic α-amylase inhibitor, was tested in normal volunteers (101). AO-128, a valiolamine derivative α-glucosidase inhibitor, has antiobesity activity in animal models (102). It also suppressed postprandial glucose and insulin levels in lean and obese patients and improved glucose tolerance in obese patients in the absence of weight changes after two weeks of t.i.d. dosing (103,104). The clinical utility of these agents for treating obesity remains to be determined.

INSULIN LOWERING AGENTS

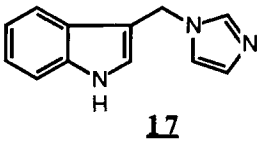
The hyperinsulinemia associated with obesity is thought to play a role in the development and or maintenance of excess adiposity. Ro 22-3581 (16), a novel inhibitor of *in vitro* rat pancreatic insulin secretion, reduces weight gain, lipid deposition and circulating insulin levels in obese rodents independent of its effect on food intake (105,106). Several other classes of compounds, of which the most notable are 3-substituted indoles represented by 17, are also inhibitors of pancreatic insulin secretion, circulating insulin levels, food intake and body weight gain (105,107).



15



16



17

THERMOGENIC AGENTS

Ephedrine, both an indirectly-acting sympathomimetic amine and a mixed adrenergic agonist, reduces body weight and body fat with minimal effects on body protein in a number of genetic, hypothalamic, and dietary rodent models of obesity (82,108-111). The antiobesity activity results mainly from an increase in energy expenditure rather than a decrease in energy intake (82,109,111). Ephedrine activates the unique heat producing mechanism in brown adipose tissue (BAT), since it stimulates respiration in isolated brown adipocytes and increases both GDP binding by BAT mitochondria and the concentration of the BAT mitochondrial uncoupling protein following chronic treatment (112-114).

Calorimetry studies show that ephedrine increases energy expenditure in humans

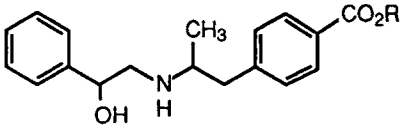
following acute administration (115-118). Ephedrine-induced thermogenesis is further enhanced with chronic treatment and is accompanied by an increase in lipid metabolism (119,120). In normal adults skeletal muscle is the major site for acute ephedrine-induced thermogenesis (116). Ephedrine significantly reduced body weight in a group of moderately overweight women maintaining their normal eating habits (119) and in a group of low-energy-adapted overweight and obese women, consuming an energy restricted diet (121). However, at comparable doses of ephedrine, weight loss of unselected obese male and female patients on a hypocaloric diet did not differ from that of the placebo group (122). The dose dependent cardiovascular and CNS related side effects associated with ephedrine were attenuated during prolonged treatment (119,122).

Ephedrine Combinations - Recent studies have examined the thermogenic and antiobesity potential of mixtures of methylxanthines and ephedrine in hypothalamic and genetically obese rodents. Although chronic administration of caffeine plus theophylline to hypothalamically obese mice is not effective, either compound or both in combination with ephedrine markedly potentiate its thermogenic and antiobesity effects without altering energy intake (111). A normalization of body composition also occurs when genetically obese rats are given preparations containing ephedrine, caffeine and theophylline. In this case the antiobesity activity of the mixture is attributed both to its anorectic effect and its ability to minimize the decline in metabolic rate accompanying the hypophagia (123).

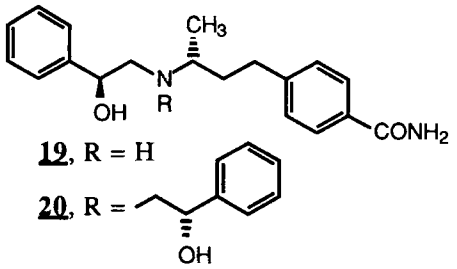
The thermogenic response to norepinephrine is associated with an increase in intracellular concentrations of cAMP. Both phosphodiesterase and adenosine reduce cAMP levels. The former acts by enzymatic breakdown and the latter by inhibition of adenylate cyclase activity and by prevention of norepinephrine release from nerve terminals. A sustained elevation of cAMP in target tissues, due to the inhibitory effects of methylxanthines on both phosphodiesterase and endogenous adenosine, has been proposed as a mechanism for the ability of methylxanthines to increase ephedrine-induced thermogenesis (111). Since prostaglandins inhibit release of noradrenaline, a decrease in prostaglandin biosynthesis may be expected to promote the thermogenic action of ephedrine. Indeed, aspirin potentiates the thermogenic and antiobesity activities of ephedrine in hypothalamically obese mice, although by itself it has no effect on oxygen consumption or body composition (124). The decrease in body fat content of ephedrine/aspirin treated mice is not accompanied by changes in body protein and is entirely due to an increase in energy expenditure.

As observed in animals, mixtures of ephedrine, caffeine and theophylline stimulate metabolic rate in humans to a greater extent than ephedrine alone (118). Measurement of 24-hour energy intake and expenditure indicate that individuals with a predisposition to obesity are more sensitive to the anorectic and thermogenic properties of the ephedrine/methylxanthine mixture than lean individuals (118). Clinical studies comparing the activity of ephedrine to a mixture of ephedrine and methylxanthines have not been reported.

Adrenergic Agonists - The phenethanolamines, BRL 26830A (**18**), LY104119 (**19**), and Ro 16-8714 (**20**) are ß-adrenergic agonists with potent thermogenic properties. Long term treatment of lean, diet-induced obese or genetically obese rodents with any of these drugs leads to a loss of body fat without depletion of body protein (1,2,125,126). This effect, which is more pronounced in obese animals, is due primarily to enhanced energy expenditure and not suppressed energy intake. In fact, a drug-induced increase in energy intake appears to account for the attenuation of the antiobesity activity observed in the lean animals. Although BAT is an important effector tissue for the thermogenic action of these compounds (1,125-127), it is likely that other sites participate in the overall thermogenic response. Measurement of hind limb oxygen uptake *in situ* suggests that muscle may contribute to the increased metabolic rate of rats dosed with **18** (128). The obligatory fuel for the thermic effect of **18** in rats is lipid, since administration of methyl palmoxirate, (an inhibitor of long-chain fatty acid oxidation) prevents the **18**-induced increase in oxygen consumption (129). Lipid is also the major substrate for **18** and **20** induced thermogenesis in BAT (129,130).A potential liability in designing ß-adrenergic agents for the treatment of obesity is nonselectivity between the thermogenic effects and effects on the cardiovascular system. However, the ß-adrenoreceptor that mediates the antiobesity activity of these compounds appears to be neither the $ß_1$- nor the $ß_2$-subtype.



18, R = CH_3
21, R = H

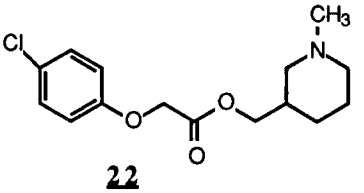


19, R = H
20, R =

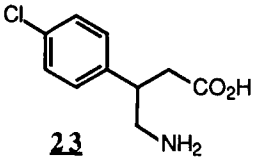
In vitro studies with BRL 28410 (**21**), a biologically active metabolite of **18**, show selective stimulation of an atypical ß-adrenoreceptor for lipolysis in white and brown adipocytes (131,132) and for glycogen synthesis in soleus muscle (133). The relative potencies of **18**, denopamine ($ß_1$-agonist) and salbutamol ($ß_2$-agonist) at stimulating metabolic rate, heart rate and uterine relaxation in pithed rats provide further evidence for a novel ß-adrenoreceptor (6).

The capacity of **20** to stimulate metabolic rate has been assessed by indirect calorimetry in lean and obese subjects (134,135). A single administration of **20** produced a dose related increase in energy expenditure of lean individuals (134). However, the thermogenic response to **20** was not separated from the drug-induced increase in heart rate and systolic blood pressure. Furthermore, there were no differences between lean and obese individuals for the thermogenic and cardiovascular effects of this drug (135). Clinical studies done with **18** produced conflicting results (136-138). The normal decline in metabolic rate that accompanies weight loss during dieting was prevented by **18** (138). These patients demonstrated a decrease in overnight energy expenditure during a two week followup, after withdrawal of treatment. Two recent clinical trials have confirmed the antiobesity effects of **18** in obese subjects on low energy diets and extended the previous findings by showing that **18** plus diet continue to be more efficacious than diet alone (139), and that drug-treated subjects lose more body fat while retaining more protein than the placebo group (140).

PM 170 (**22**) displays antiobesity activity in hypothalamically and genetically obese rodents, but has little effect on lean controls (141). The loss of body weight or reduced rate of weight gain of these animals is associated with a decrease in energy intake. A sustained increase in oxygen consumption of lean rats following both single and multiple dosing indicates that, in addition to its anorectic properties, **22** also induces thermogenesis (142). Moreover, **22** stimulates oxygen consumption of isolated liver mitochondria through a partial uncoupling of respiration from oxidative phosphorylation (142).



22



23

Acute administration of baclofen, (**23**), a derivative of the neurotransmitter GABA, stimulates oxygen consumption of rats and activates BAT thermogenesis as assessed by an increase in both tissue temperature and mitochondrial GDP binding (143). When given chronically, **23** suppresses body weight gain of lean rats without affecting food intake and increases GDP binding by BAT mitochondria. The effect of **23** on BAT appears to be mediated by the sympathetic nervous system through its action on the ventromedial hypothalamus (VMH), since the increase in BAT thermogenesis following injection of **23** into the VMH is abolished by ganglionic or ß-adrenergic blockade or denervation of the tissue. A novel mechanism of action of **23** is proposed since GABA does not alter thermogenesis in BAT.

Conclusion - The study of several agents with differing mechanisms of action have moved from the preclinical to the clinical stage. The results of those studies should demonstrate the value of these approaches for the treatment of obesity. The promising results from clinical studies under way with appetite suppressants and thermogenic agents predict the introduction, in the near future, of effective therapeutics for the treatment of obesity.

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Chapter 21. Gastrointestinal Motility Enhancing Agents

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Introduction - A previous review of this area concentrated on the anatomical organisation of the gastrointestinal tract and pharmacology of the motility stimulants known at that time (1). The present review covers subsequent literature of those compounds which are still of interest, and more recent compounds, particularly with reference to their mechanism of action.

A number of books have been published in the area of gastrointestinal diseases (2-6) including a useful basic text book on gastrointestinal motility (7). In addition a number of relevant reviews and reports of symposia have appeared (8-17).

Benzamides - Metoclopramide, **1**, is an upper gastrointestinal motility stimulant which is both a central and peripheral dopamine D_2 receptor antagonist (1). For many years it was believed that this antagonism was responsible for its gastric motility stimulant activity, but it is now generally accepted that the majority of its gut prokinetic activity results from enhanced acetylcholine release from cholinergic neurons within the gut (2). This effect is believed to be related to an action on 5-hydroxytryptamine (5-HT) receptors (18), although the nature of this receptor is still unresolved. Both *in vivo* and *in vitro*, endogenous 5-HT acts as a gastric motility stimulant either by enhancing gut cholinergic activity or by directly stimulating smooth muscle contractility (19). In both rat and human, exogenous 5-HT has little or no ability to affect cholinergic activity. However, use of 5-hydroxytryptophan, the bio-precursor of 5-HT, causes increased turnover of endogenous 5-HT and a resultant increase in gut cholinergic activity (20). In guinea pig ileum, metoclopramide induces a maintained increase in electrically evoked, cholinergically mediated contractions by activation of a 5-HT-like receptor which was shown not to be a 5-HT_3 receptor, but otherwise remained uncharacterized (21).

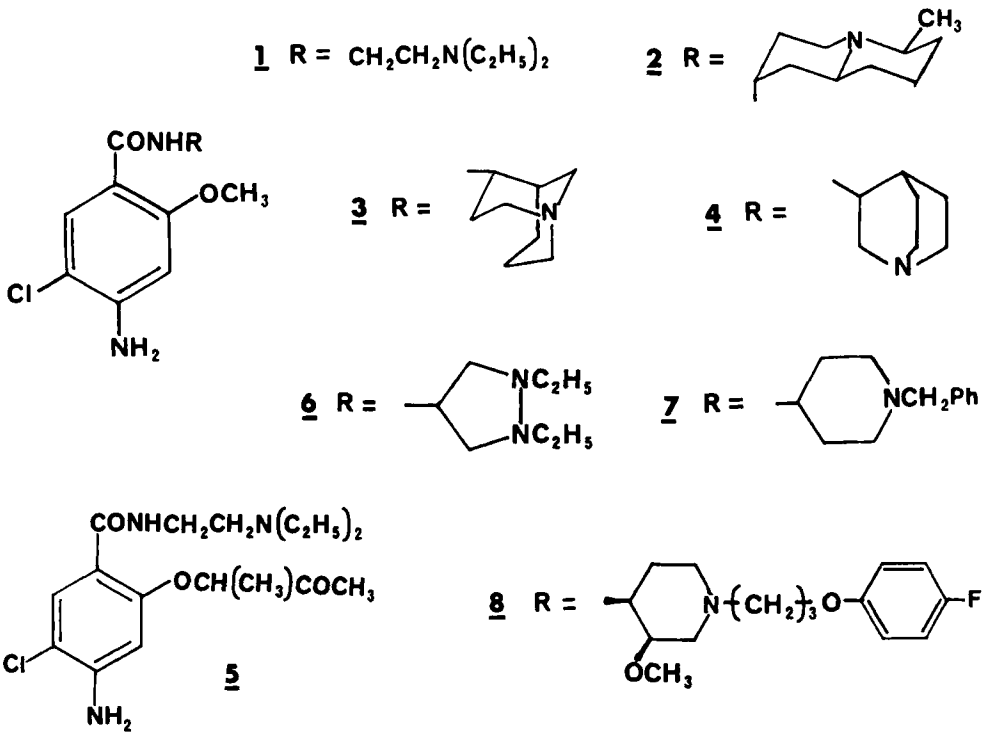
In cancer patients, high-dose metoclopramide has been shown to reduce emesis evoked by cytotoxic drugs, especially cisplatin (22). This activity has been linked to its gastric prokinetic activity by some authors (23), but to its 5-HT_3 receptor antagonism by others (24).

A number of compounds derived from metoclopramide have appeared in which structural modification has eliminated dopamine receptor antagonist activity, but retained the gastric motility stimulation. The apparent efficacy of these compounds in man supports the evidence from animal models that dopamine probably plays a relatively minor role in motility disorders of the upper gut (17).

The previously reported BRL 20627, **2**, shows only weak dopamine receptor antagonist activity (ED_{50} 25 mg/kg sc for inhibition of apomorphine-induced climbing in mice) and only weakly displaces

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[^{3}H]-spiroperidol from rat striatal tissue (IC_{50} 2.5 μM) (25). Stimulation of gastric emptying has been demonstrated in man, with a potency similar to metoclopramide (26-28). BRL 20627 is only a weak antagonist of the 5-HT evoked Bezold-Jarish (B-J) reflex in anaesthetised rat, indicative of poor potency as a 5-HT_3 receptor antagonist (29). Within a series of quinolizidines, activity was associated with substitution at the 2-position of the quinolizidine ring and selectivity with the axial orientation (25).



A more potent, conformationally restricted, gut motility stimulant, BRL 24924, **3**, has subsequently emerged (30,31). Although it is an equatorial isomer, BRL 24924 has no effect on dopamine D_2, nor 5-HT_1 or 5-HT_2 binding sites (32,33). BRL 24924 is 10x more potent than metoclopramide as a stimulant of gastrointestinal motility in rats and is a potent stimulant of electrically-evoked, cholinergically mediated contractions in guinea-pig isolated ileum (30,32). In conscious rats, BRL 24924 increases the irregular myoelectrical spiking activity in the small intestine (33). In man, it increases the rate of gastric emptying (34), and decreases oral-cecal transit time at doses as low as 0.3 mg (35). Both these actions were achieved without a rise in blood plasma prolactin concentrations, indicative of an absence of dopamine D_2 receptor antagonism (36). BRL 24924 is also a potent 5-HT_3 receptor antagonist (B-J reflex, ID_{50} 3.7 μg/kg *vs.* 5-HT) (32) and is effective at inhibiting cytotoxic drug and X-irradiation induced emesis in the ferret (37-39). However, *in vitro*, 5-HT_3 receptor antagonism does not significantly contribute to its gut stimulatory properties (32).

The structurally related benzamide, zacopride, **4**, is a gastric motility stimulant (40) and a potent 5-HT_3 antagonist which is being studied as an anti-emetic (41,42).

An alternative approach to achieve selectivity has been to alter the ortho-alkyloxy group of metoclopramide. From this work a number of gastric prokinetics and anti-emetics were discovered (43), and the ketone, **5**, is undergoing clinical trials as an anti-emetic (44).

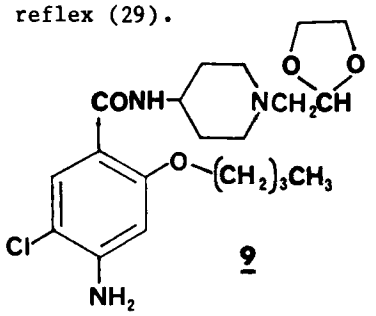
Dazopride, **6**, is a selective gastric motility stimulant which is a weak dopamine D_2 receptor antagonist, displacing [^{3}H]domperidone from rat striatal sites only at high concentrations (23). It is less potent than metoclopramide at inhibiting emesis evoked by cisplatin in the dog, but 6x more potent at stimulating gastric emptying (23). Dazopride is a weak 5-HT_3 receptor antagonist, less potent than metoclopramide *in vitro* (45). In preliminary clinical trials dazopride is thought to stimulate gastric motility, but only at very high doses (80-100 mg) (17).

Introduction of a 3-methoxy group into the piperidine side-chain of clebopride, **7**, gave compounds which were both potent dopamine D_2 receptor antagonists and gastric motility stimulants (46). Subsequent chain-extension of the N-substituent gave cisapride, **8**, a potent gastric prokinetic agent, now in an advanced stage of clinical development (46-47). This compound is a relatively weak dopamine receptor antagonist both peripherally and centrally (12x and 24x less potent than metoclopramide , respectively) (48). Unlike metoclopramide (49), cisapride, at doses which stimulate upper gut motility, markedly stimulates colonic motility (50). Although it has been shown to increase both the lower esophageal sphincter pressure (LESP) and the force of esophageal peristalsis, it was ineffective with regard to motor dysfunction associated with postprandial gastroesophageal reflux, but gave good results for symptomatic relief (47). Cisapride was also effective in the treatment of gastroesophageal reflux in children without central nervous system abnormalities (51). It has been shown to be effective in the treatment of chronic dyspepsia (52). Acceleration of emptying of a solid meal was noted in dyspeptic patients (53), although in another study significant acceleration was only observed when pre-dose rates were abnormally low (54). In human volunteers, mouth to cecum transit time of a semi-solid meal was reduced, but with little effect on the rate of gastric emptying (55).

In animal models, cisapride enhances non-adrenergic, non-cholinergic excitatory activity in the gastric antrum of anesthetized ferrets (56). In the small intestine, it increases antro-duodenal coordination in a dose-dependent manner *via* an increase in acetylcholine release (57). Cisapride has been shown to mimic the effects of 5-HTP in the intestine of conscious dogs (58). In guinea pig, but not human, proximal colon, it evokes contractions predominantly *via* activation of a non-neuronally mediated mechanism which is subject to tachyphylaxis (59,60). In humans, cisapride enhances propulsive colonic peristaltic movements by a non-cholinergic mechanism (61). It also normalises the threshold of the inhibitory reflex of the internal anal sphincter and may therefore be of possible therapeutic use in the treatment of constipation (62).

Cisapride antagonises 5-HT evoked, cholinergically mediated, contractions in guinea pig ileum at a threshold concentration of less than 10^{-7}M, indicative of 5-HT_3 receptor antagonist activity (63). In contrast, it is only a weak antagonist of those 5-HT_3 receptors mediating the B-J reflex (ED_{50} 0.24 mg/kg iv) (29). In rat caudal artery, cisapride antagonizes the effects of 5-HT (IC_{50} 4.7 x 10^{-5}M), suggesting activity as a 5-HT_2 receptor antagonist (64). Similar 5-HT_2-like

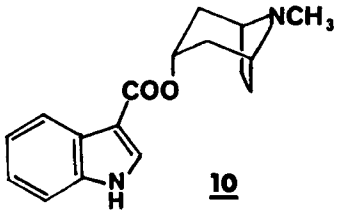
antagonist activity was seen in rat intestinal mucosa in vitro (65). In guinea pig ileum, the potency of cisapride, BRL 20627, clebopride, dazopride and metoclopramide as antagonists of 5-HT evoked contractions did not correlate with their ability to increase cholinergically mediated contractions (6). Similarly in a direct comparison of BRL 24924, BRL 20627 and cisapride there was no correlation between their ability to act as gut motility stimulants and their potency as antagonists of the B-J reflex (29).



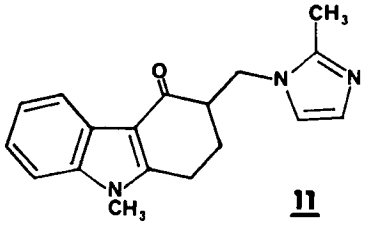
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The piperidinyl benzamide, dobupride, **9**, is reported to be in Phase I clinical trials as a gastroprokinetic with no demonstrable ability to block dopamine receptors (31,66).

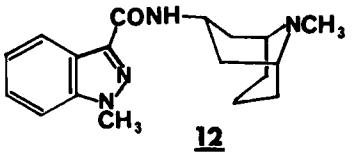
Non-Benzamide 5-HT_3 Receptor Antagonists - As described earlier, some benzamides are potent 5-HT_3 receptor antagonists. However other series of compounds have shown good 5-HT_3 receptor antagonist activity. The central actions of these compounds are discussed in Chapter 6. The apparent considerable differences in the ability of individual compounds to antagonise 5-HT_3 mediated responses in different isolated tissues has led to the proposal of 5-HT_3 receptor sub-types (67). In particular the 5-HT_3 receptor associated with the guinea pig ileum is much more difficult to block than those associated with vagal and heart responses (68).



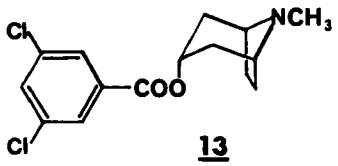
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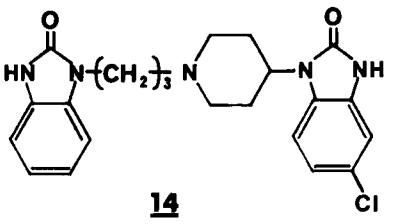
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Both ICS 205-930, **10**, (B-J reflex, ID_{50} 0.4 or 4 μg/kg iv vs. 5-HT) (67,69) and the structurally distinct, GR 38032F, **11** (B-J reflex, ID_{50} 0.42 μg/kg, but vs. 2-methyl-5-HT) (70) are potent 5-HT_3 receptor antagonists and increase gastric emptying of radio-opaque beads in the conscious guinea pig, with potencies of 50-100x that of metoclopramide (71). It was suggested that the effects of ICS 205-930 may be due to an action either within the central nervous system or by a mechanism which facilitates gastric cholinergic activity. However, ICS 205-930 does not potentiate cholinergically mediated contractions evoked by electrical field stimulation in guinea pig ileum (32). Neither BRL 43694, **12**, (B-J

reflex, ID_{50} 0.7 μg/kg iv vs. 5-HT) (72) nor MDL 72222, **13**, the first selective 5-HT_3 receptor antagonist reported (B-J reflex, ID_{50} 22 μg/kg iv vs. 5-HT) increase cholinergic activity in guinea pig ileum (73,74). In the conscious rat and dog, neither BRL 43694 nor ICS 205-930 mimic the gastric motility stimulant activity of either metoclopramide or BRL 24924 (75). In conscious fasting or fed dogs ICS 205-930 did not affect small intestinal myoelectric activity (76). In a preliminary report in man, ICS 205-930 decreased oral-cecal transit time by 23% at a relatively high dose of 20 mg by iv infusion (77). This contrasts with similar studies in which the much weaker 5-HT_3 receptor antagonists metoclopramide and BRL 20627, at doses of 10 mg iv, decreased oral-cecal transit time by 42% and 26% respectively (28).

In animals and man most 5-HT_3 receptor antagonists reduce or prevent chemotherapy or radiation induced emesis (42, 78-80).

Domperidone - Domperidone, **14**, is a peripherally selective dopamine D_2 receptor antagonist which is structurally different from the benzamides and is marketed as a prokinetic and anti-emetic (1, 17). In functional tests the ED_{50} for central dopamine receptor antagonist activity is 40 mg/kg sc in the rat (81), but 2.5 mg/kg sc in the mouse (2). In the dog, domperidone and metoclopramide are equipotent as gastric motility stimulants (82).



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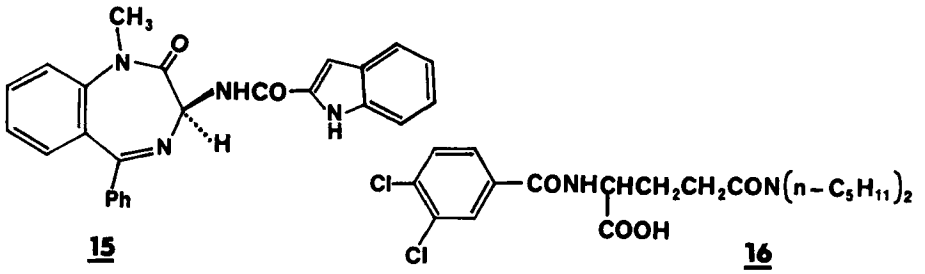
Unlike metoclopramide, domperidone did not increase electrically-evoked, cholinergically mediated contractions in human isolated stomach (83). In the Heidenhain pouch preparation of conscious dogs, domperidone evokes only a small increase in the amplitude of phasic intragastric pressure waves which was mimicked and obscured by pre-injection of the α_2-adrenoreceptor antagonist phentolamine (84). In the rat, it failed to stimulate either interdigestive intragastric pressure changes or the emptying of a liquid meal in the dose range 0.25-10 mg/kg sc (2). Domperidone increases peristaltic activity and amplitude in the antrum and duodenum; however, it is less potent than metoclopramide at increasing gastric emptying in patients with various gastric stasis syndromes (17). Domperidone is a potent stimulator of prolactin secretion and hyperprolactaemia is more frequently associated with domperidone treatment than with metoclopramide (17). In the colon, domperidone has been shown to inhibit the stimulation of motility evoked by dopamine (85).

Peptides and Peptide Antagonists - Endogenous peptides undoubtedly play an important role in the control of gastrointestinal function (86). Studies of the neurochemistry of enteric neurons and of the pharmacology of transmission from these neurons have identified more than 20 potential peptidic neurotransmitters, most of which have demonstrable effects on gastrointestinal function. Understanding the role of neuropeptides is exceedingly difficult, with the effects of particular peptides sometimes depending on *in vivo* and *in vitro* methodology (87) or on whether they are administered into the brain or periphery (88). The development of selective agonists and antagonists is, however, helping to clarify the situation.

Somatostatin has been shown to both stimulate (89) and inhibit (90) gastric emptying in man. The observed effects in animals are thought to be dependent upon a variety of factors, including concentration, species, nature of the meal and even the physical position of the animal. The somatostatin analogue, SMS 201,995, [H-(D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr(ol)], has been shown to increase emptying of a mixed meal in man (91).

The effects of morphine and opiate peptides on gut motility is dependent upon the opiate receptor to which the drug primarily binds, but overall effects on gut neuronal activity are generally inhibitory (86). The synthetic met-enkephalin analogue HOE 825, [Tyr-(D)Lys-Gly-Phe-(L)homocysteine-thiolactone], has been shown to increase motility in all parts of the intestine in both conscious and anesthetised animals (92). It is reported to be a mixed δ / μ agonist with an exclusive peripheral mode of action. It is thought to act by inhibition of an inhibitory neuron (93).

The advent of a number of selective cholecystokinin (CCK) antagonists has stimulated further study into the effects of CCK on the gut (94). Specific CCK binding sites have been identified in rat upper gastrointestinal tract (95). CCK is thought to have an inhibitory effect on gastric emptying, possibly mimicking the delay caused by food intake (96). The non-peptidic, peripherally selective, CCK antagonist L-364,718, **15**, reverses the inhibitory effect of CCK octapeptide (CCK-8) on gastric emptying in animal models (97). In rats, the delay in the gastric emptying of a protein-rich liquid meal was inhibited by L-364,718. No effect was observed on delay evoked by either saline or acid (98). The structure-activity relationships of a series of CCK antagonists closely related to L-364,718 has been published in which the amide link has been replaced by a methylene (99).

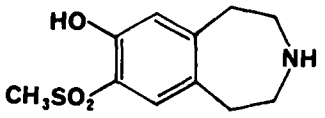


15 **16**

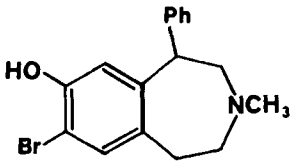
The glutamic acid derivative lorglumide (CR 1409, **16**) has been shown to be a potent and selective CCK antagonist in guinea pig ileum (100,101). In the same tissue, CR 1409 did not affect capsaicin, electrically-evoked contractions or the peristaltic reflex.

Miscellaneous - The macrolide antibiotic, erythromycin, has been shown to initiate interdigestive migrating contractions in both dog (102) and man (103). Suggestions that the effects of erythromycin mimic the effects of exogenous motilin (104) have been disputed (105). This activity is specific for the 14-membered macrolides in which the dimethyl-amino sugar, desosamine, and the neutral sugar are linked in parallel (102). A number of quaternary amino derivatives of erythromycin have shown gastric motility stimulant activity in the absence of anti-microbial activity (106).

A series of 3-benzazepines, which include SK&F 103,829, **17**, and SK&F R-83566, **18**, increase LESP in the dog by a stimulation of 5-HT receptors. This activity was blocked by the 5-HT_2 receptor antagonist, ketanserin (107).

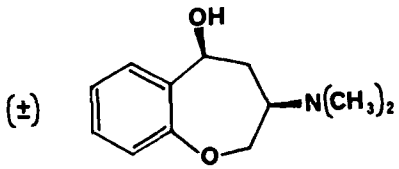


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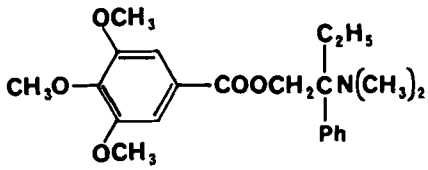


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The benzoxepine-5-ol derivative (KC 2450, exepanol, **19**), has peripheral muscarinic agonist like activity (108) and has been shown to increase the amplitude of gastric motility and of phasic activity in the rat stomach (109), and also to increase LESP in anesthetised dogs (110). *In vitro*, KC 2450 demonstrated equal affinity with acetylcholine for muscarinic binding sites; it was postulated that it acts by a dual mechanism, a direct action on smooth muscle and *via* a local enteric neuron (110).



19



20

Trimebutine, **20**, has been shown to be effective in the treatment of irritable bowel syndrome (IBS) (111) and dyspepsia (112). Inhibition of Phase 3 (P3) of the migrating motor complex in the lower gut has been associated with clinical symptoms of IBS (113). Trimebutine has been shown to induce the P3 migrating motor complex in man, possibly by acting at opioid receptors (114).

Conclusion - There are a multitude of test systems for assessing a compound's potential to stimulate gastrointestinal motility. Many compounds, for example dopamine D_2 and 5-HT_3 receptor antagonists, are active in only a few systems, whereas others, such as metoclopramide, cisapride and BRL 24924, consistently show good activity in many more. It is only when more compounds are progressed to the clinic that the relative merits of the test systems will emerge.

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Chapter 22. Luteinizing Hormone Releasing Hormone Analogs In Control of Fertility and Gonadal Hormone Dependent Disease

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Introduction - Luteinizing hormone releasing hormone (LHRH, GnRH, pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2) is released in a pulsatile manner from the hypothalamus and stimulates the secretion of luteinizing hormone and follicle stimulating hormone from the pituitary. LHRH has diagnostic and profertility applications, but the preponderance of therapeutic uses (1,2) for LHRH analogs depends on gonadal suppression. Occupancy of 10% of LHRH receptors (3) on a gonadotrope can stimulate maximal gonadotropin release, but high and continued receptor activation results in tachyphylaxis (4). LHRH antagonists compete with endogenous LHRH for receptors but do not release gonadotropins. Earlier developments in the chemistry and pharmacology of LHRH analogs have been reviewed (1,2,5-8). This chapter covers developments since 1984, with additional material for perspective.

Chemistry

Agonists - The LHRH agonists in clinical development (1) have a relatively hydrophobic, D-amino acid in position 6 and (usually) a further substitution in position 10 (Table 1). These correspond to known catabolic sites at 5-6 (9) and at 9-10 (10), and might result in slower degradation. However, the potency of these analogs also correlates with their *in vitro* receptor affinities (11) and with their hydrophobicities (12). Polypeptide hormones may bind to the phospholipid membrane prior to the occupation of membrane-bound receptors (13) and more hydrophobic analogs might do so more readily (8). The most potent LHRH analogs (nafarelin and **7**) are also the most hydrophobic (14). These analogs are more potent *in vivo* than predicted from *in vitro* data due in part to a prolonged $t_{1/2}$ attributed to "hydrophobic depoting" in the body.

A class of positively charged amino acids [N^G,$N^{G'}$-dialkylhomoarginines, hArg(R_2)] designed for interaction with the negatively charged phospholipid membrane (15) (see under Antagonists) was a very effective substitution (16). While [D-hArg(Et_2)^6,Pro^9-NHEt]LHRH and [D-hArg-(CH_2CF_3)$_2^6$,Pro^9-NHEt]LHRH, 140X and 180X LHRH potency (17), respectively, are among the most potent LHRH agonists known, they are far less hydrophobic than the previous agonists of similar potency. Facilitation of membrane binding may result in higher receptor affinity and "hydrophilic depoting" (15) in the body.

The incorporation of L-Asp through the ß-carboxyl function generated the only superactive L-amino acid^6 analogs (i.e. [ß-Asp(α-OMe)^6,Pro^9-NHEt]LHRH and [ß-Asp(α-OBzl)^6,Pro^9-NHEt]LHRH, 12-20X LHRH potency) (18). Chimeric analogs bearing elements of LHRH and substitutions found in the corresponding fish and fowl hormones (2) were prepared (19). Full LHRH potency was found for [His^5]LHRH and [His^5,Trp^7]LHRH.

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Table 1. LHRH Agonists in Clinical Development[a]

No.	Structure	Name	Use[b]	Route
1	[D-Leu^6,Pro^9-NHEt]LHRH	leuprorelin	P	injection
2	[D-Ser(tBu)^6,Pro^9-NHEt]LHRH	buserelin	P E,L,PP	nasal ; injection nasal; implant injection
3	[D-Ser(tBu)^6,aza-Gly^{10}]LHRH	goserelin	P B	rod
4	[D-Trp^6]LHRH	tryptorelin	P,PP	microspheres
5	[D-His(Bzl)^6,Pro^9-NHEt]LHRH	histrelin	E	nasal
6	[D-Nal(2)^6]LHRH[c]	nafarelin	E L,PP, IVF	nasal; microspheres
7	[D-Nal(2)^6,aza-Gly^{10}]LHRH		V	implants

[a]Compounds listed are in clinical development or on sale in some country. They are listed in order of potency (data from animal models) from lowest (1; 20X LHRH) to highest (7; ∿220X LHRH).
[b]Applications for which clinical trials have been performed. P, prostatic carcinoma; B, breast cancer; E, endometriosis; L, leiomyomata; PP, precocious puberty; IVF, in vitro fertilization; V, veterinary.
[c]Nal(2), 3-(2-naphthylalanine).

Antagonists - Following the discovery that the replacement of the His^2 residue of LHRH led to antagonists, extensive structure-activity studies resulted in a class of potent, extremely hydrophobic analogs (e.g. 8, Table 2). Such structures have substitutions at multiple sites (1,2,6) which can be analyzed as follows: position 1, N-blocked amino acid, typically hydrophobic, which protects N-terminus from proteolysis and modulates global hydrophobicity of molecule; position 2, D-aromatic amino acid which determines antagonistic character of molecule; position 3, aromatic D-amino acid which removes residual agonistic activity, blocks proteolysis and modulates the global hydrophobicity; position 6, aromatic D-amino acid which blocks proteolysis and increases global hydrophobicity; position 10, blockade of proteolysis, most successfully by D-Ala but aza-Gly and -NHEt are occasionally useful.

Amino acids with charged side chains were introduced into position 6 for increased water solubility (20). Surprisingly 9, with a positively charged side chain replacing the very hydrophobic, aromatic side chain typically seen at this position, had high potency and a prolonged duration of action (21). Potency was further improved (22,23) by the incorporation of the hydrophobic D-Nal(2) into position 1, e.g. 10, 11 (entered clinical trials). Further potency increases (12, 13) resulted from substitution of Leu^7 by the more hydrophobic Trp (24) or Phe (25), following the pattern of chicken LHRH (form II). Amide bond replacement in an analog of 13 by $\psi[CH_2NH]$ greatly reduced potency (26).

The positively charged "D-Arg^6 class" was hypothesized to prolong

Table 2. Representative LHRH Antagonists

No.	Compound	AO ED_{50} µg/animal	MCD EC_{50} µg/ml
8	[N-Ac-Pro^1,D-pF-Phe^2,D-Nal(2)^{3,6}]LHRH	3.3	
9	[N-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]LHRH	1.8	
10	[N-Ac-D-Nal(2)^1,D-pF-Phe^2,D-Trp^3,D-Arg^6]LHRH	2.4	
11	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]LHRH	<1	
12	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Pal(3)^3,D-Arg^6,Trp^7,D-Ala^{10}]LHRH	<0.25	
13	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Trp^3,D-Arg^6,Phe^7,D-Ala^{10}]LHRH	<0.5	
14	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Trp^3,D-hArg(Et$_2$)^6,D-Ala^{10}]LHRH	0.6	0.2
15	[N-Ac-D-Nal(2)^1,D-α-Me,pCl-Phe^2,D-Trp^3,D-Arg^5,D-Tyr^6,D-Ala^{10}]LHRH	<2.5	3.7
16	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Pal(3)^3,Arg^5,D-Glu(pMeOPh)^6,D-Ala^{10}]LHRH	<1.5	1.6
17	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Pal(3)^{3,6},Arg^5,D-Ala^{10}]LHRH	<1.0	2.9
18	[N-Ac-D-Nal(2)^1,D-α-Me,pCl-Phe^2,D-Pal(3)^3,Arg^6,Lys(iPr)^8,D-Ala^{10}]LHRH	<1.0	4.0
19	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Trp^3,D-Lys(iPr)^6,Lys(iPr)^8,D-Ala^{10}]LHRH	<1.0	6.6
20	[N-Ac-D-Nal(2)^1,D-α-Me,pCl-Phe^2,D-Pal(3)^3,D-Arg^6,hArg(Et$_2$)^8, D-Ala^{10}]LHRH	<1.0	4.9
21	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Pal(3)^3,Lys(Nic)^5,D-Lys(Nic)^6, Lys(iPr)^8,D-Ala^{10}]LHRH	<3.0	>300
22	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Pal(3)^3,D-hArg(Et$_2$)^6,hArg(Et$_2$)^8, D-Ala^{10}]LHRH	0.3	13
23	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Pal(3)^{3,6},hArg(Et$_2$)^8,D-Ala^{10}]LHRH	0.6	196
24	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Pal(3)^{3,6},Pal(3)^5,hArg(CH$_2$CF$_3$)$_2^8$, D-Ala^{10}]LHRH	>1.0	>1000

action by depoting on phospholipid membranes (27). A class of amino acids [hArg(R_2)] was designed to enhance this binding through a combination of electrostatic and hydrophobic interactions (15). Further increases in duration of action were demonstrated with 14 (detirelix; $t_{1/2}$ >48 hr, 5 mg dose SC, men).

An important side effect of the "D-Arg^6 class", first noted for 10 at dose levels required for clinical efficacy, was hypotension and edema (28) caused by mast cell degranulation (MCD). Potent LHRH antagonists with decreased MCD became a major focus of synthetic efforts (6). Hydrophobicity and multiple positive charges are associated with potent MCD (29), so analogs with increased distance between the native Arg^8 residue and the D-Arg^6 substitution were prepared. The transposition to Arg^5, D-Tyr^6 yielded 15, with ∿40 fold lower MCD EC_{50} (30) than 11 but lower antiovulatory activity (AOA) as well. The more hydrophilic Pal(3)^3 and more hydrophobic Trp^6 substitutions yielded an analog with higher AOA and lower MCD potency (30). A related series of analogs (31) resulted in 16, which has low MCD potency and is in clinical trials. A change to Pal(3)^6 resulted in a further AOA increase (17) with unaffected MCD (32).

In addition to hArg(R_2), another amino acid thought to have membrane affinity is N^{ε}-isopropyl-lysine [Lys(iPr)] (33). Analogs containing Lys(iPr), designed to improve gut wall transit, showed high potency with low MCD (18, 19). Changing to D-hArg(Et$_2$)^8 gave an analog with a similar potency/side effect profile (20) (33). The combination of Lys(iPr)^8 with a side chain modified amino acid, N^{ε}-nicotinyl-Lys [Lys(Nic)], yielded 21, which has a very low MCD (34) but somewhat decreased AOA (clinical trial). *In situ* reductive alkylation of Lys in [N-Ac-D-Nal(2)^1, D-pCl-Phe^{2,3},D-Lys^6,Phe^7,D-Ala^{10}]LHRH produced analogs (35) with significantly reduced MCD for one substitution [Lys(pClBzl)^6] but also with decreased AOA. Extension to 6,8-disubstitution produced analogs with much reduced MCD but with similarly lowered AOA (36).

The Arg-Pro sequence is common to peptides with potent MCD activity (37), suggesting that Arg^8 is pivotal for this side effect. Study (38) of $hArg(R_2)^8$ substitutions resulted in 22 and 23, with reduced MCD potency and high AOA. Compound 24 has a position 8 substitution with reduced basicity and had exceedingly low MCD potency. The decreased MCD potency of $hArg(R_2)^8$ or $Lys(iPr)^8$ substituted analogs is probably related to steric hindrance of the basic N-function. Compounds 22 and 23 are in advanced pharmacology studies.

Comparative Pharmacology

Agonists - Continual treatment with potent LHRH agonists elicits a decreasing stimulatory response and results within 4 weeks in complete tachyphylaxis to production of biologically active gonadotropins. The resulting reversible "medical castration" is desired in several therapeutic situations (prostatic cancer, precocious puberty, endometriosis, in vitro fertilization programs, premenstrual syndrome, breast cancer, etc.). While the initial stimulatory phase is frequently unimportant, in metastatic cancer the associated "flare" may restrict agonist use (39). Pre-pubertal treatment causes no symptomatic stimulation and puberty may be postponed indefinitely (40). Starting treatment in the mid-luteal phase also minimizes hyperestrogenicity (41). The hyperprolactinemia of lactation prevents symptomatic stimulation, and this may be a good time to initiate contraceptive use of LHRH agonists (40,42).

During this hyperstimulated, tachyphylactic state there is continued secretion of immunologically detectable but non-biologically active gonadotropin (43,44), apparently due to the free α-subunit (45,46). High α-subunit production is reported for other hyperstimulatory/hypersecretory states such as primary gonadal failure and gonadotropinoma (47). Continuous LHRH treatment is associated with elevated α-subunit but lowered ß-LH subunit mRNA in pituitary cells (48). These observations may account for the hyper- and neoplastic changes seen in the pituitaries of rats and mice on lifetime toxicity studies.

Antagonists - LHRH antagonistic analogs compete with endogenous LHRH for its receptor and therefore must be constantly present to successfully block gonadotrope function. They do not exhibit a stimulatory phase and do not cause receptor down-regulation (49). Profound pituitary and gonadal suppression is seen within hours. Lowering gonadal steroid output with LHRH antagonists may increase endogenous LHRH secretion (8). The minimal effective single dose for 24 hours suppression thus may not suppress chronically. Lack of receptor down-regulation and increased LHRH synthesis may be reflected by the more rapid return of reproductive function following antagonist than agonist treatment (40) and a marked overshoot of testosterone and sperm levels (50).

Formulations

The nasal bioavailability (BA) of LHRH from aqueous solutions is about 1% (51-54). Absorption of nafarelin was dose and concentration dependent (54). At ∿2 mg/mL, a 400 µg intranasal (IN) dose of nafarelin has BA of 5 to 8% (54). Because of the low BA and the limited IN dose, high potency is required for gonadal suppression by this route. Thus, while the most potent analogs can be used as solely nasal therapy in endometriosis (55), uterine leiomyomata (56) and precocious puberty (57), therapy with less potent analogs must be initiated by daily injection (58). Addition of 5% α-cyclodextrin to an aqueous solution of

leuprorelin increases absorption 2-3.7 fold in rats, dogs and humans (59). Sodium glycocholate (a bile acid salt), EDTA, Brij 56® and Azone™ all significantly enhanced absorption of nafarelin in rhesus monkeys (60) and an "enhanced" nasal formulation (1% sodium glycocholate) was tested in prostatic cancer patients (61).

A limiting factor for BA and total dose administered is the solubility of the compound. Solid (powder) formulations of nafarelin were studied (62) and an equivalent BA compared to the optimized aqueous formulation was achieved using a powder formulation of 1/2 to 2/3 the dose.

Controlled Release - Controlled release of peptides was reviewed (63). Goserelin (3-6 mg), formulated for once monthly treatment as a combination with a 1:1 copolymer of DL-lactic and glycolic acids (PLGA) and extruded as a rod for subcutaneous implantation (64), was tested for premenopausal breast cancer (65) and is sold for prostatic cancer (66).

Injectable microspheres (∿40 μ) were developed for nafarelin (1% in a 45:55 copolymer of PLGA) and were more effective than daily injection (67). At 2-7% loading, more prompt, sustained (2 mo.) gonadal suppression was found (68). Tryptorelin incorporated into microspheres (28 day duration) by the above process is sold in France for precocious puberty (69,70).

A 3-5 month PLGA implant containing buserelin has been described (71). Polyorthoester systems which surface degrade at physiological pH provide close to linear release (72). Porous capsules containing [D-Trp^{6},Pro^{9}-NHEt]LHRH released 20-400 μg/day for 125 days (63).

Two non-biodegradable, very long-term controlled delivery devices (silastic matrix and hydrogel reservoir) incorporate [D-Nal(2)^{6}, aza-Gly^{10}]LHRH (73). Both systems deliver >16 μg per day for at least a year when loaded with 20 mg of peptide.

Clinical Applications

Cancer - Three LHRH agonists are commercially available for prostatic cancer. Suppression of the flare and adrenal androgens by combination treatment with antiandrogens (e.g., flutamide) was proposed (74), but a recent double blind trial showed only a modest increase in mean time to progression (from 12 to 14 mos.) (75).

Remission rates in premenopausal breast cancer patients treated with LHRH agonists approximate those from oophorectomy and correlate with the expression of estrogen receptors (76,77). Combination treatment with the antiestrogen, tamoxifen, may be deleterious (78).

Early reports suggesting that gonadal suppression could protect against the testiculotoxicity of cancer chemotherapy were not borne out in animal or clinical trials (79). Preliminary studies with LHRH antagonists also were not successful.

Non-malignant disease - Phase III studies with LHRH agonists in endometriosis are being completed and NDA filings are imminent. The peptides are as efficacious as danazol, and lack the side effects of the steroid (80,81). Endometriosis presents as severe pelvic pain and/or unexplained infertility. During LHRH agonist treatment the pain relief is dramatic,

and post-treatment conception rates are at least equivalent to danazol (81). Over the course of 6 mo. treatment, the estrogen withdrawal causes decreased bone density (0-6%) (82,83), but a return to normal within 6 to 12 mos. of stopping treatment (84) is seen.

Reports that these symptoms at true menopause can be minimized by progestogen therapy led to the evaluation of combination therapy for both endometriosis and uterine leiomyomata. Medroxyprogesterone acetate (85, 86) negates both beneficial and adverse effects of LHRH agonists, but norethindrone minimized hot flashes and bone changes without affecting therapeutic efficacy (87).

Rapid regression of uterine leiomyomata (fibroids) is seen with LHRH agonist treatment (1), but the rapid post-treatment regrowth indicates the major use will be to shrink fibroids prior to surgery (88).

LHRH agonists have been studied in hundreds of precocious puberty patients for up to 7 years (89). They cause regression of pubertal signs in both sexes and normalization of growth rate and of adult height (90). They are effective only for central precocious puberty; the adrenal syndrome (McCune-Albright) is unresponsive (91).

In benign prostatic hypertrophy (92-94), regression of prostate size was noted (95,96), and some patients had symptomatic relief (increased urinary flow). Hot flashes and impotence were common, however, and the acceptability of surgery suggests that the LHRH analogs would be prescribed only for high surgical risk patients.

Uncommon conditions associated with cyclical menstrual changes (97) are alleviated by LHRH agonists (acute intermittent porphyria, meno/metrorrhagia, menstrual related migraines, and progressive cyclical luteal hearing loss). Premenstrual syndrome was blocked by LHRH agonist (98). LHRH agonists regress hirsutism in women (99), often a symptomatic presentation of polycystic ovarian disease (PCO). Normal menstrual and ovulatory cyclicity was not restored in PCO, but cosmetic improvement from lowered ovarian androgens is valuable.

Contraception - While LHRH agonists are effective female contraceptives at low doses without marked ovarian suppression (100,101), the resulting unopposed estrogen state raised concerns of possible endometrial hyper- and neoplasia (102). Combination regimens with progestogens were evaluated (103), but advantages over existing contraceptive methodology are not clear. WHO sponsored trials are underway using the agonists for 3 to 6 months in nursing mothers (42), in whom lactational amenorrhea is already common. Agonists were studied as interceptives (luteolysis), but the effect was blocked by chorionic gonadotropin. A similar use of LHRH antagonists alone (104) or with a prostaglandin (105) looks favorable and should reach clinical trial.

Early hopes that the agonist analogs would be effective male contraceptives receded when concomitant administration of testosterone, necessary to prevent the hot flashes and impotence that follow use of the agonist analogs alone, interfered with the establishment of azoospermia. Since then the dose level has been increased, continuous administration from depot preparations has been utilized, and the duration of treatment extended, each with positive results (106,107). Even in the presence of testosterone supplementation continuous administration for at least a year results in azoospermia in all subjects (108). However, such a dosage schedule would not appear to generate widespread use.

The LHRH antagonists may have a better prognosis. The favorable pharmacokinetics noted for newer antagonists, resulting in lower doses required to block pituitary function in men, increases the probability of success (109). Animal studies suggest that the required supplementation with testosterone does slow the evolution of azoospermia, but this state has routinely been reached within 2-3 spermatogenic cycles (110,111).

Profertility - Pituitary suppression by LHRH agonists coupled with exogenous gonadotropins is being studied in secondary infertility. Increased fertility and conception rates have been found during in vitro fertilization/ovum transfer procedures and in infertility syndromes (112) such as PCO and oligomenorrhea (idiopathic or chemotherapy), particularly in clomiphene-insensitive patients. The rapidity of LHRH antagonist action may be preferred for these indications.

Diagnostic Uses - Injectable synthetic LHRH (Factrel) is used for the differentiation between hypothalamic or hypophyseal/gonadal infertility. While IV administration of LHRH to amenorrheic patients was of no help for classification or prediction of outcome of LHRH treatment (113), a single injection of nafarelin provided a rapid test for pituitary and gonadal function in sexual precocity (114).

An LHRH antagonist was predictive of the degree of calcium loss by individual monkeys when they were subsequently ovariectomized (115). A short course of treatment premenopausally to diagnose the predisposition to postmenopausal osteoporosis in women might be useful since its incidence and severity shows individual variation. High risk women would most benefit from early hormonal replacement therapy.

Conclusions - LHRH agonists are available for treatment of prostatic cancer in several countries, and approval for endometriosis, precocious puberty and uterine leiomyomata is expected over the next two years. Widespread use for contraception does not seem likely. The application of antagonists in some niches not occupied by agonists, for example, for male contraception and pregnancy termination, appears feasible. While the primary clinical focus for the last ten years has been the agonists, the next period will see a concentration on the clinical evaluation of LHRH antagonists.

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Section V - Topics in Biology

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Chapter 23. Structure and Function of G-Protein Coupled Receptors

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Introduction - Many hormones and cellular mediators exert their influence on target cells through interactions with cell surface receptors. The mechanism of action of receptors for a number of different hormones have suggested that these receptors could be grouped into families that utilize common systems for their signal transmission. These classes include the growth factor receptors (EGF, insulin) (1), the transporter receptors which internalize their ligands (LDL, transferrin) (2), ion channels (nicotinic acetylcholine, GABA) (3), and receptors linked to second messenger production through guanine nucleotide binding regulatory proteins (G-proteins; rhodopsin, adrenergic, cholinergic) (4). Further support for the grouping of receptors into families has come from the recent cloning of the genes for a variety of receptors in each of the functional families (1-4). In general, those receptors which use a common signaling pathway have similar structures and share significant amino acid similarity. Thus, it is becoming possible to deduce for a putative receptor of unknown function both the class of ligands bound by the receptor and the mechanism of signal transduction used based on its amino acid sequence. Receptors which couple to G-proteins have been cloned and possess structural and functional relatedness as a family (4). In this review, we will discuss the recent data which support this conclusion and present a model that predicts the ligand binding site, G-protein interaction site, and sites involved in desensitization of these receptors. This model should prove useful for the medicinal chemist attempting to rationally design drugs which will interact with members of this receptor class.

Overview of G-Protein Coupled Receptor Action - The components involved in signal transduction mediated by G-protein coupled receptors are shown in figure 1, with each of the components located relative to the membrane bilayer.

The first step in this signaling pathway is the interaction of an extracellular message with a receptor which spans the membrane. Table 1 presents a partial list of those hormones that have been shown to use G-protein coupled receptors. Those receptors whose primary sequence has been determined are indicated by reference numbers in parentheses.

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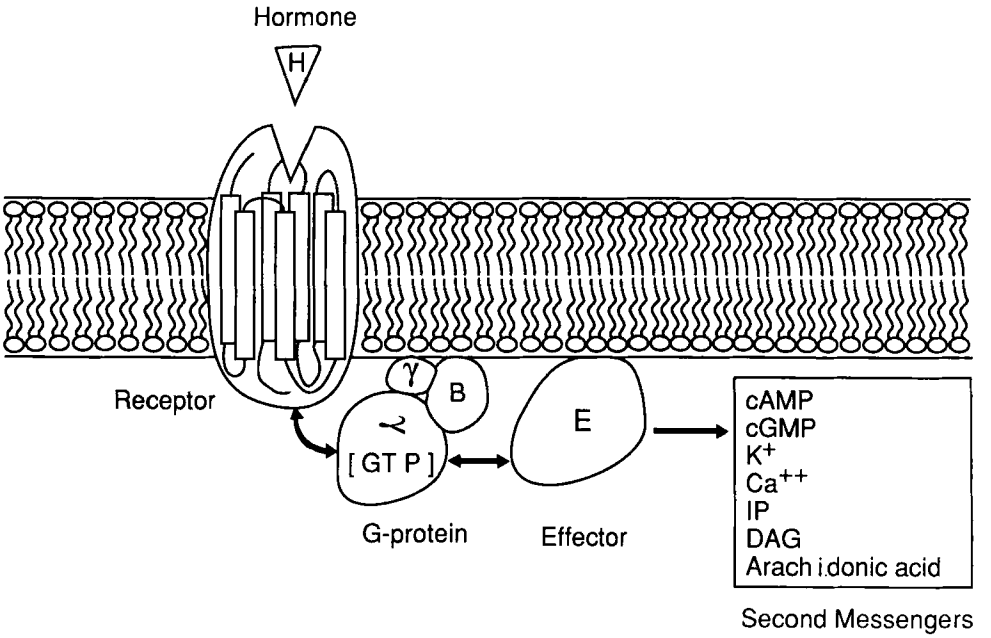


Figure 1. Schematic Representation of Signal Transduction Pathway.

Table 1. G-Protein Coupled Receptors

Small Molecule	Peptide	Sensory
α-Adrenergic (5)	Enkephalin	Rhodopsin (14,15)
β-Adrenergic (6-8)	Endorphin	Olfactory
Muscarinic Cholinergic (9-13)	Cholecystokinin	
Dopamine	Bombesin	
Histamine	Substance K (16)	
Adenosine	Substance P	
Serotonin	Neuromedin	
Prostaglandin	Bradykinin	
Leukotriene	fMetLeuPhe	
Thromboxane	C5a,C3a	
Prostacyclin	Thrombin	
PAF	Vasopressin	
cAMP	Oxytocin	
	Angiotensin II	
	VIP	
	Parathyroid Hormone	
	Calcitonin	
	Neurotensin	
	TRH	
	Somatostatin	

Once bound to an agonist the ligand receptor complex becomes activated. Antagonist ligands do not activate the receptor. The details of receptor activation are unknown, but probably involve conformational changes within the receptor. A number of conformational intermediates of the visual opsins have been identified following the light catalyzed isomerization of 11-cis-retinal to all-trans-retinal (14, 15). By analogy, the same type of changes may occur in other G-protein linked receptors. Following hormone binding, the activated receptor interacts with a G-protein leading to activation of that G-protein (17, see chapter 24). The G-protein then interacts with one of a variety of effectors to cause a change in the intracellular level of a second messenger (17). While the best studied effector system is the hormone sensitive adenylyl cyclase (18), several other systems have been shown to require G-proteins for activation including phospholipases A_2 and C (17, 19, 20), some ion channels (17, 21-23), and the cGMP phosphodiesterase (24). The diversity of the effectors that are modulated through a G-protein pathway allows for the control of the intracellular levels of a wide variety of second messengers, as indicated in figure 1. Most of the pathways that have been identified to use G-proteins exhibit a unidirectional modulation, i.e. the effector is either stimulated or inhibited following agonist stimulation of the receptor. Adenylyl cyclase can be either stimulated to raise the intracellular level of cAMP or inhibited by G-proteins to lower the level of cAMP depending on which receptor is activated (18). Whether this dual control is operative for other pathways will require further investigation. In addition several receptors have been proposed to interact with more than one pathway. The muscarinic cholinergic receptors have been demostrated to inhibit adenylyl cyclase, stimulate phospholipase C and modulate K^+ channels (25). While this divergence of responses are due in part to different subtypes of the receptor which interact with different G-proteins, it was recently demonstrated that at least one of the subtypes (M2) can interact with two different pathways, presumably by interacting with two different G-proteins (26).

Many of the components of these pathways have been identified and the steps involved in activation of each of the components are becoming clear; however, virtually nothing is known about the processes that transmit the signal from one component to the other. The fact that signal transduction systems as varied as photoreception and hormone stimulation of adenylyl cyclase use very similar pathways led to the idea that there might be structural and functional similarity between the individual components of the different pathways (4). In the cases of the stimulatory and inhibitory G-proteins and the visual transduction system this suggestion was proven by the demonstration *in vitro* that purified G_i could interact with the ß-adrenergic receptor (ßAR, normally a G_s coupled receptor) (27) and rhodopsin (28), and that transducin could interact with the α_2-adrenergic receptor (α_2AR, normally a G_i coupled receptor) (28). Therefore, the interactions required for a receptor to activate its homologous G-protein could be mimicked by an heterologous combination. This observation foreshadowed the later determination of structural similarity among the receptors of these signaling pathways and supported the *in vivo* observations described above for the muscarinic receptors.

Structure of G-Protein Coupled Receptors - The first G-protein linked hormone receptor genes to be cloned were for the β_1 and β_2 adrenergic receptors from turkey and hamster, respectively (6, 7). The predicted amino acid sequences for these proteins were found to share significant sequence similarity. Based on hydrophobicity and hydrophobic moment plots, and secondary structure analysis these receptors were predicted to

have very similar overall structures. An initial surprise was that both proteins also share significant amino acid sequence similarity with the visual opsins (4, 6, 7). Subsequently, the mammalian β_1- (8) and α_2- (5) adrenergic receptor genes, four muscarinic cholinergic receptor genes (9-13), and substance K receptor gene (16) have been cloned and their predicted protein sequences were shown to be similar both to each other and to the visual opsins. It should be noted that cloning of the genes for known receptors has resulted in the identification of previously unknown subtypes of these receptors. Screening of libraries with the known M1 and M2 muscarinic cholinergic gene probes revealed the existance of two new muscarinic receptor subtypes (12, 13). Identification of additional sequence related to the α_2AR suggests that novel subtypes of this receptor may also exist (5). In all cases examined, receptor subtypes which are identified on the basis of pharmacological differences have proven to be due to the presence of different gene products.

The proposed transmembrane topologies for those G-protein linked receptors whose primary sequences have been determined are schematicaly shown in figure 2. The horizontal lines represent the boundaries of the membrane bilayer. The cylinders represent the proposed hydrophobic transmembrane α-helices, while the thin lines represent the hydrophilic residues connecting the transmembrane domains. The extentions from the N-terminal region are carbohydrate residues. Each of the classes of receptors are shown relative to the G-protein to which they couple. The G-protein used by the substance K receptor is currently unknown ($G_?$).

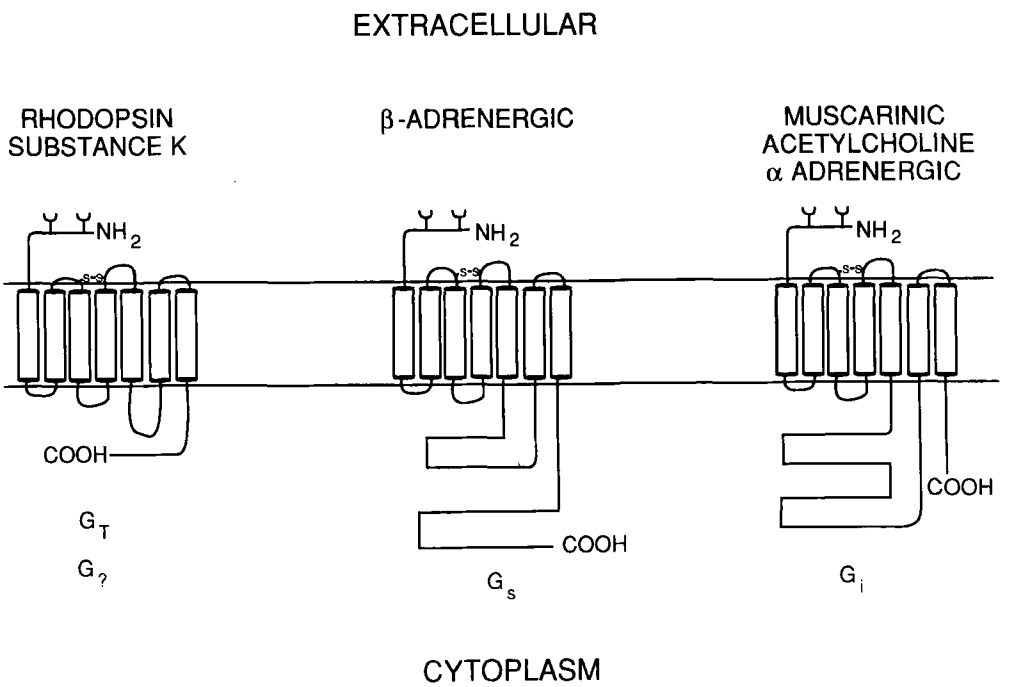


Figure 2. Schematic Diagram of G-Protein Coupled Receptor Structure.

All of the proteins have seven regions comprised of hydrophobic amino acids that would be predicted to form transmembranous alpha helices, connected by short stretches of hydrophilic residues. The greatest degree of amino acid similarity among the receptors is in the transmembrane helices. This sequence similarity varies from 20-90% with the greatest conservation of sequence observed between subtypes of a particular receptor class (4, 11). In contrast, very little sequence homology is found in the loop regions connecting the transmembrane helices.

Several other interesting structural features are conserved among the receptor proteins. All of the proteins contain between one and four consensus sites for N-linked sugar addition. Only the sites present at the N-terminus of the protein are glycosylated in rhodopsin and the βAR (29, 30). The role of the added carbohydrate is unclear as removal of the sugar, enzymatically or by mutation, does not affect the function of the protein (31-33), although it may affect the trafficing of the protein through the cell (15). All of the receptors contain proline residues at conserved positions within the predicted transmembrane regions. These residues have been postulated to be involved in distorting the helical structure to allow for the binding of ligands (15, 17, 32). One of these proline residues in the βAR is required for correct protein folding and processing (32). A number of charged residues are present within the hydrophobic regions which are conserved among the various proteins and may be involved in the binding of ligands to the receptor or in the determination of the absorption spectrum of retinal in rhodopsin (14, 15, 34).

The major structural differences between the receptors are the variable sizes of the third intracellular domain and the C-terminal domain. Interestingly, the sizes of these regions seem to be similar for those receptors which interact with the same G-protein and different for those interacting with other G-proteins. The receptors which couple to Gs have a relatively smaller third intracellular loop and a long C-terminal domain. Those that couple to Gi have large third intracellular loops and short C-terminal domains. Rhodopsin which couples to transducin and the substance K receptor which couples to an as yet undescribed G-protein have small third intracellular loops and short C-termini. The generality of this observation will await the determination of additional receptor sequences. This observation however suggests that these internal regions may determine the specificity of the receptor:G-protein interaction.

The structure of rhodopsin is biochemically the best characterized. Protease protection and chemical probe experiments have determined the location of individual residues relative to the membrane. Low angle X-ray and neutron diffraction studies have indicated that the bulk of the protein is contained within the membrane bilayer (35, 36). The protein has been found to possess 50-60% alpha helical content and spectroscopic data suggest that the helices are oriented perpendicular to the membrane (37-39). This data, coupled with structural predictions using hydrophobicity and hydrophobic moment plots, supports the seven transmembrane helix model (14, 15). However, a detailed protein structure from X-ray crystallography or NMR has not been obtained. The structures suggested for the other receptors are based primarily on amino acid sequence and predicted structural similarity to rhodopsin. Recently, protease protection experiments have been performed by several groups on the βAR. Using partial protease and endoglycosidase treatment,

it has been demonstrated that the N-terminus of the βAR is extracellular and that the C-terminus is intracellular (30). Proteolytic cleavage sites were found within two of the regions predicted to be hydrophilic loops. These data suggest that the receptor passes through the membrane at least once (although the authors speculate more transmembrane passages) and that it is oriented similarly to rhodopsin with respect to the membrane. Limit proteolysis has been used to define the structure of the avian β_1 adrenergic receptor (40). Two protease resistant domains were identified following extensive digestion of the receptor. One composed of the first five hydrophobic regions and the second composed of helices six and seven. Similar results have been obtained following proteolytic digestion of rhodopsin, supporting the suggested similarity between the protein structures (41). The proteolysis experiments are consistent with the proposed positions of the N-terminus, C-terminus and at least two of the predicted interhelical loops are oriented in the membrane as predicted by the model. The determination of the location and distribution of the remainder of the protein will await further detailed biochemical analysis.

The structures depicted in figure 2 are shown with a single disulfide bond between outer loops 2 and 3. Definitive biochemical support for the location of this bond is currently lacking. The two cysteine residues that would be involved in this bridge are conserved in all of the receptors and opsins (5-17) and genetic removal of either or both of them from the βAR results in the identical dramatic alteration in ligand binding by the receptor (32). The importance of disulfide bonds in both the structure and function of the βAR has been demonstrated by several groups where reduction of the receptor alters its electrophoretic mobility and acts to augment the ability of agonists to activate the receptor (42). A suggestion has been made that rearrangement of intramolecular disulfide bridges may be involved in receptor activation (40, 42, 43).

Stucture-Function Relationships of G-Protein Coupled Receptors - A combination of biochemical and genetic analysis has allowed the identification of the ligand binding site, sites of G-protein interaction, and regulatory sites on several of the G-protein linked receptors. Again, rhodopsin is biochemically the best studied receptor of this class. Several studies have determined that the ligand 11-cis-retinal is bound to the apoprotein opsin through the formation of a Schiff's base with lysine 296, which is within the seventh hydrophobic transmembrane helix (44). The bound ligand interchelates between the other transmembrane helices and is believed to lie within a helical bundle parallel to the plane of the membrane(45). While it is clear that a number of conformationally different states of rhodopsin arise during the activation and regeneration stages of the photocycle, little is known about the details of these different states (14, 15). By analogy with rhodopsin, it has been proposed that the ligand binding sites of other G-protein linked receptors will also involve the transmembrane helices (46).

Genetic and biochemical analysis of the βAR support this model. Figure 3 summarizes what is known about the structural requirements for ligand binding to the βAR (32). The individual amino acid residues of the hamster β_2AR are indicated using the single letter amino acid code. The solid squares indicate the location of N-linked carbohydrate addition.

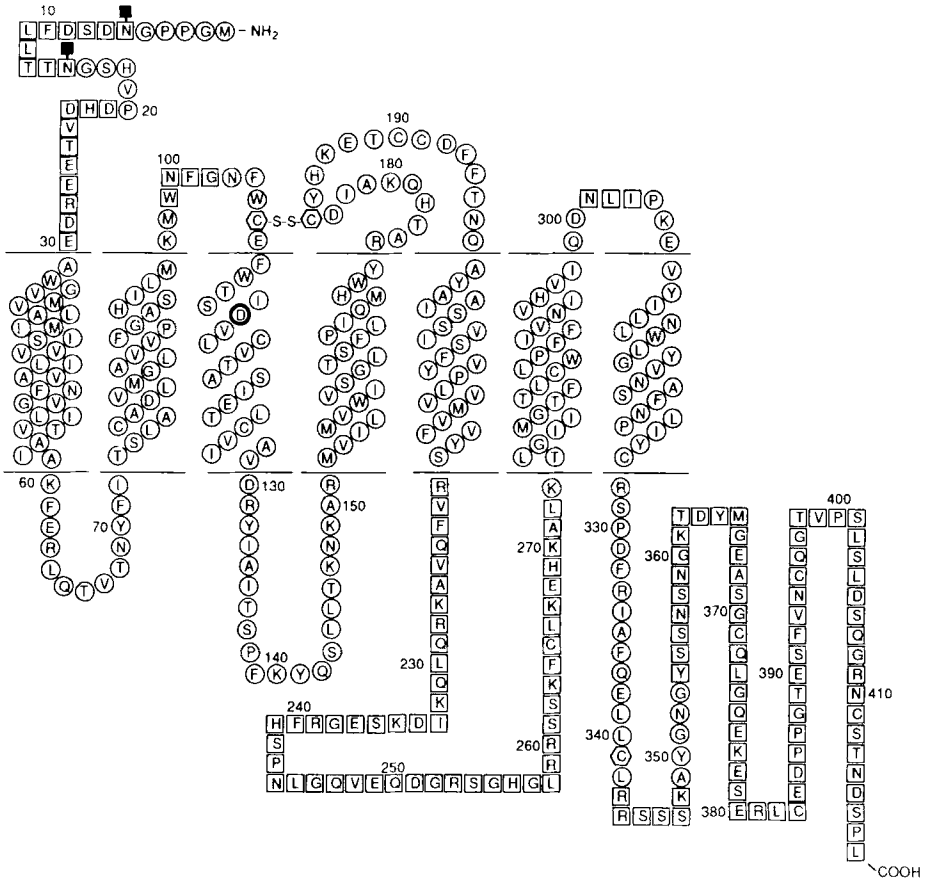


Figure 3. Proposed Transmembrane Toplogy of the Hamster β_2-Adrenergic Receptor (from ref. 32 with permission).

Extensive deletion mutagenesis has revealed that most of the hydrophilic loop segments between the transmembrane helices may be removed without affecting the ability of the receptor to bind ligands (32, 46). The removal of any one of the transmembrane domains, however, resulted in a loss of ligand binding (32, 46). Those residues shown in figure 3 as open squares can be removed without altering the ligand binding properties of the receptor. The remainder have either not been tested or resulted in improperly folded protein. Similarly, limit proteolysis of the βAR which results in removal of the N-terminus, the C-terminus and the large third internal loop did not alter the ligand binding properties of the proteolyzed receptor (40).

A number of single amino acid substitutions in the transmembrane regions were found to affect the ability of the βAR to bind ligands (32, 34). A systematic replacement of charged residues within the transmembrane helices revealed that aspartic acid 113 (shown in the bold-faced circle in figure 3) in the third helix was critically important for ligand binding, while aspartic acid 79 in the second helix was important only for the binding of agonists, but not for antagonists (34). Replacement of other charged residues either had no effect on

ligand binding or affected the folding of the protein. Replacement of residues in the seventh helix also affected agonist and antagonist binding. No single amino acid substitutions outside of the transmembrane helices (with the exception of the two cysteines shown as hexagons in figure 3 and postulated to form a disulfide bond between outer loops 1 and 2) have been found which affect the ligand binding ability of the βAR (32). It should be noted that there are mutations which remove portions of the intracellular loops and appear to increase the binding affinity of agonists for the receptor (46, 47). These mutations, however, uncouple the receptor from the G-protein and are believed to affect ligand binding indirectly. Conversely, the mutations described above that are within the helices do not affect the coupling of the receptor to G-proteins. Those mutant receptors which have altered agonist binding properties still exhibit decreases in agonist affinity in the presence of GTP (47). These data, therefore, support a model where the transmembrane helices not only provide a structural framework for the receptor, but are, in fact, the ligand binding domain of the receptor. As the transmembrane helices are also the portions of the receptors where the highest degree of sequence similarity is observed, it is proposed that this model will hold true for all of the receptors in this class. Ligand selectivity would then be determined by the limited sequence divergence among the receptors and conformational differences found within the transmembrane regions.

The substitution of aspartic acid residue 113 of the βAR with a variety of other residues dramatically alters the affinity of the βAR for both agonists and antagonists. When substituted by a glutamic acid residue, a 100 fold decrease in affinity was found, while an asparigine substitution exhibited a 10,000 fold decrease (48). As the relative affinities for various agonists and antagonists were unaltered in these mutant proteins, it was proposed that the carboxylate group of the aspartic acid side chain interacts directly with the protonated amine group of the ligand (48).

A proposed model for the ligand binding domain of the βAR receptor is shown in figure 4 (48). This model illustrates the receptor as if viewed from the surface of the membrane. The arrangment of the seven helices is by analogy to opsin. The proposed ionic interaction between the carboxylate group of the aspartic acid 113 side chain with the protonated amine group of epinephrine is indicated. The positioning of the remainder of the ligand is drawn by analogy to the position of retinal when bound to opsin. Therefore like retinal and opsin, the ligand is proposed to lie within a bundle of the seven helices in the plane of the membrane, interacting directly with residues in several of the helices. All of those receptors whose genes have been cloned and which bind cationic amine ligands contain this aspartic acid residue, while the opsins and the substance K receptor do not (4, 5-16). It has been suggested that this residue will be found in other cationic amine binding receptors as the sequences become available (48). By extension, it would be predicted that similar conserved ligand contacts will be found in the receptors for other classes of ligands. Thus, it was proposed that the class of compounds bound by a receptor of unknown function may be predicted from the primary sequence of the receptor protein (48). The use of molecular modeling to describe the receptor-ligand complex should prove useful for the design of new ligands which will take advantage of the structure of the target. The similarity of sequences between subclasses of receptors such as the adrenergic or cholinergic is greatest within the proposed ligand binding

pocket or transmembrane region, while diverging in the extramembranous loops. It might be possible to design new classes of compounds to interact with the divergent regions of the receptors in order to obtain a higher degree of subtype specificity. Likewise, an understanding of the environment around the ligand contacts may allow for the design of tighter binding ligands.

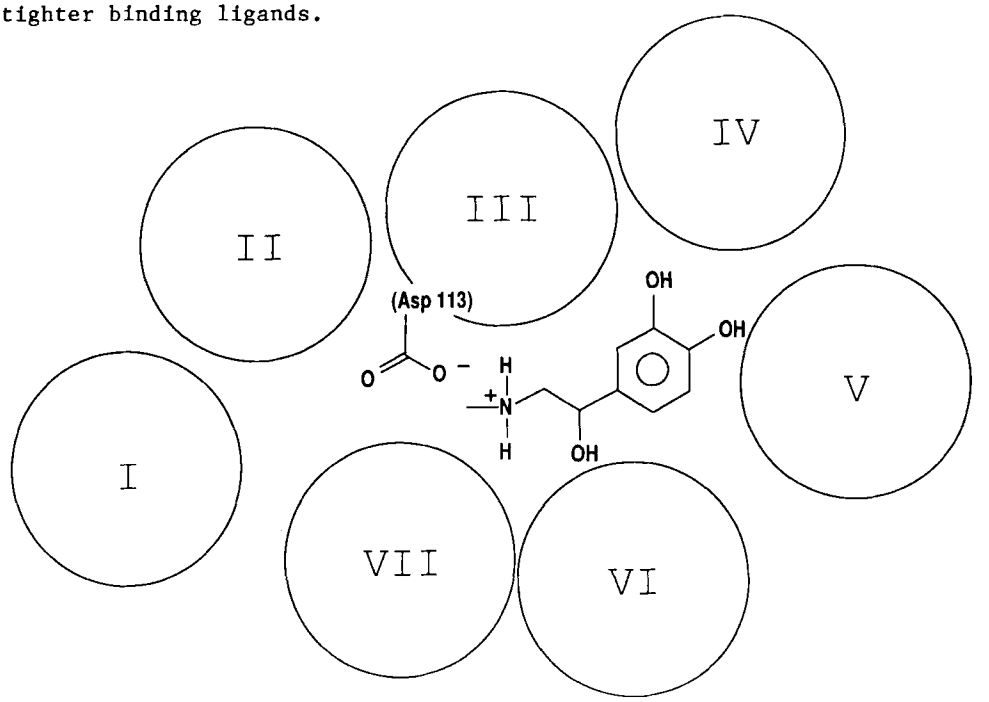


Figure 4. Model for the Ligand Binding Domain of the β-Adrenergic Receptor (from ref. 48 with permission).

Several regions of the receptors have been defined which seem to be important for their interaction with G-proteins. As would be predicted from the structural models shown in figure 2, most of the evidence implicates the proposed internal loop regions of the receptors in G-protein interaction (14, 15). Antibodies directed toward each of the cytoplasmic loop regions of rhodopsin have been prepared (49). Their interaction with rhodopsin bound to its G-protein transducin indicated that internal loops 1 and 2 were inaccessible to the antibodies, while inner loop 3 and the C-terminus were more exposed. In addition, proteolysis of rhodopsin demonstrated that the C-terminus was not essential for interaction with transducin, but that the third cytoplasmic loop was required (41). These data suggest that several regions of the receptor are involved in the contacts between rhodopsin and transducin.

Genetic analysis of the βAR has defined two regions in the third inner loop as being important for G-protein coupling. These regions are located at the boundries of the transmembrane helices and the cytoplasmic domains. In contrast, the outer loops and the C-terminus of the βAR could be removed without destroying the G-protein interactions (46, 47). As mentioned above the sizes of the third intracellular loop and the C-terminus have been suggested to be important for determining the specificity of the receptor:G-protein interaction. Genetic and

proteolytic analysis of the βAR, however, indicated that this is probably not correct, since removing most of the third loop (leaving intact the regions shown to be required for coupling) and C-terminus did not alter the specificity of the interaction (40, 50). An examination of the amino acid sequences predicted to be within the cytoplasmic loops for each of the receptors reveals only limited sequence similarity. The most strikingly conserved region is inner loop 1 where several residues are identical in all of the receptors (4-16). However, substitution of these residues individually did not affect G-protein coupling by the βAR. The regions of inner loop 3 of the βAR which have been demonstrated to be important for coupling are not very similar in sequence to the corresponding regions from other receptors. The only feature that these regions appear to have in common is the predicted ability to form charged amphiphilic alpha helices (47, 50). It has, therefore, been postulated that the distribution of charge along these helices rather than primary sequence is important for activation of the G-proteins (47, 50). The validity of these predictions remains to be fully investigated. If the predictions are correct these regions of the receptor might be used in the design of a new class of receptor antagonists which would specificly disrupt the interactions of the receptor and the G-protein independant of the normal ligand binding site.

Mechanism of Receptor Desensitization - Receptors exhibit a reduction in their responsiveness to further agonist challenge upon prolonged exposure to agonists. This functional desensitization has important physiological and pharmacological consequences. In recent years studies on rhodopsin and the βAR have revealed many of the details of the desensitization mechanisms. Two basic types of desensitizations have been described for G-protein linked receptors (51). Homologous desensitization refers to the loss of activity of a single class of receptors upon exposure of cells to a specific agonist. In contrast, heterologous desensitization is a more general phenomenon, involving the loss of activity of several types of receptors after exposure to an agonist specific for one of them.

The earliest functional consequence of homologous βAR desensitization is an uncoupling of agonist binding from adenylyl cyclase activation (52). This uncoupling event is accompanied or rapidly followed by sequestration of receptors away from the cell surface to an intracellular compartment (53). Upon longer agonist exposure, downregulation occurs, whereby receptors are lost from the cell by degradation (54). The events leading to heterologous desensitization are less well defined. Receptor sequestration has not been implicated in heterologous desensitization, and the general nature of the loss of receptor activity suggests the involvment of an alteration in G-protein function.

Rhodopsin undergoes a rapid desensitization upon light activation which involves the uncoupling of rhodopsin from transducin. Sequestration of the proteins is not observed for this system. The loss of rhodopsin function correlates with the phosphorylation of serine and threonine residues at the C-terminus of the protein by rhodopsin kinase (55-57). This kinase only phosphorylates the light activated form of rhodopsin. Phosphorylated rhodopsin then interacts with a 48kDa protein, arrestin, which competes with transducin for a proposed common binding site on rhodopsin. The formation of the rhodopsin-arrestin complex leads to the inactivation of the visual response (57).

Phosphorylation of the βAR has also been correlated with both homologous and heterologous desensitizaton (58). It has been proposed that heterologous desensitizaton is mediated by phosphorylation of the βAR by the cAMP-dependant protein kinase. In analogy with the rhodopsin system, both a kinase specific for the agonist-occupied form of the βAR (59) and the involvement of an arrestin-like activity have been implicated in homologous desensitization (60). This βAR specific kinase (βARK) can also phosphorylate light activated rhodopsin and agonist occupied α_2AR, suggesting a more general specificity for the enzyme (61, 62). The presence of a serine-threonine rich C-terminus on the βAR, as in rhodopsin, suggests that this region of the receptor may serve as the substrate for βARK. While the α_2AR does not possess a serine-threonine rich C-terminus, its third intracellular loop contains several serines and threonines which could serve as the substrate. Deletion mutational analysis of the βAR, however, has revealed that the C-terminus of the receptor and several other proposed phosphorylation sites can be removed without affecting the ability of the receptor to undergo rapid agonist mediated sequestration or long term desensitizaton (63). These data bring into question the role of phosphorylation in the sequestration event. Further studies will be required to fully assess the role of phosphorylation in the uncoupling response.

In contrast, mutational analysis of the βAR has demonstrated that those mutant receptors which are unable to couple to G-proteins also fail to undergo rapid, agonist dependant sequestration (63). Both G-protein coupling and sequestration require the presence of amino acid residues located at the N-terminal and C-terminal portions of inner loop 3 of the βAR. One interpretation of these data would be that the G-protein is directly involved in the desensitization process. Experiments examining the ability of the βAR to undergo sequestration in S49 cells which have alterations in Gs (cyc^-, H21a, or unc) that interrupt different steps in the receptor:G-protein:effector pathway suggested that receptor:G-protein coupling was required for long term, but not for rapid sequestration of the receptor (64, 65). An alternative explanation for the mutational data would be if the arrestin-like protein implicated in receptor uncoupling recognized the same region of the receptor as the G-protein and was required for both sequestration and uncoupling (60). Therefore, removal of the sites for interaction with the G-protein and arrestin would result in uncoupled receptors which could not be sequestered. The elucidation of the role for each of these components in the desensitization response will have to await additional experimentation.

Conclusions - It has become clear that those receptors which interact with G-proteins share a variety of structural and functional similarities. In particular, there appears to be a common motif for the ligand binding domains of the receptors that involves critical residues within the transmembrane regions. In the near future it should be possible to identify all of the points of contact between a receptor and its ligands, and to use this knowledge to design better ligands for that receptor. A role of amphiphilic helices in activation of G-proteins has been suggested and may provide a target for modulating receptor G-protein interactions independent of the ligand binding site of the receptor. Finally, the role of G-proteins, arrestin, and phosphorylation in receptor desensitization has been mentioned. Significant advances have been made in understanding of G-protein linked receptors, and it is anticipated that this trend will continue in the future with these and other receptors.

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Chapter 24. G-proteins as Receptor-Effector Couplers

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Introduction - Guanine nucleotide binding proteins subserve many functions in the cell. Some, such as tubulin, are cytoskeletal elements; others, such as eIF-2 and EF-II, are soluble proteins involved in the initiation and elongation steps, respectively, of protein synthesis (1). "G-proteins" is the term used to designate a distinct subset of the family of guanine nucleotide binding proteins. Like other members of the family, G-proteins bind guanine nucleotides with high affinity and specificity, possess intrinsic GTPase activity, and undergo a functionally important change in conformation, depending upon which guanine nucleotide, GDP or GTP, is bound. G-proteins are distinct from other guanine nucleotide binding proteins in their heterotrimeric structure, their association with the cytoplasmic surface of the plasma membrane, and their role in transmembrane signalling, and have been recently reviewed in detail (1,2).

Numerous G-proteins have been identified through classic protein purification, and through cDNA cloning. The number of receptors and effectors known to be regulated by G-proteins has also increased substantially. This chapter will cover the features of structure and function shared by all G-proteins, and detail those features that distinguish individual G-proteins. It is already clear that quantitative and qualitative changes in G-proteins may profoundly influence transmembrane signalling. This underscores the possibility that G-proteins could be important targets for therapeutic intervention.

G-protein Coupled Receptors and Effectors - G-proteins serve as receptor-effector couplers. Receptors for many different types of "first messenger" are coupled to G-proteins (Table I). Examples include the photon receptor, rhodopsin, neurotransmitter receptors such as the β-adrenergic and muscarinic cholinergic receptors, classical hormone receptors such as those for gonadotropins, glucagon, and parathyroid hormone, receptors for prostaglandins, and receptors for chemotactic factors such as f-Met-Leu-Phe. The structures of several of these receptors have recently been elucidated (3) and have been reviewed in detail in the preceding chapter by Dixon. These receptors share a common structure: a single polypeptide chain with seven discrete hydrophobic regions that may represent membrane-spanning domains. Effectors regulated by G-proteins are structurally and functionally diverse, and include enzymes of "second messenger" metabolism such as adenylyl cyclase and phospholipase C, as well as certain types of ion channels. The primary structure of G-protein-coupled effectors has not yet been defined.

In two cases, structurally defined G-proteins have been correlated with specific functions. G_s couples receptors for agents that stimulate cAMP production by adenylyl cyclase, and transducin couples rhodopsin to cGMP

Table I. Classification of G-protein-coupled receptors and effectors.

Receptor[+]	G-protein	Effector
β-adrenergic, many polypeptide hormones	G_s	adenylyl cyclase stimulation
α_2-adrenergic, muscarinic cholinergic, opiate, somatostatin	G_i	adenylyl cyclase inhibition
α_1-adrenergic, TRH, muscarinic cholinergic, f-Met-Leu-Phe	G_{PI}*	phospholipase C stimulation
rhodopsin, cone opsins	transducin	cGMP phosphodiesterase stimulation
muscarinic cholinergic, somatostatin	G_K	stimulation of inward rectifying K^+ channel
opiate, somatostatin	G_o	Ca^{++} channel inhibition
chemical odorants	G_s, G_{PI}	stimulation of adenylyl cyclase, phospholipase C

[+]Representative examples of a class of receptors are given.
*There are pertussis toxin-sensitive and -insensitive forms.

phosphodiesterase in photoreceptor rod outer segments (1,2). Recent evidence suggests that G_s may also act directly to facilitate opening of voltage-sensitive Ca^{++} channels (4). The specific G-proteins involved in regulating other effector functions have not been definitively identified. In several cases, including inhibition of adenylyl cyclase (5), stimulation of an inwardly-rectifying K^+ channel (6), and inhibition of a Ca^{++} channel (7,8), the implicated G-protein is a pertussis toxin substrate (see below). Pertussis toxin sensitivity alone, however, does not permit unequivocal identification of a particular G-protein, since multiple pertussis-toxin-sensitive G-proteins have been identified (1,2). Phospholipase C, the enzyme that generates dual "second messengers," inositol trisphosphate and diacylglycerol, is also G-protein-regulated (9). In a few cell types, notably neutrophils (10), the relevant G-protein is a pertussis toxin substrate; in many other cells (e.g. pituitary, liver, pancreatic acinar) the G-protein coupled to phospholipase C (G_{PI}) may be structurally distinct, as it is insensitive to pertussis toxin (9). The identity of this pertussis toxin-insensitive G-protein is at present unknown.

In addition to visual transduction, which is mediated by the retinal G-protein, transducin, sensory transduction involving smell (11) and taste (12) appears to be mediated by G-proteins. The relevant effectors are adenylyl cyclase and phospholipase C. Receptors on lymphocytes and monocytes

involved in immune regulation may also be coupled to G-proteins. These include receptors for bacterial lipopolysaccharide (13), the T-cell antigen receptor (14), and the interleukin-2 receptor (15). Further evidence is needed to confirm these findings and identify the relevant G-protein(s).

Experiments involving reconstitution of purified G-proteins with other components in plasma membranes or artificial phospholipid vesicles have been used to define the specificity of receptor and effector coupling. The β-adrenergic receptor preferentially couples with G_s rather than transducin, and the reverse is true for rhodopsin (16). Rhodopsin will, however, couple effectively with G_i and G_o which are closer in structure (see below) to transducin than is G_s. In terms of effector interaction, only transducin stimulates cGMP phosphodiesterase, and only G_s stimulates adenylyl cyclase (1,2). Both G_i and G_o, purified from brain, have been reported to stimulate phospholipase C activity in HL-60 membranes (17). Such results should be viewed with caution, since "purified" G-protein preparations may in fact be mixtures of structurally, and functionally, distinct proteins. Future experiments relying upon expression of defined cDNA encoded proteins should prove helpful in delineating the function of specific G-proteins.

Activation of G-proteins - G-protein coupling to receptor and effector involves a GTPase cycle (Fig. 1). Inactive G-proteins contain tightly-bound GDP (18). Interaction of G-protein with an agonist-bound, "active," receptor promotes release of bound GDP and permits GTP, (cellular concentration about 10-fold higher than GDP), to bind to, and activate the G-protein. The very slow spontaneous dissociation rate for bound GDP minimizes spontaneous G-protein activation. In the GTP-bound, activated state, the G-protein interacts with, and regulates, effector activity. Depending upon the specific G-protein and effector involved, G-protein-effector interaction may lead to stimulation or inhibition of effector activity. Intrinsic G-protein GTPase activity leads to hydrolysis of the γ-phosphate of GTP. This leaves

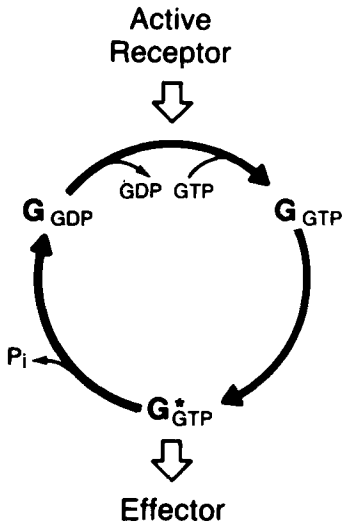


Figure 1. The G-protein GTPase Cycle

GDP bound to the G-protein, and serves to terminate G-protein activation and thereby interaction with effector (1,2,19). The GDP-bound G-protein must again encounter an activated receptor in order to release GDP, bind GTP, and

reenter the cycle. Receptors function catalytically in this cycle, i.e. a single active receptor can activate multiple G-proteins (1,2). This provides for amplification of the original signal. Interaction of G-protein with an effector enzyme catalyzing the formation (or destruction) of "second messenger" provides yet further signal amplification.

The binding of agonists and antagonists to G-protein-coupled receptors shows important differences. Agonists, but not antagonists, promote formation of a high-affinity ternary complex between receptor, agonist, and G-protein (1,2). Binding of GTP to the G-protein dissociates the latter from the complex and reduces the affinity of the isolated receptor for agonist. Antagonist affinity is the same whether receptor is coupled to G-protein or not. Antagonists also do not promote release of bound GDP, and therefore do not stimulate GTP hydrolysis. In membrane preparations free of endogenous guanine nucleotide, measurement of ligand affinity with and without added guanine nucleotide can be used to distinguish agonists from antagonists. Guanine nucleotide-induced changes in binding affinity are used to indicate that a given receptor is coupled to a G-protein.

Another general feature of G-proteins is their ability to serve as specific substrates for covalent modification by bacterial toxins. Toxins such as those of *V.Cholerae* and *B.Pertussis* are enzymes that cleave endogenous NAD and transfer the ADP-ribose moiety to specific amino acids on G-protein substrates. Cholera toxin will modify G_s, the G-protein that stimulates adenylyl cyclase, whereas pertussis toxin does not modify G_s but will modify several other G-proteins(1,2). The retinal photoreceptor G-protein, transducin, can serve as a substrate for both toxins. The amino acid substrate is arginine for cholera toxin (20) and cysteine for pertussis toxin (21). ADP-ribosylation alters G-protein function in a different way for each toxin. Cholera toxin-catalyzed ADP-ribosylation reduces GTPase activity; as a result the G-protein is persistently activated in a receptor-independent manner (19). Pertussis toxin has the opposite functional effect. ADP-ribosylation catalyzed by pertussis toxin leads to uncoupling of G-protein from receptor (5). This effectively blocks transmission of an agonist signal for receptors coupled to G-protein pertussis toxin substrates.

Subunit Structure and Function - G-proteins consist of three distinct protein subunits termed α, β, and γ. The α-subunit binds guanine nucleotide, is a GTPase, and serves as the bacterial toxin substrate. Each G-protein contains a structurally unique α-subunit (1,2). This has led to the assumption that it is the α-subunit that confers specificity in G-protein interaction with receptors and effectors.

β-subunits show less structural variation than α-subunits. Peptide mapping (22) and immunochemical (23) studies have shown that the β-subunits of different G-proteins are very similar. Cloning of cDNAs encoding G-protein β-subunits indicates that the 36kDa β-subunit of several different G-proteins is an identical 340 amino acid protein (24,25). A 35kDa β-subunit is found in other G-proteins. Its structure, again predicted from the sequence of a cDNA (26), differs by 34 (10 nonconserved) residues out of 340 from the 36kDa form of β-subunit. With the exception of transducin which consists exclusively of a 36kDa β-subunit, it is not clear whether the 36 or 35kDa β-subunits are preferentially associated with a given G-protein. Also unclear is whether the slight difference in structure of the two forms has any functional significance.

β-subunits are tightly, but noncovalently, linked to low molecular weight (8-11kDa) γ-subunits to form the β/γ complex. The γ-subunit of transducin has been cloned, and is a hydrophilic 84 amino acid protein (27). Transducin-γ is immunochemically distinct from the γ-subunits of other G-proteins (23), and

there is immunochemical evidence (28) for further differences between other G-protein γ-subunits. This raises the possibility that β/γ complexes of various G-proteins could differ structurally and functionally. If β/γ complexes play a direct role in effector regulation (see below), structural differences in the γ-subunit could in theory account for specificity in β/γ subunit action. There is general agreement that the G-holoprotein rather than individual subunits is uniquely capable of interacting with receptors (29,30). The situation with respect to effector interaction is less clear.

All G-proteins are persistently activated by nonhydrolyzable analogs of GTP such as guanosine (3'-O-thio) triphosphate (GTP-γ-S), and by millimolar concentrations of fluoride anion (1,2). AlF_4^- may interact with bound GDP, and by mimicking the γ-phosphate of GTP, activate the G-protein (31). Activation of solubilized G-protein by GTP-γ-S or fluoride causes dissociation of α-subunits from β/γ subunits (32). It is not yet clear, however, whether physical dissociation of α and β/γ subunits occurs upon activation of membrane-bound G-proteins by the physiologic nucleotide, GTP. The activated α-subunits of G_s and transducin are fully capable of interacting with, and activating, their respective effectors (1,2). One model of G-protein action postulates that a common pool of β/γ subunits modulates the activation of G-protein by receptor (32). According to this model, inhibition of adenylyl cyclase does not occur via interaction of the α-subunit of G_i (G-protein responsible for adenylyl cyclase inhibition) with effector. The inhibition is caused by release of free β/γ subunits upon G_i activation. The released β/γ subunits in turn block adenylyl cyclase activation by retarding activation and dissociation of G_s (33). β/γ subunits may also regulate other types of effector activity, including potassium channels (34) and phospholipase A_2 (35). This data is controversial, since others suggest that regulation of effector activity is always mediated by α-subunits (36). Further data, particularly relating to G-protein action within the context of the cell membrane, will be necessary to clarify the mechanism(s) of effector regulation by G-proteins.

Cloning of cDNAs Encoding G-protein α-Subunits - The sequences of cDNAs encoding seven different G-protein α-subunits, each a distinct gene product, have been determined (1,2). For G_s, four distinct forms of mRNA, presumptively derived by alternative splicing from a single gene, have been described (37). These differ in the region after residue 71 by the presence or absence of a 15 amino acid peptide, and the presence or absence of an additional serine residue. The resultant polypeptides of 379, 380, 394, and 395 amino acids correspond to multiple forms of G_s(approximately 45 and 52kDa) that are identified by cholera toxin-catalyzed ADP-ribosylation (38). Functional differences between various forms of G_s have not yet been reported. The G_s mRNA and protein are ubiquitously distributed (39). Two distinct retina-specific cDNAs encoding 350 and 354 amino acid forms of transducin-a have been cloned. The former corresponds to the 39kDa protein purified from rod outer segment membranes. The latter has been tentatively identified as a retinal cone-specific form of transducin based on immunohistochemistry with peptide-specific antibodies (40).

A 39kDa α-subunit, the most abundant pertussis toxin substrate in brain, has been termed G_o, since its function is unknown (41). Recent evidence suggests that G_o may couple opiate receptors to inhibition of Ca^{++} channels in NG-108 neuroblastoma-glioma hybrid cells (7). G_o is encoded by a cDNA whose sequence predicts a 354 amino acid protein (42). Immunochemical (43), *in situ* hybridization, and Northern analysis (39) studies suggest that G_o is particularly abundant in brain, but is absent, or present at very much lower concentration, in peripheral tissues. Distinct cDNAs for three other pertussis toxin substrates have been cloned (44-46). These have been arbitrarily designated G_{i1}, G_{i2}, and G_{i3}. It is unclear which of the proteins encoded by

these three cDNAs corresponds to the pertussis toxin-sensitive G-proteins implicated in such functions as adenylyl cyclase inhibition, phospholipase C stimulation, and K^+ channel stimulation. G_{i1} cDNA encodes a 41kDa protein abundant in brain (47), and present in lower concentrations, if at all, in peripheral tissues (39). G_{i2} cDNA encodes a 40kDa protein corresponding to the major pertussis toxin substrate in neutrophils (48). This protein is also found in brain (perhaps in glial cells), and in other peripheral tissues (39,48). G_{i3} cDNA encodes a 41kDa protein distinct from the 41kDa protein in brain. This protein is present in HL-60 cells (49) and in lymphocytes (50).

Homology Between G-protein α-Subunits - The amino acid sequences of G-protein α-subunits show between 40% (G_s vs. all others) and >90% (e.g. G_{i1} vs. G_{i3}) homology. Certain highly conserved domains are characteristic, not only of G-proteins, but are also highly conserved in other GTP-binding proteins such as the p21 *ras* oncogene products, and even the bacterial elongation factor, EF-tu (51). These regions correspond to domains involved in binding guanine nucleotide as determined by x-ray crystallographic analysis of EF-tu (52) and p21 *ras* (53). The least conserved (<60%) regions of G-protein α-subunits are located at approximately residues 100-140 and 300-320 (for an average 354 amino acid α-subunit). Although the sequence of these regions is highly variable in different G-proteins, for a given G-protein, amino acid sequence conservation is extraordinarily high (>95%) for different mammalian species (44). One may speculate that regions of sequence diversity correspond to domains that confer specificity in G-protein coupling to receptors and effectors (51).

At present, no firm evidence exists to identify putative effector interaction domains of G-protein α-subunits. There is evidence that the carboxy-terminus of the α-subunit is involved in receptor interaction (58). Uncoupling of G-proteins from receptors, for example, is due to pertussis toxin-catalyzed ADP-ribosylation of a cysteine near the carboxy-terminus (5,21). The amino-terminus may represent a domain for α-subunit interaction with the β/γ complex (54). The same region has been implicated in tethering the hydrophilic α-subunit to the plasma membrane (55). Myristoylation of an amino-terminal glycine may be important in membrane attachment (56). This is one of several features that distinguish G-proteins from the p21 *ras* oncogene products (1). The latter, like G-proteins, bind guanine nucleotides specifically, possess GTPase activity, and are associated with the cytoplasmic surface of the cell membrane. Unlike G-protein alpha subunits, palmitoylation of p21 at a cysteine near the carboxy-terminus is responsible for membrane attachment (57). There is no evidence for p21 interaction with G-protein β/γ subunits or their functional equivalent. Whether or not mammalian *ras* proteins function as receptor-effector couplers remains an open question.

Altered G-proteins as a Cause of Altered Signal Transduction-Quantitative and qualitative changes in G-proteins may be an important cause of alterations in target cell response to extracellular "first messengers." Covalent modifications of G-proteins such as ADP-ribosylation catalyzed by bacterial toxins provide a striking example. The possibility that endogenous cellular enzymes covalently modify (e.g. phosphorylate) G-proteins remains unproven, but deserves further investigation. Mutations in G_s-α in the S49 mouse lymphoma cell line offer dramatic evidence of the effects of G-protein alterations on signal transduction. In the UNC mutant, G_s-α is present and can be activated by GTP analogs and fluoride, but is uncoupled from receptors. As a result, β-adrenergic agonists and PGE_1, stimulators of cAMP formation in wild type cells, fail to stimulate UNC cells. The mutant phenotype is due to a single amino acid substitution (arginine to proline) in the 6th position from the carboxy-terminus (58). In the CYC^- mutant, G_s-α

protein and mRNA are totally lacking (59). The consequence is that not only receptor agonists, but also fluoride and GTP analogs, fail to stimulate adenylyl cyclase. In a human genetic disorder, pseudohypoparathyroidism, an approximate 50% reduction in G_s-α protein and mRNA have been found in affected subjects (60,61). G_s deficiency may explain the resistance to parathyroid hormone and other agents acting via cAMP stimulation that is characteristic of this disorder. In contrast, a constitutively activated form of G_s has been described in growth hormone-secreting tumors from certain subjects with acromegaly (62). The abnormality in regulation of cell growth and hormone secretion was ascribed to the altered G_s. Reduced expression of G_i in livers of diabetic rats may explain some of the metabolic abnormalities associated with insulin-deficient diabetes (63). It seems likely that further studies will uncover other examples of pathologic states in which altered G-proteins are the cause of altered signal transduction.

Conclusions - The foregoing should make it clear that modulation of G-protein function by drugs could, in theory, be an effective strategy for either potentiating or blocking signal transduction. How realistic is such a goal? Available data do not permit a definitive conclusion. Obstacles to such a strategy would include the requirement that to target G-proteins, drugs must be capable of penetrating the cell membrane; also, a strategy based on interference with GTP binding or hydrolysis might be too nonspecific, i.e. it would likely interfere with the function of other intracellular GTP-binding proteins. Lithium (at submillimolar concentrations) has been reported to interfere with receptor-G-protein coupling involving G_s, G_i, and perhaps other G-proteins (64). Whether this observation can explain the unique efficacy of lithium in manic-depressive illness is as yet uncertain, but it raises the possibility that even a relatively nonselective action on G-proteins could be therapeutically important. More selective targeting of G-proteins might take advantage of their unique subunit structure (alpha and beta/gamma subunit interaction), and membrane localization. Recognition of the heterogeneity within the G-protein family, and identification of the domains conferring specificity in G-protein-receptor and -effector coupling may eventually offer appropriate targets for therapeutic intervention.

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Chapter 25. Isozymes of Protein Kinase C

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Introduction - The Ca^{2+}, phospholipid-dependent protein kinase, protein kinase C (PKC), is of major importance for regulation of cell growth and expression of differentiated functions (1,2). A variety of hormones, growth factors, and neurotransmitters stimulate the phospholipase C-mediated hydrolysis of phosphatidylinositolbisphosphate (PIP_2) in tissues to produce two second messenger molecules, diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 causes release of Ca^{2+} from intracellular stores, while DAG is a neutral lipid cofactor for PKC and is an endogenous regulator of PKC activity. Although other neutral lipids, including fatty acids, can also stimulate PKC *in vitro*, a role for these agents *in vivo* has not yet been demonstrated (3).

Activation of PKC is associated with increased growth, enhancement of terminal differentiation, increased exocytosis, and tumor promotion (1). Part of the reason for the wide-range of biological consequences of PKC activation may be explained by the recent evidence that PKC is actually a family of proteins encoded by several genes. In this chapter we will discuss the structure of the PKC isozymes, the biochemical properties of the coded proteins, and the use and importance of PKC type-specific reagents for exploring PKC type-specific stimulus-response coupling.

Measurement of PKC activity - Much of our understanding of the role of PKC in biological processes stems from experiments with phorbol esters, plant-derived diterpenes which bind to and activate PKC. Both *in vitro* and *in vivo* experiments show that phorbol esters mimic the effects of DAG in activating PKC (4). PKC has been identified as the major cellular phorbol ester receptor and therefore, phorbol esters are often used as specific tools to explore PKC function in cells (5). Furthermore, because phorbol esters (particularly phorbol dibutyrate, PDBu), are less lipophilic than DAGs, they are much more suitable ligands for binding assays. [^{3}H]PDBu binding, therefore, represents an additional approach for measuring PKC (6,7). Although the possibility for other PDBu binding proteins exists, none has yet been identified.

Phorbol esters and DAG appear to interact with the same site on PKC because DAG is a competitive inhibitor of phorbol ester binding (8). In general, cellular responses elicited by phorbol esters are similar to responses elicited by activators of PIP_2 hydrolysis. However, one major difference between activation of PKC by phorbol esters and DAG generated from receptor-mediated PIP_2 hydrolysis should be emphasized. In many cases of agonist stimulation, the duration of the increased DAG (and as a consequence, PKC activation) is less than five minutes. In contrast, activation by phorbol

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esters, which are relatively metabolically stable, is prolonged. Possibly the prolonged PKC activation by phorbol esters contributes to their activity as tumor promoters.

The initial event in cellular activation of PKC is a redistribution of PKC from cytosol to membrane fractions (9). Both phorbol esters and agonists that stimulate PIP_2 hydrolysis increase the chelator-stable membrane association of PKC. This redistribution has become a hallmark for measurement of PKC activation. Other potential reporter systems for measurement of PKC activation involve assessment of the phosphorylation state of PKC substrates. The best characterized of these are the EGF receptor, for which phosphorylation is associated with decreased affinity for EGF (10), and an 80 kDa protein which appears to be a major PKC substrate in several tissues (11). Other assays for PKC activation state are required to fully address the role of PKC in cellular events.

Molecular Biology of PKC - A more complete understanding of PKC structure has emerged with the cloning and sequencing of the genes for PKC. Mammalian PKC cDNA clones (12-17) have been isolated from brain cDNA libraries. These gene cloning studies demonstrated that PKC is actually a family of genes. Currently, at least six separate genes have been isolated (18). For this review, we will use the nomenclature α, β, γ, δ, ε and ζ (19). Based on the nucleotide sequences, all the isolated mammalian cDNA clones code for proteins of approximately 77 kDa. Amino acid sequence conservation is greater than 90% among the bovine, rat and human α PKCs. The genes for α, β and γ map to human chromosomes 16, 17 and 19, respectively, suggesting that different control mechanisms may be involved in the expression of each of the genes (13).

Evidence that the isolated clones code for PKC has been presented. In vitro translation of the α clone resulted in a 76 kDa protein that was immunoprecipitated by a polyclonal antibody to PKC (13). Another cDNA clone has been used to produce a β-galactosidase fusion protein (20). Antibodies raised against that protein recognized PKC by several criteria. Other clones have been expressed in COS cells (15,16). The transfected cells had three- to ten-fold higher levels of [^{3}H]PDBu binding and PKC activity than the control cells.

The proteins encoded by the α, β and γ genes are highly homologous and are identical in four conserved regions (C1-C4) (see Fig. 1). Each protein contains a carboxy-terminal kinase domain and an amino-terminal regulatory domain. Each isozyme also contains five interspersed variable regions (V1-V5) which are unique for each of the proteins, but are highly conserved across species (12,13). This suggests that the variable regions are not simply structural spacers, but that they may serve unique functional roles for the various PKC isoforms. Thus, the variable regions may provide the foundation for PKC biochemical heterogeneity.

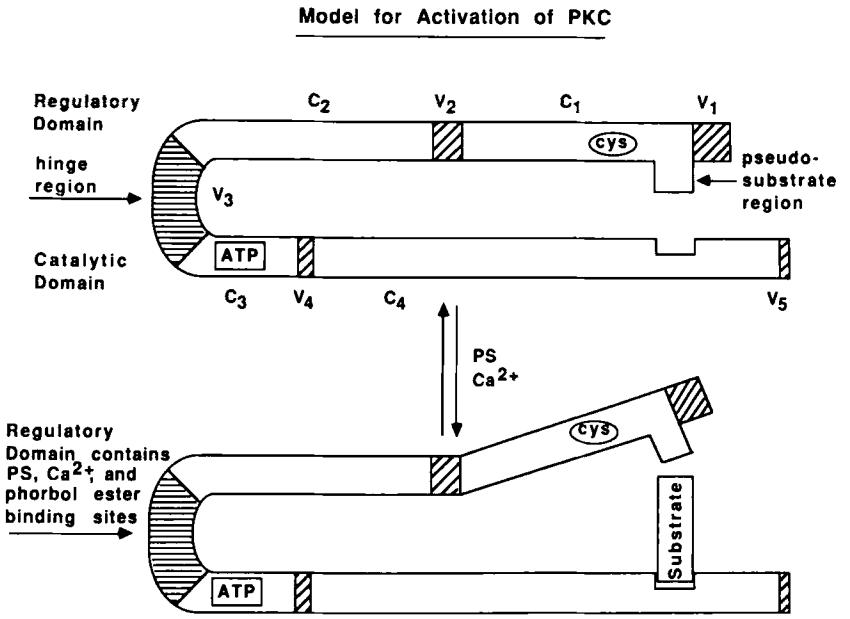


Fig. 1. Consensus structure of PKC isozymes. PKC is depicted in an inactive conformation in the absence of Ca^{2+} and phosphatidylserine (PS). Positions of the constant (C) and variable (V) regions, ATP binding site, protease-sensitive hinge region, and the pseudosubstrate site are indicated. Conformational changes associated with Ca^{2+} and PS binding produce an active PKC molecule which can interact with substrates. PS, but not Ca^{2+}, is also essential for phorbol ester binding. The position of sites associated with Ca^{2+}, PS, phorbol ester, or substrate binding have not yet been identified and are drawn only to reflect their general positions in either the regulatory or the catalytic domain.

The carboxy-terminal half of PKC contains the C3 and C4 regions which code for the kinase domain. C3 contains an ATP binding sequence, GXGXXG---K, which is found in many protein kinases (12). The most extensive region of homology, C4, extends over the C-terminal end. The C1 and C2 regions in the amino-terminal half of PKC comprise the regulatory domain that interacts with Ca^{2+}, phospholipid, and DAG or phorbol esters. The C1 region contains a repeating unit of six cysteine residues. This sequence is also found in various DNA binding proteins such as the glucocorticoid receptor, as well as some metalloproteins (12). The C1 region also contains a sequence which shares homology with PKC substrates except that it is lacking a serine or threonine phosphate acceptor. This "pseudosubstrate" region may keep PKC in an inactive state in the absence of cofactors (21). The C2 region is missing from δ, ε, ζ PKC (18). Potential binding regions for phospholipid, Ca^{2+} or phorbol esters were not identified based on sequence homology to other lipid and Ca^{2+} binding proteins. The exact demonstration of the binding sites for these PKC cofactors may require experiments involving site-directed mutagenesis and a suitable expression system. Epitope mapping studies with antibodies that interact with PKC phospholipid and Ca^{2+} binding regions will also be valuable.

The β PKC gene can undergo alternative splicing leading to the expression of two distinct PKC species (14-16). These clones, βI and βII, encode 671 and 673 amino acids, respectively, share an identical N-terminal sequence, and differ only in the carboxy-terminal region of approximately 50 amino acids. Southern analysis of rabbit genomic DNA showed a single 9 kb band for both the βI and βII clones, supporting the proposal that the clones are derived from one gene by splicing (16). The expression of the various PKC proteins may involve a number of complex control mechanisms.

The α, β and γ cDNA clones have been used to examine the expression of PKC mRNA in various tissues (13,15,16). All the clones hybridize most strongly to brain tissue. These results are consistent with earlier tissue distribution studies in which phorbol ester binding and PKC were measured and found to be greatest in brain tissue (22-25). The γ clone is found only in neuronal tissue, while the α and β clones are expressed in nonneuronal tissues as well (26). Northern analysis of bovine and rat brain RNA using the α, β and γ clones detected multiple RNA species, which were different for each clone. It has not yet been determined whether each of the RNA species is translated into a unique protein. However, the demonstration of more than one RNA species for each gene suggests that additional, as yet unidentified PKC proteins may be expressed.

Biochemical properties of PKC isozymes - Three isozymes of PKC can be separated by chromatography on hydroxylapatite (HAP) (19,27,28). They are named to reflect their order of elution from HAP as Types 1, 2, and 3 PKC. Expression of rat cDNA clones in COS cells was used to determine the relationship between the biochemically fractionated proteins and their corresponding genes (19). Type 1 PKC is coded for by the γ sequence, Type 2 PKC is a mixture of proteins coded by the βI and βII sequences, and Type 3 PKC is coded by the α sequence.

Each of the isolated proteins binds PDBu and has a molecular weight of ~80 kDa. Each of the coded proteins contains an amino-terminal regulatory domain and a carboxy-terminal kinase domain. These two domains are separated by a "hinge" region which is sensitive to attack by trypsin (12). Thus, trypsin cleavage produces a 35-38 kDa Ca^{2+}, phospholipid-dependent PDBu receptor molecule (the regulatory domain) and a 43-45 kDa Ca^{2+}, phospholipid-independent kinase (the catalytic domain), (29,30, Fig. 1).

There are differences among the isozymes with respect to cofactor requirements (28). The presence of Ca^{2+} increased Vmax 1.4-fold for Type 1, 6-fold for Type 2, and 14-fold for Type 3 as compared to the Vmax measured without Ca^{2+}. PDBu stimulated Type 1 by 1.8-fold, Type 2 by 2.6-fold, and Type 3 by 3.5-fold under low Ca^{2+} conditions in which PDBu is most effective at stimulating PKC activity. The differences in enzymatic activity bear out the predictions from analyses of the isolated genes which suggest that type-specific sequences may result in type-specific biochemical properties.

Non-DAG neutral lipid activators, such as fatty acids, stimulate the various isozymes with different efficacies. Type 3 activity was stimulated to 80% of maximum by 400 μM arachidonic acid, whereas Type 1 activity was increased to only 25% of maximum under the same conditions (31). These differences in fatty acid sensitivity may lead to type-specific stimulus-response coupling during

later phases of cellular responses when large amounts of unsaturated fatty acids and calcium are mobilized (31).

Phosphorylation of the EGF receptor by the three isozymes was compared. Under the assay conditions used, Type 3 was most effective in phosphorylating the EGF receptor. Type 1 was least effective; however, it was most effective at autophosphorylation (32). Further experiments are required to determine if there is type-specificity for phosphorylation of other physiologically relevant PKC substrates.

PKC antibodies - Understanding the role of individual PKC isozymes in biological processes requires the development of PKC type-specific reagents. To date, no major differences in physical, enzymatic, or binding properties of the proteins, which could serve as the basis for a "type-specific" assay, have been noted. However, type-specific polyclonal and monoclonal antibodies (mAbs) to PKC have been made. Immunization with purified Type 2 or Type 3 PKC was used to produce type-specific polyclonal (28) and monoclonal antibodies (33). Type specific Abs have been produced by immunization with a mixture of isozymes followed by immunopurification with individual isozymes (27,34). The production of type-specific Abs indicates that PKC variable regions are important Ab recognition sites. Consequently, these Abs should be useful in epitope mapping studies for defining the function of the variable regions.

Monoclonal Abs to Type 3 PKC have been produced (33). Tryptic cleavage of PKC was used to generate the PKC fragments in order to determine the domain specificity of the mAbs. The mAbs can be divided into three classes: Class 1 recognize Type 3 PKC catalytic domain, Class 2 recognize Type 2 and Type 3 catalytic domains, and Class 3 recognize Type 3 regulatory domain. All of these mAbs inhibit PKC activity, but only the Class 3 mAbs inhibit phorbol ester binding activity. Inhibition of PKC and PDBu binding activities by Class 3 (but not Class 1) mAbs was competitive with phospholipid, indicating that these mAbs may be binding at or near PKC phospholipid binding sites. These and other well-characterized Abs will be valuable for PKC structure-function studies.

PKC Abs have been used to determine tissue distribution of PKC isozymes. Three distinct Ab-staining patterns were found in rat brain tissue slices using various mAbs (35). The type-specificity of these mAbs has not yet been described. Type-specific polyclonal Abs were used to determine isozyme content in various regions of brain and in cultured cells using a quantitative immunoblot assay (36,37). It is important to note that the complexity of PKC contributes to difficulty in determining cell and tissue isozyme content. For example, one immunopurified polyclonal Ab recognizes several molecular weight species <80 kDa on immunoblots (38). The relative abundance of these PKC fragments varied among the different tissues and cell types studied. In contrast, only 80 kDa PKC was found in tissue distribution studies using another immunopurified polyclonal Ab (34). Differences in methodologies or Ab properties probably account for the discrepancy. Because of the strong possibility that there are tissue-specific PKCs (e.g. Type 1 PKC is found only in the brain, 26), it will probably be necessary to coordinate measurements of enzymatic activity and immunoreactivity in order to fully characterize tissue PKC isozyme content. Two studies emphasize the importance of this approach. PKC from rabbit reticulocytes is associated with a 92 kDa protein (39), while three 68 kDa proteins,

each with Ca^{2+}, phospholipid-dependent kinase activity, have been purified from liver (40).

The presence of PKC fragments in various tissues (38) raises the possibility that endogenous proteolytic activity may be an important regulator of PKC. In vitro studies have shown that treatment of cells with phorbol esters for prolonged periods causes depletion of 80 kDa PKC by increasing turnover (41). Proteolysis can produce two functional PKC fragments: the 45-50 kDa Ca^{2+}, phospholipid-independent kinase (PKM), and the 35-38 kDa phorbol ester binding fragment. It is possible that in some cases these fragments are long-lived and play a role in the PKC signal transduction process (42).

Inhibitors of PKC - The use of inhibitors of PKC activity is one approach to studying the role of PKC in cellular function. A variety of compounds have been reported to inhibit PKC activity. However, none of the agents is totally specific for PKC. No isozyme-specific inhibitors have been reported.

Compounds that affect the phospholipid requirement of PKC can act as inhibitors. These include triphenylethylenes (43), melittin (44) and polymyxin B (45). The antipsychotic agents trifluoperazine, chlorpromazine and haloperidol inhibited partially purified PKC (46). These compounds also act as calmodulin antagonists, and thus are not specific for PKC. Several antineoplastic drugs including doxorubicin, vincristine, and vinblastine inhibit in vitro PKC activity (47). Concentrations of 150-300 uM were required to inhibit activity by 50%. Whether these compounds can inhibit a PKC-stimulated biological response in intact cells has not been tested. The anti-tumor agent alkylysophospholipid inhibited PKC activity with a K_i value of approximately 6 uM in extracts from acute monocytic leukemia cells (48). Phosphorylation of endogenous proteins in both HL60 and K562 cells was also inhibited by alkyllysophospholipid. The inhibition of PKC activity by all these agents is competitive with phospholipid. Since a variety of enzymes and cellular processes are sensitive to agents that interact with the phospholipid environment, none of these inhibitors is specific for PKC.

Several isoquinolinesulfonamides, such as H-7 and H-9, have been synthesized (49). These inhibit both cyclic nucleotide-dependent protein kinases and PKC. Several investigators have used these inhibitors in intact cell experiments. In HL-60 cells, H-7 inhibited phorbol ester induced differentiation, as well as differentiation induced by 1,25-dihydroxyvitamin D_3 and all trans-B-retinoic acid (50). H-7 also inhibited phorbol ester induced 40 kDa protein phosphorylation in platelets (51). The isoquinolinesulfonamide inhibitors are unique because their inhibition of kinase activity is competitive with respect to ATP. However, as discussed above, the kinase domains of cGMP and cAMP dependent kinases are highly homologous to PKC, and therefore, the K_i values for inhibition of these three kinases by H-7 and H-9 are not substantially different. This limits their usefulness as specific PKC inhibitors.

Two microbial alkaloids, staurosporine and K252, have been shown to inhibit PKC activity (52, 53). They are more potent than other reported inhibitors, and their K_i values are in the nanomolar range. As with the isoquinolinesulfonamides, though, they also inhibit cAMP- and cGMP-dependent protein kinases.

Regulation of PKC activity by the endogenous lipid environment has been suggested by several groups. The inhibition of PKC activity by sphingosine and sphinganine is competitive with respect to calcium, PDBu and diacylglycerol (54, 56). Sphingosine inhibited the association of PDBu with the PKC/Ca^{2+}/Triton X-100/PS complex. Therefore, displacement of the activating DAG or phorbol ester from the PKC-lipid complex seems to be the mechanism of sphingosine inhibition. Sphingosine blocks thrombin-induced protein phosphorylation of the 40 kDa substrate in platelets. Both sphingosine and sphinganine block PMA-induced cell adherence and inhibition of growth in HL-60 cells as well as the oxidative burst of neutrophils induced by a variety of agonists. Regulation of *in vitro* PKC activity by gangliosides has been demonstrated (57). The inhibitory effect may be the result of a direct interaction with PKC, since inhibition was not affected by substrate, calcium, phospholipid or diolein. The severe toxicity of sphingosine and sphinganine to some cells may limit their general utility as PKC inhibitors (58).

Endogenous protein inhibitors of PKC have also been reported. These inhibitors have been identified in brain tissue, but are also present in most cell types. In most systems the inhibitor(s) of PKC prevents the direct determination of kinase activity in cell lysates, and measurement of cellular PKC activity often requires partial purification of the enzyme by ion exchange chromatography. A heat-sensitive inhibitor of approximately 20 kDa was partially purified from rat brain (59). The inhibition was competitive with the kinase substrate, and was not affected by changes in ATP, Ca^{2+} and phospholipid. A 40 kDa inhibitor has been described which is heat-stable and is composed of two polypeptide chains of 19 kDa (60). A protein inhibitor of 17 kDa has also been purified (61). A 12 kDa protein, which is a proteolytic fragment of the 17 kDa protein, also has inhibitory activity (62). These inhibitors share a number of properties with calmodulin, such as heat stability in the presence of Ca^{2+}. The physiological role of the PKC inhibitors is not yet known, but these proteins may represent an endogenous mechanism for PKC inactivation.

A recent analysis of the PKC amino acid sequence revealed a "pseudosubstrate" homolog in the C1 region (21, Fig. 1). The sequence is missing the required threonine or serine to make it a PKC substrate. However, the synthetic peptide corresponding to this PKC sequence of 11 amino acids was shown to be among the most potent of PKC inhibitors reported to date with IC_{50} of 0.18 uM. Inhibition was specific for PKC. This peptide will therefore be very valuable in *in vitro* studies of PKC.

Down modulation of PKC may serve as an endogenous mechanism for decreasing PKC activity. Many studies have demonstrated that prolonged treatment of cells with phorbol esters results in a decrease in phorbol ester receptors (63, 64). More recently, the ability of phorbol esters to cause down modulation has been used to produce cells that are functionally deficient in PKC. In this way, the role of PKC as a step in the activation pathway of various cellular effectors can be examined. For example, in 3T3 cells, an 80 kDa protein has been shown to be a substrate for PKC. Treatment of the cells with 16 uM PDBu for 16 hr leads to a loss of [^{3}H]PDBu binding activity, PKC activity and PKC immunoreactivity (65). In these down modulated cultures, stimulated phosphorylation of the 80 kDa protein by PDBu, as well as PDGF and EGF, does not occur. These studies

suggest that phosphorylation of the 80 kDa protein by the growth factors is mediated via the PKC pathway. This down modulation protocol has been used by a number of researchers to study the role of PKC in growth of 3T3 cells (66, 67) and human fibroblasts (68), regulation of c-myc (69) and c-fos levels (70) and ornithine decarboxylase activity (71).

The mechanism of down modulation has not been defined, although degradation of PKC appears to be a major factor in decreasing PKC activity (72). Treatment of FRSK rat keratinocytes with phorbol esters causes a rapid redistribution of PKC to the membrane. After 30 min, however, membrane-associated PKC activity decreases. The decrease in activity is associated with loss of PDBu receptors and the production of a 40-60 kDa cytosolic protein with Ca^{2+}, phospholipid-independent kinase activity. Treatment with the protease inhibitors TPCK, TLCK or leupeptin prevents the loss of PKC as well as the down regulation of PDBu binding (72).

In keeping with this proposal, there are now several reports of phorbol esters causing accelerated loss of immunoreactive PKC. In GH3 cells, phorbol esters caused a decrease in immunoprecipitable PKC which roughly correlated with loss of PKC activity (41). Phorbol ester treatment of 328 rat glioma cells also caused a decrease in immunoprecipitable PKC without influencing PKC synthesis (73). However, degradation of PKC was increased as reflected in the half-life of the protein: 6.7 hrs versus 2.4 hrs for control and PMA-treated cells, respectively. Similar results were reported in mouse B82L cells in which PMA-treatment caused greater than 24-fold decrease in the half-life of PKC (74).

SUMMARY - PKC is now recognized to be a heterogeneous family of proteins. Although type-specific functions have not yet been described, it is tempting to consider that the type-specific sequences that comprise the variable regions provide the foundation for type-specific sequences that comprise the variable regions provide the foundation for type-specific biochemical properties. This could in turn result in type-specific stimulus-response coupling. Future studies will be directed towards understanding the role of individual isozymes in specific processes. These studies will require the use of type-specific reagents, such as antibodies. At the present time, there is no demonstration of a large difference among the isozymes with respect to phorbol ester binding properties, substrate specificity, or susceptibility to inhibitors or activators which could serve as the basis for a type-specific assay.

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Chapter 26. Therapeutic Strategies in the Treatment of AIDS

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Introduction - The recent epidemic of acquired immunodeficiency syndrome (AIDS) has prompted a broadly based effort to find means of preventing and treating this disease. There have been over 50,000 cases of AIDS in the United States, and the disease is now present throughout the world. In addition, there are estimated to be well over 1 million persons in the United States alone infected with human immunodeficiency virus (HIV). Many of these individuals have some symptoms at present, and they are at risk at developing AIDS over the next decade (1).

The discovery of human immunodeficiency virus (HIV) as the etiologic agent for this disease (2,3) enabled the consideration of therapy directed at the causative organism. As will be discussed in this chapter, a number of agents have been found to inhibit the replication of HIV in vitro, and one of these agents, 3'-azido-2',3'-dideoxythymidine, also known as azidothymidine, AZT, or zidovudine, has been shown to induce clinical improvement and prolong survival in patients with fulminant AIDS (4,5,6). This was the first demonstration that an established infection with a pathogenic human retrovirus was amenable to anti-viral therapy, and it has focused efforts to develop other anti-retroviral drugs for the treatment of this disorder.

Stages in the Life-Cycle of HIV: Targets for Antiviral Therapy

HIV is perhaps the most complicated retrovirus studied. In common with previously studied animal retroviruses, it contains genes encoding the core structural protein (gag), the outer envelope glycoprotein (env), and reverse transcriptase, or viral DNA polymerase (pol) (Figure 1) (7). It also contains sequences at either end of the genome called the long terminal repeats (LTR) which contain regulatory elements associated with the replication of the virus (7,8). HIV is quite unusual, however, in that it contains 5 non-structural genes tat-III, art/trs, 3'-orf, sor, and R, some of which are known to have important regulatory functions for viral replication (7,9,10). The very complexity of this virus and its life-cycle (Figure 2) provides the medicinal chemist with a variety of potential targets for antiviral agents.

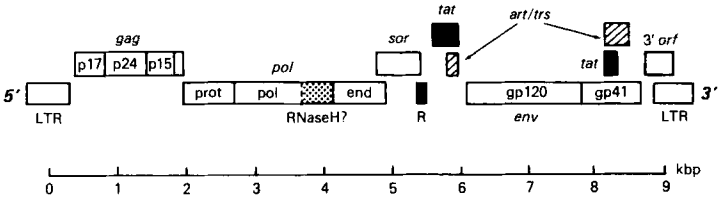


Figure 1. Gene structure of HIV.

Figure 2. Stages of the HIV Life-cycle Which May Be Targets for Therapy

Binding to CD4 receptor of Target Cell
Fusion of virus (possibly involves env gp41)
Entry into target cell
Uncoating of RNA
Reverse transcription (RNA→DNA, then DNA→DNA)
Degradation of RNA by RNase activity
Integration of DNA into host genome (by integrase)
Efficient transcription and translation of RNA
Ribosomal frameshifting
Viral component production and assembly
Viral budding
Infection by cell-free virus

Viral Binding - The first step of infection involves binding of an HIV virion to a receptor on a target cell. There is strong evidence that the T cell surface glycoprotein CD4 acts as a receptor for the viral gp120 env glycoprotein (11,12). After binding, HIV can form syncytia with target cells (3,7), i.e. fuse with the cells. This latter step is believed to be mediated by the fusion domain of the gp41 env glycoprotein. It has been shown that HIV gp120 env glycoprotein alone can kill $CD4^+$ target cells through syncytia formation without the cells being infected (13,14), and for this reason interventions which act at these early steps may be particularly useful.

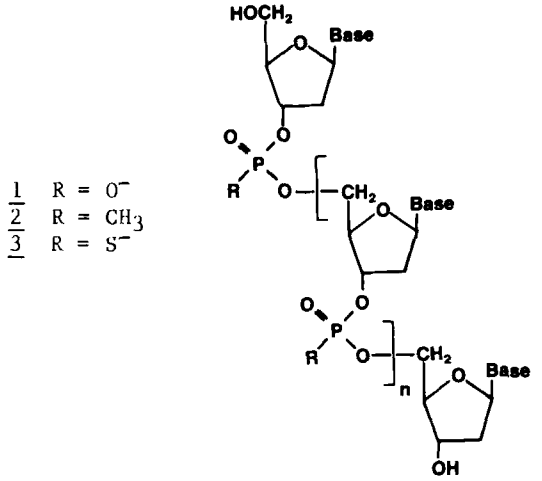
Antibodies to HIV can block viral binding and thus prevent infection (15,16); however, persons infected with HIV, for reasons which are not completely clear, develop only low titers of neutralizing antibody, possibly presenting problems for vaccine development. A solublized form of the CD4 receptor glycoprotein lacking the cytoplasmic and transmembrane domains can inhibit HIV binding and replication in target cells (17-21), and clinical trials with this agent are now being contemplated. Dextran sulfate (MW 7000 - 8000, sulphur content 17 - 20%) inhibits HIV replication at concentrations of 1 uM or higher, at least in part by blocking binding of the virus to a target cell (22-24). Finally, small peptide sequences of the env gp120 glycoprotein of HIV (including an octapeptide called "peptide T") have been reported by one group to inhibit viral binding and replication (25); other laboratories, however, have had considerable difficulty in confirming these results (26).

Viral Entry, Uncoating, and Reverse Transcription - After binding to the receptor, HIV enters a cell and the RNA is uncoated. The anti-influenza drug, amantadine, is believed to work by blocking viral uncoating (27), and it is possible that a similar approach will be effective against HIV. Following the uncoating of the virus, a complementary strand of DNA is copied from viral RNA by HIV DNA polymerase (reverse transcriptase). Subsequently, a second (positive strand) copy of DNA is made so that the genetic information is encoded in a double-strand form of DNA (7). The viral RNA is degraded by RNase activity which is believed to be mediated by a special domain in the reverse transcriptase (28). An orderly degradation of viral RNA is critical to the successful formation of proviral DNA, and drugs that block this step would be expected to impair viral replication. Reverse transcriptase is for all practical purposes a unique viral enzyme, and as will be discussed below, many of the drugs now being studied (including AZT and other dideoxynucleosides) act at this step.

Integration of DNA, Transcription, and Translation - HIV DNA may exist in a circularized form or may become integrated into the host genome through viral integrase (also believed to be a pol-encoded product) (29). At some time (in certain cases after a delay during which the virus can be considered latent), the viral DNA is transcribed to mRNA and genomic RNA and ultimately translated to form viral proteins and infectious virions. These steps utilize the biochemical machinery of the host cell. However, they are regulated by at least two viral gene products (tat-III and art/trs) (30,31). These two products are essential for efficient viral replication (32). In addition, at least one host cell protein (NF-κB) can bind to a sequence on the viral long terminal repeat (LTR) called κB and, through its interaction with this sequence, increase production of HIV (33). κB is an 11-base pair sequence which is also found in the κ-immunoglobulin gene enhancer region. The NF-κB product is produced in activated B and T cells, and can act synergistically with tat-III to increase viral production (33,34). It thus may be a biochemical mechanism by which T cell activation may lead to induction of latent virus.

The tat-III and art/trs gene products are, like reverse transcriptase, unique to the virus and as such represent potential targets for antiviral therapy. For example, tat-III encodes a small (86 amino acid) protein (30); it may be possible to determine its structure by x-ray crystallography and by this approach design drugs or molecular constructs which may block its active site. It may also theoretically be possible to block the binding of NF- B on the viral LTR and in this manner interfere with efficient viral production in activated T cells.

Viral replication may be affected by the use of "anti-sense" oligodeoxynucleosides. These are short sequences of DNA (1), or DNA which has been chemically modified (e.g. methylphosphonate oligodeoxynucleosides [2] or phosphorothioate oligodeoxynucleosides [3]) with base pairs which are complementary to a segment of the viral genome (37-40). (See also Chapter 30.) Such oligodeoxynucleosides could theoretically work by several mechanisms including hybridization arrest of mRNA or directly interfering with binding of a regulatory protein (such as tat-III) to its acceptor site. Unmodified DNA may not be effective because it is sensitive to degradation by nucleases. Phosphorothioate ana-



1 R = O^-
2 R = CH_3
3 R = S^-

logues of DNA (3), which have sulfur substitutions for one of the non-bridging oxygens, are resistant to these nucleases, and such analogues have recently been shown to inhibit the replication of HIV in vitro (39, 41). There are likely to be several mechanisms responsible for the action of these compounds, possibly including competition for template-primer at the level of reverse transcriptase. Interestingly, both sequence-specific and sequence non-specific inhibition by phosphorothioates may be observed (presumably because they operate through several mechanisms of action) (39,41).

Ribosomal Frameshifting - Ribosomal frameshifting is involved in the production of the gag-pol polyprotein (as a precursor fusion product) of certain retroviruses (35,36). This is a process by which ribosomes shift from one reading frame to another (as they move along mRNA) and therefore avoid premature termination of polypeptide synthesis. Gag-pol subsequently undergoes posttranslational cleavage to form the gag and pol gene products. Specific inhibitors which block ribosomal frameshifting could be effective as anti-HIV agents.

Post-translational Modification - The virally encoded proteins undergo secondary processing involving viral proteases, host myristylating enzymes, and host glycosylating enzymes (42-44). The protease responsible for cleavage of the viral gag polypeptide into species of approximately 24, 16, and 14 kD, for example, has been shown to be a pol gene product (44). Such post-translational steps can be targets for antiviral therapy (45-47). Viruses produced in the presence of inhibitors of trimming glucosidase (e.g. castanospermine) are defective in their syncytia formation and have reduced infectivity of target cells (46,47). Syncytia formation and infectivity are critically dependent on a normal pattern of glycosylation of the env proteins.

Viral Budding and Infectivitity - The last steps of viral replication involve viral assembly at the surface and release by budding. Interferons (particularly alpha interferon) are believed to act (at least partly) at this step (48,49), and interferon inducers (such as mismatched double-stranded RNA, or ampligen) may thus affect viral budding (50,51). However, ampligen may have other mechanisms of action against virus as well. A preliminary clinical trial has shown ampligen to be well tolerated in patients with AIDS or AIDS-related complex (ARC)(52), and larger studies to define its clinical activity are underway. Interferons may have a direct anti-tumor effect against Kaposi's sarcoma, a tumor which often complicates HIV infection, and they should not be viewed exclusively as anti-retroviral agents (49).

HIV has at least 8 genes, and the function of three of these viral genes, sor, R, and 3'-orf, are at present unknown or only partially understood (10,53,54). Studies with viral mutants have shown that the sor gene ("short open reading frame"), while not absolutely necessary for HIV virion formation or cell-to-cell transmission, is crucial for the efficient generation of infectious cell-free virions (53,55). As we know more about this and the other genes of HIV, it is possible that new approaches to therapy will become apparent.

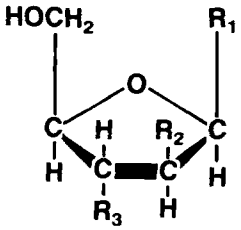
Compounds Acting at the Level of Reverse Transcriptase

As noted above, HIV DNA polymerase (reverse transcriptase) can for all practical purposes be considered a unique viral enzyme, and many of

the drugs which have been shown to inhibit HIV replication act by inhibiting reverse transcriptase. (See also Chapter 17.) In particular, a number of dideoxynucleosides (4-19), after being phosphorylated to a 5'-triphosphate moiety, are potent and selective inhibitors of reverse transcriptase and inhibit HIV replication in human T cells and macrophages (Figure 3) (56-66). In addition, several related compounds (for example certain didehydro-dideoxynucleosides [20,21]), have potent activity against HIV in vitro (Figure 4) (67-70). While there is an emerging body of structure-activity data, at present, the anti-retroviral activity of nucleoside congeners must be determined on a case-by-case basis.

Figure 3. Some dideoxynucleosides with anti-HIV activity in vitro.

	R_1	R_2	R_3	Approximate conc. with in vitro anti-HIV activity (μM)	Ref.
4	Thymine	H	N_3	1 - 5	56
5	Thymine	H	H	200	57
6	Thymine	H	F	4 - 20	58,59
7	Cytosine	H	H	0.5	57
8	5-F-cytosine	H	H	0.5	60
9	Adenine	H	H	10	57
10	Adenine	H	N_3	10	58,59
11	Adenine	F	H	10 - 100	61
12	2-F-adenine	H	H	10	62
13	2-Cl-adenine	H	H	10	62
14	2-Br-adenine	H	H	10	62
15	2-Amino-adenine	H	H	--	63
16	Hypoxanthine	H	H	10	57
17	Guanine	H	H	10	57
18	Guanine	H	N_3	5	64
19	Uracil	H	N_3	0.2	65

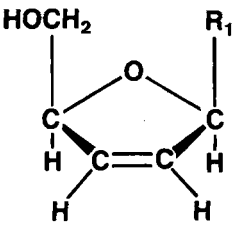


It is worth noting that the apparent activity, toxicity (and therapeutic index) of any antiviral drug in vitro will depend on the precise assay system used and will vary significantly according to the target cells, multiplicity of virus, and othr specific features. In particular, drugs will generally be more active (and yield a higher in vitro "therapeutic index") in assay systems that employ a low multiplicity of infection (58). Also, for reasons which are not clear, assay systems which employ MT-4 target cells are much more sensitive to thymidine analogues than are other target cells (59). For these reasons, one cannot directly compare the results obtained by different groups in different systems, and the anti-HIV concentrations given in Figures 3 and 4 must be viewed as only approximate.

Dideoxynucleosides - Dideoxynucleosides are 2'-deoxynucleoside analogues which have the 3' hydroxy (-OH) group replaced by a group which cannot form 3'→5' phosphodiester linkages. One mechanism by which they are thought to inhibit viral replication is by DNA chain termination (58,71). In addition, they may act as competitive inhibitors with normal nucleoside triphosphates for viral reverse transcriptase, with, in certain cases, K_i's in the range of 0.04 to 0.2 μM (72-74). The prototype compounds of this group (4, 7, 9, 12, and 14), which were not new compounds (75,76), were observed in 1985 to be potent inhibitors of HIV replication in human T cells (56,57). Some of the compounds (particularly com-

pounds 4, 5, and 17) had been shown to be inhibitory for certain murine retroviruses in murine cell cultures before pathogenic human retroviruses were recognized or before AIDS was a known medical entity (76-78). As detailed below, however, because of the differential phosphorylation of these compounds in different cells and other features which are still not understood, one cannot draw conclusions about their activity against HIV in human cells based on such animal models, nor can one extrapolate toxicity from such animal models at this time (79,80).

Figure 4. 2',3'-didehydro-2',3'-dideoxynucleosides



	R_1	Approximate concentration with in vitro anti-HIV activity (μM)	Refs.
20	Thymine	1 - 3	67-69
21	Cytosine	0.2 - 0.3	70,67

Dideoxynucleosides must undergo anabolic phosphorylation (activation) in target cells to a 5'-triphosphate active moiety. It is necessary for these compounds to be phosphorylated by the kinases of the target cells because HIV does not appear to encode for nucleoside kinases. This puts additional constraints on the structure of a potential drug. The kinases of different species may have marked differences in their ability to phosphorylate these drugs which may in turn affect their observed activity. Compound 7, for example is poorly phosphorylated in murine cells and has weak antiretroviral activity in such cells, while it is one of the most potent substances tested in human cells (79,80). Compound 5 is well phosphorylated in murine cells and has antiretroviral activity in such cells (78); however, it is a relatively poor substrate for human thymidine kinase and is only active in human T cells at 200 uM (57). Insertion of an 3'-azido group, however, yields AZT (4) which has substantial activity in human cells (56) and which induces immunologic, virologic, and clinical improvements (including enhanced survival) in persons with AIDS (4,6,81). AZT penetrates into the cerebro-spinal fluid (4,5,82) and can reverse certain cases of HIV-induced dementia (5,83). However, AZT can also induce substantial toxicity, particularly bone marrow suppression, and many AIDS patients cannot tolerate it for long periods of time (4,84).

Pyrimidine Dideoxynucleosides - Compound 4 is efficiently phosphorylated by human thymidine kinase (K_m = 3.0 μM); however, the rate-limiting step for its activation in human (but not mouse) cells appears to be the phosphorylation of the monophosphate (to diphosphate) by thymidylate kinase (K_m = 8.6 μM, V_{max} = 0.3% of V_{max} for dTMP) (72). It has been reported that human T cells exposed to 4 have decreased levels of dTTP and dCTP (72); however, the principal author of that report recently reversed the latter conclusion (85). dCTP levels, if anything, appear to be increased in T cells exposed to AZT (85). The effect of AZT on normal nucleoside metabolism remains an important topic for further research. It is possible that there may be considerable cell-to-cell differences in these effects.

As 4 is phosphorylated more rapidly than 5 in human cells, it has been hypothesized that the substitution in the 3'-position might be im-

portant in determining the rate of phosphorylation of thymidine analogues by human cells. In this regard, 6 has been found to have potent anti-HIV activity, although it appears to be more toxic to human T cells than is 4 (58,59). Other 3'-thymidine analogues (with a chloro-, bromo-, O-methyl-, or thiocyanato- group in the 3'-position), however, were not found to have anti-HIV activity in vitro (59). A didehydro-dideoxy thymidine analogue (20) is well phosphorylated by human T cells and inhibits HIV replication (67-69).

2',3'-dideoxycytidine (ddC, 7) is perhaps the most potent anti-HIV nucleoside tested so far in human T cells (57). ddC is resistant to cytidine deaminase (79,86), is well absorbed when given orally in humans and lower animals, and is mostly excreted unchanged in the urine (87,88). ddC penetrates into the cerebrospinal fluid of humans, although not as effectively as AZT (88). 5-fluoro-ddC (8), synthesized with the goal of conferring slightly increased penetration into the cerebrospinal fluid, also has potent anti-HIV activity in vitro (60). In the presence of thymidine, human T cells have increased phosphorylation of ddC (in part because of effects of thymidine-5'-triphosphate on ribonucleotide reductase) (89). Co-administration of these two substances might thus possibly yield a better therapeutic effect than ddC used alone.

Phase I studies of ddC in patients with AIDS or ARC have shown that it can decrease serum HIV p24 antigen (a measure of the viral load) and induce short term increases in T4 cells (a measure of immune function) (88). The dose-limiting toxicity appears to be a painful peripheral neuropathy which develops after 6 to 14 weeks on high doses of drug. Lower doses of ddC are presently being tested in patients to determine whether an anti-HIV effect can be observed without neuropathy developing. ddC forms a choline derivative in human cells (79), and it is a matter of speculation whether this contributes to the neuropathy. Because the toxicity of ddC is different than that of AZT, an alternating regimen of the two drugs has been tested (AZT one week alternating with ddC for one week). Preliminary results suggest that this regimen can be tolerated for over 8 months without inducing peripheral neuropathy or significant bone marrow suppression in some patients (88). It thus appears that periodic rest periods can provide a way to reduce the neurotoxicity associated with ddC.

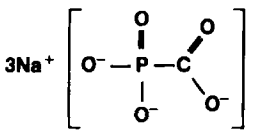
Purine Dideoxynucleosides - A number of purine analogues (9-18) also have activity against HIV in vitro (57-59,61-64). 2',3'-dideoxyadenosine (ddA, 9), which inhibits HIV in vitro at 10 uM (57), is now entering Phase I testing at the National Cancer Institute. The "in vitro therapeutic index" of ddA in human T cells is greater than that of AZT (57), and it has relatively little toxicity against bone marrow precursor cells in vitro (90). ddA is rapidly deaminated in human plasma by adenosine deaminase to form 16 (2',3'-dideoxyinosine, ddI) (91); however, ddI is also active against HIV in vitro (57). ddI can be metabolized back to ddA-5'-triphosphate in cells, and ddA can thus be a "pro-drug" for itself (92). Several 2-halo derivatives of ddA (12-14) are resistant to deamination by adenosine deaminase and preserve their activity against HIV (62). Also, a 2-amino derivative (2,6-diaminopurine-2',3'-dideoxyriboside, 15) is only slowly metabolized by adenosine deaminase and acts as a competitive inhibitor of this enzyme (63); 15 and its metabolic product 17 both have activity against HIV (57,63).

ddA is rapidly cleaved (to adenine and dideoxyribose) by exposure to acid conditions (91), a property which may reduce oral absorption in

humans. A ddA analogue, 11, with a 2'-F in the sugar moiety, is resistant to acid degradation and has anti-HIV activity in vitro (61). However, it is likely that relatively simple clinical maneuvers (such as the concomittant administration of antacids or the use of enteric coated capsules) may circumvent many problems associated with the acid lability of the N-glycosidic bond of ddA. For long-term oral use, ddI may conceivably be preferrable to ddA because free adenine could theoretically exert some renal toxicity, while hypoxanthine (the acid cleavage product of ddI) is not thought to be toxic.

Other Compounds - Dideoxynucleosides are not the only family of compounds which act at the level of reverse transcriptase. Phosphonoformate (22), a pyrophosphate analogue with activity against a number of DNA viruses including cytomegalovirus (CMV), inhibits HIV replication in vitro (93) and is undergoing clinical testing at present. Also, ansamycin, a rifamycin analogue, has some in vitro activity against HIV (94) and is being tested in vivo.

22



Combination Therapy

A general approach which is presently being explored is the use of combination therapy against HIV. As noted above, drugs (e.g. ddC and AZT) with anti-HIV activity and different toxicities may yield reduced toxicity when given sequentially (88). Also, certain drug combinations may yield improved therapy when given at the same time. For example, acyclovir (an anti-herpes drug with little anti-HIV activity per se) can potentiate the in vitro activity of AZT against HIV without potentiating its toxicity (95). The mechanism for this effect is not known. A pilot study showed that the pharmacokinetics of these two drugs are independent of each other and that a regimen of 100 mg AZT and 800 mg acyclovir given to AIDS or ARC patients every 4 hrs was tolerated for a 10 week period of time (96). While clinical, immunologic, and virologic effects on this regimen were similar to that observed with higher doses of AZT given alone (96,97), one cannot conclude at this time whether this regimen offers any clinical advantages over AZT as a single agent.

A number of other agents, including dextran sulfate, ampligen, and interferon alpha, have also been shown to have greater-than-additive activity against HIV when tested in combination with AZT in vitro (22,51, 98). As discussed above, these agents may act at different steps of the HIV replicative cycle than AZT, and this may be the basis for their synergistic effects. Not all drugs, however, have such activity. Ribavirin, a nucleoside analogue with activity against a number of viruses and partial activity against HIV in vitro (27,99), was recently shown to inhibit the activity of AZT, possibly because it reduces AZT phosphorylation (100). Ribavirin as a single agent has not been proven to suppress HIV replication in vivo (as assessed by serum HIV p24 antigen), nor has it been proven to confer a clinical benefit to patients with AIDS (101).

Conclusion

Through an intense collaboration involving an agency of the federal government, a private sector firm, and several academic medical centers, AZT was brought from a laboratory observation to prescription drug status in a little over two years. While AZT is not a cure for AIDS and it does have prominent side effects in some patients, its development has increased the survival and quality of life of many patients and reduced some of the pessimism surrounding the AIDS epidemic. It also demonstrated for the first time that an established retroviral infection of humans was amenable to therapy. Drug development in this area can proceed quickly, and medicinal chemists can potentially see new compounds brought into clinical use more rapidly in AIDS than any other area of study. At present, the most immediate promise for effective therapy of advanced HIV infection is a combination of dideoxynucleosides with non-overlapping toxicity. At the same time, a number of stages of the viral life-cycle of HIV are worth exploring as possible targets for therapeutic intervention.

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Chapter 27. Enzyme Changes Linked to Anticancer Drug Resistance

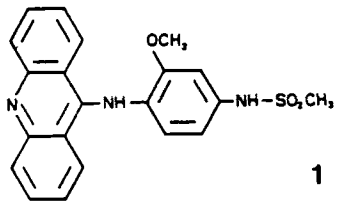
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Introduction - Cancer chemotherapeutic agents are effective inducers of cytostasis and are eventually cytotoxic. Unfortunately, therapeutic responses to these agents are most frequently transient or inadequate, where an initial disease remission will be followed by relapse and ultimately progression. Subsequent attempts to control the cancer with additional drug therapy are, sooner or later, ineffective. The underlying reasons for ineffectual chemotherapy lie in the tumor cells' propensity to express or develop resistance. The "ability" of tumor cells to adapt to a selective pressure is frequently the result of their overexpressing certain enzymes which can protect against subsequent drug exposure. It is the intent of this review to summarize a number of adaptive enzyme changes which have recently been shown to be of relevance to anticancer drug resistance.

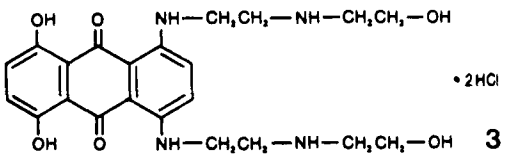
Topoisomerases - DNA topoisomerases can break and reseal one or more strands of DNA in such a manner that transient DNA unwinding, decatenation or unknotting can occur. The integrity of the broken strand is maintained by the topoisomerases forming an intermediate complex with the DNA, covalently linked to a single terminus of the break site by a phosphotyrosyl residue. The distinction between two groups of mammalian topoisomerases has some relevance to their potential to act as drug targets. Topoisomerase I is monomeric with a molecular mass of approximately 100 kD and functions by breaking and resealing a single strand of DNA without a requirement for ATP (1,2). The enzyme is covalently bound to the 3' end of the break site. Topoisomerase II is a homologous dimer with each subunit having a molecular mass of approximately 175,000 kD. It requires ATP for full activity and cleaves both strands of DNA. The enzyme becomes covalently bound to the 5' termini of break sites (1,2). Both types of topoisomerase are predominantly located in the nucleus with approximately 75% of topoisomerase II associated with the nuclear protein scaffold of metaphase chromosomes (3-5), while in interphase cells the enzyme is tightly associated with the nuclear matrix (6) and closely associated with the multi-enzyme replication complex at the anchorage sites of nascent DNA onto the three-dimensional structural protein lattice (7-9).

Topoisomerase II and Drug Resistance - A diverse range of anticancer drugs are known to have intercalative properties. Such agents include actinomycins; acridines (4'-(9-acridinylamino)methanesulfon-m-anisidide, m-AMSA, 1); anthracyclines (adriamycin, 2); anthracenediones (mitoxantrone, 3); elipticines (2-methyl-9-hydroxy-ellipticinium) and epipodophyllotoxins (4-(4,6-O-ethylidene-beta-D-glucopyranoside; VP-16, 4). Recently, these drugs have been shown to induce protein-associated DNA strand breaks, by stabilizing, perhaps through intercalation, a "cleavable complex" between topoisomerase II and DNA. Specific studies using m-AMSA have shown a correlation between formation of DNA-topoisomerase II cleavable complexes and subsequent protein-associated DNA strand breaks and cytotoxicity (10,11). The induction of DNA

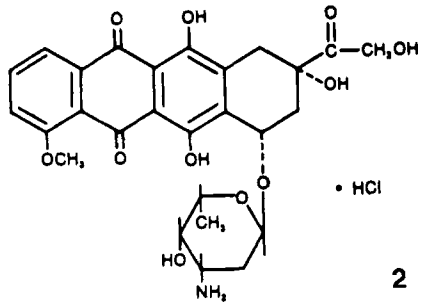
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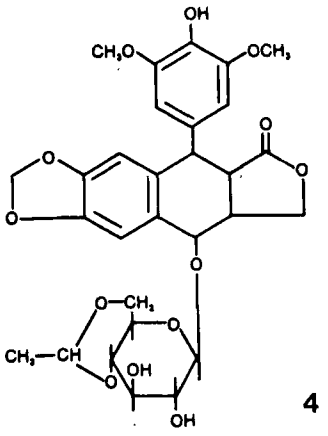
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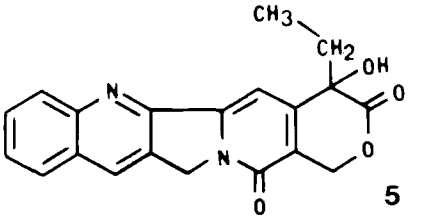
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cleavage by each of the different classes of intercalators is a non-random event. Each of the drug classes mentioned above produces a characteristic and unique pattern of cleavage sites (12,13). The specific association of topoisomerase II with the cleavage site was confirmed by purifying the protein from a calf thymus DNA-cleavage complex and showing both biochemical and immunological identity with a purified topoisomerase II (10,13-16).

Phenotypic expression of topoisomerase II activity may be altered either quantitatively or qualitatively (17). Modified expression of topoisomerase II has been identified in a number of tumor cell lines expressing resistance to m-AMSA (17-19); VP-16 (17,20-22); 9-hydroxyellipticine (23,24); adriamycin (25) and nitrogen mustard (26). The eukaryotic situation has an analogy with the development of bacterial resistance to prokaryotic topoisomerase (DNA gyrase) inhibitors such as nalidixic acid (27).

In contrast to the majority of examples where drug resistance can be correlated with increased amounts of specific enzymes, resistance to topoisomerase II acting drugs is mediated by a decrease in its levels. The amount of intracellular topoisomerase II will vary contingent upon the proliferative status of the cell culture. Resting or plateau phase cells have minimal enzyme activity, while cells in mid-log phase can have 10^5 to 10^6 copies/cell (28). These differences correlate with drug sensitivity profiles. Cells in mid-log are considerably more susceptible to epipodophyllotoxins than cells in resting phase (29). Topoisomerase II levels in B lymphocytes from chronic lymphocytic leukemia (CLL) patients or in B lymphocytes from normal patients were below the levels of detectability by standard methodologies. The fact that such cells are refractory to treatment with adriamycin is consistent with these low levels (30). The rat Dunning prostate tumor has been found to be comparatively low in topoisomerase II levels when compared to an anaplastic, more aggressively growing derivative of this tumor (31). Thus, there appears to be a relationship between the tumor cell's physiological growth state and topoisomerase II activity. In tissue culture, the more quiescent the cells, the less enzyme activity and the least likely the cells to succumb to cytotoxicity by topoisomerase active drugs. In

animals and in human leukemics the slower growing or least anaplastic tumors fail to respond to these same drugs.



5

Topoisomerase I and Drug Resistance - Cultured and normal tumor cells appear to maintain steady-state levels of topoisomerase I throughout cell growth and division (32,33). This would suggest that cells should be equally sensitive to any drug which inhibits topoisomerase I at all stages of growth. Camptothecin, 5, an antitumor agent in both animals and man (34,35), has been shown to induce site-specific single strand cleavage of DNA in the presence of DNA topoisomerase I (36). Camptothecin's cytotoxic activity has been correlated with its effects upon topoisomerase I in mammalian cells (37-39). It has been proposed that camptothecin causes topoisomerase I to be covalently attached to the 3' end of DNA which is undergoing replication (28). This has the effect of preventing normal replication both through a direct inhibition of the breakage-resealing activity of topoisomerase I and by producing an enzyme-DNA complex which would have the potential to interfere with the orderly progression of the replication fork.

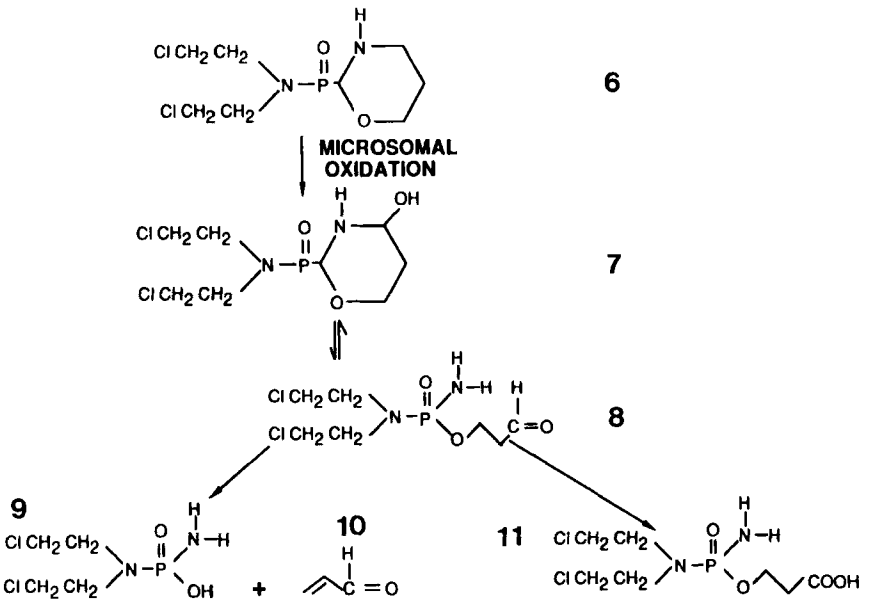
Drug resistant topoisomerase mutants have been isolated both in bacteria (17) and in mammalian cells (40). Alteration of the binding properties of camptothecin for the modified topoisomerase has been established as the mechanism of resistance in the eukaryotic cells (40). These preliminary observations suggest that similar topoisomerase I changes will be found in other camptothecin resistant mutants.

Alkyl Guanine Transferase - Cellular mechanisms which repair DNA damage have long been considered significant to the expression of drug resistance. Although a logical concept, the relationship between increased DNA repair potential and resistance to nitrogen mustards remains somewhat equivocal (41). A relatively unusual form of DNA repair has been definitively linked to resistance to nitrosoureas. Both chloroethyl and methyl nitrosoureas decompose spontaneously to yield isocyanate species and halogenated alkylcarbonium ions. The latter reacts to form a monoadduct with the oxygen at the 6-position of guanine, creating an O^6 chlorinated alkyl guanine residue (42). Dechlorination of the modified guanine residue leads to the formation of a second electrophile which can alkylate a second nucleophile in such a way as to produce a DNA crosslink, a lesion considerably more toxic to a cell than the preliminarily formed monoalkylation. Many cells have an enzymatic system (O^6 alkylguanine transferase) designed to repair the monoadduct before it can produce a crosslink. Cells which possess O^6 alkylguanine transferase activity have been designated Mer+ or Mex+, whereas those that are deficient are Mer- or Mex- (43). The removal of the alkylation is achieved through an exchange, whereby the alkyl moiety is transferred from the O^6 position of guanine to a cysteine residue of the enzyme. Once this exchange is achieved the enzyme is inactivated. The process is thus a stoichiometric "suicide" exchange reaction.

Although nitrosoureas are effective in the treatment of murine neoplasms, these agents have proved to be of limited value in the management of human cancer (44). A rationale for this observation has recently emerged. The majority of murine tumors used for screening purposes have been found to express Mer- phenotypes, while human tumors are uniformly Mer+ (45). In addition, only one in five human tumor cell lines were found to be deficient in O^6 alkylguanine transferase (43,46,47). Because

the enzyme is a "suicide substrate" for alkyl groups, it will be inactivated in a stoichiometric manner in the presence of alkylated DNA (47,48). Preclinical studies have shown that pretreatment of resistant human tumor cell lines with a DNA methylating agent such as streptozotocin produces O^6 methylguanine lesions in the DNA of target cells. These methylations are capable of saturating the O^6 alkylguanine transferase system, with the result that Mer+ cells will then be sensitive to the cytotoxic effects of a subsequent treatment with a chloroethylnitrosourea (49,50). Such studies have been extended to include other classes of drugs such as alkyltriazenes (51), since these agents possess a pharmacology which produces active species similar to chloroethylnitrosoureas. A rational approach to the clinical management of resistant tumors is presently underway (52). A phase I/II clinical trial has been instigated in order to test the efficacy of streptozotocin pretreatment followed by the chloroethylnitrosourea BCNU (52). Although it is too early to report antitumor effects, normal tissue toxicities have been documented. These include thrombocytopenia and hypophosphatemia at drug concentrations of 2000 mg/m^2 for streptozotocin and 125 mg/m^2 for BCNU.

Aldehyde Dehydrogenase - The expression of aldehyde dehydrogenase (ADH) is known to vary considerably within and between human populations and its role in the metabolism of alcohol has been widely appreciated (53-56). In addition to the function of the enzyme in metabolizing a common xenobiotic such as alcohol, recent studies have delineated a role for ADH in the metabolism of the anticancer drug cyclophosphamide, 6, (57). Cyclophosphamide has been utilized in high doses in combination with radiation therapy in order to reduce tumor burden and induce immunosuppression in leukemia and lymphoma patients prior to autologous or allogeneic bone marrow transplantation (58). Cyclophosphamide is a prodrug and possesses no cytotoxic potential per se (59,60). Both cyclophosphamide and a related oxazaphosphorine, ifosfamide, (61) undergo



microsomal oxidation which in the case of the former drug yields 4-hydroxycyclophosphamide, 7, and its tautomer aldophosphamide, 8. In the mixture, 7 predominates at a ratio of approximately

5:1 over its tautomer (62). 7 is not an active alkylating species, but upon decomposition, phosphoramide mustard, 9, and acrolein, 10, are produced; the former of these is an active electrophilic species. 9 is approximately four-fold less cytotoxic than 7, primarily because the latter is more effectively taken up by cells (59,60). The principle urinary metabolite of cyclophosphamide is carboxyphosphamide, 11, which is an oxidized product of aldophosphamide, 8, with the enzymatic conversion catalyzed by ADH, primarily in the liver. Carboxyphosphamide can be further metabolized to nor-nitrogen mustard, 12, a compound which is a relatively ineffective alkylating agent. Thus, there is a critical bifurcation of the metabolic pathway to the production of an active cytotoxic alkylating species, 9, or an inactive urinary excretion product, 11.

Recent evidence suggests that the quantity of ADH in either tumor or normal tissues plays a significant role in determining the sensitivity/resistance profile of that tissue to oxazaphosphorines (57,63,64). ADH activity has been found to vary quantitatively and qualitatively in rat and human tissues (55,57,65-68). A number of isoenzymes have been isolated and characterized (69). There is evidence that mutant forms of the enzyme exist (70) and that xenobiotics are capable of inducing activity in certain target tissues (67,71). Therefore, certain normal and tumor tissues may have intrinsically high levels of ADH which would make such tissues comparatively resistant to cyclophosphamide. In addition, induction of ADH by exposure to non-lethal concentrations of cyclophosphamide could lead to an acquired resistance phenotype in target tissues. A cyclophosphamide resistant mutant L1210 was isolated following repeated drug treatment of mice with non-lethal, non-curative cyclophosphamide doses (72). This mutant cell line had only limited cross-resistance to other bifunctional alkylating agents (73). More recent studies have found the cell line to be resistant to 4-hydroxycyclophosphamide compared to the parent cell line, but that sensitivity to phosphoramide mustard remains unchanged (57). DNA crosslinking, a drug effect which can be correlated with cytotoxicity, occurred only at very low levels in the resistant cell line when exposed to 4-hydroxycyclophosphamide (74). Since DNA crosslinking was found to be high in the sensitive cell line and is known to be the consequence of phosphoramide mustard alkylation, a mechanism of resistance which is a consequence of altered metabolism was inferred (57,74,75). Levels of ADH activity were measured in the L1210 cell lines. The resistant cells had an activity of 24.3 nmol/min/mg compared to 0.12 for the sensitive (75). This large difference was accompanied by a significant alteration in the metabolic products of 4-hydroxycyclophosphamide. High levels of the non-toxic carboxyphosphamide were present in extracts of resistant cells, while higher levels of the more toxic unchanged 4-hydroxycyclophosphamide and phosphoramide mustard predominate in the sensitive cells (75). Such data are strongly indicative that the elevated ADH in resistant cells detoxifies the drug and thereby protects against its cytotoxic activity. Elevations in ADH activity were found to be independent of gene amplification(s). The purified enzyme has properties identical to that of mouse liver enzyme, thus suggesting that the elevated ADH activity in resistant cells is a result of induction of mRNA, rather than an alteration in enzyme/substrate binding affinities (57). P388 murine lymphocytic leukemia has also been found resistant to cyclophosphamide through elevated ADH activity (76). Although total ADH activity per cell was only half that of the resistant L1210 cell line, this still approximated that which is found in mouse liver (57) and correlated with reduced levels of DNA crosslinking.

Treatment of bone marrow suspensions with a stable analogue of 4-hydroxycyclophosphamide, 4-hydroperoxycyclophosphamide, could kill leukemic cells without adverse effect upon the bone marrow progenitor cells (58). This procedure is now utilized in treating human leukemic and lymphoma patients prior to autologous bone marrow infusion (58). Analysis of the differential sensitivity of bone marrow progenitor cells to hydroperoxycyclophosphamide has suggested that a hierarchy of response is expressed (77-79). By using inhibitors of ADH, this differential in sensitivity could be removed (64), once again suggesting a causal link between elevated ADH and response to the oxazaphosphorines. In normal hematopoetic cells, ADH levels may decrease in certain differentiated lineages (57). This could suggest that cellular differentiation may be important in determining the expression of ADH in both normal and malignant cells.

Future considerations should be given to the potential for combining cyclophosphamide with inhibitors of ADH. The therapeutic index of such a combination would only be favorable if extra damage to normal tissues could be minimized. Alternatively, pretreatment biopsies of tumor material could be assayed for ADH. Only those patients whose tumors were low in ADH activity would then receive therapy with cyclophosphamide. It should also be noted that other potential mechanisms of acquired or intrinsic resistance to cyclophosphamide have been cited. These include altered profiles in other detoxifying enzymes such as glutathione S-transferases (41).

Glutathione S-Transferases - The relevance of glutathione (GSH) to alkylating agent resistance in both prokaryotes and eukaryotes has been appreciated for some time (80). Glutathione S-transferase (GST) isoenzymes have been analyzed with respect to their role in xenobiotic metabolism in liver and kidney as well as in a number of other normal tissues (81). These multifunctional enzymes catalyze the conjugation of hydrophobic and electrophilic compounds, as well as endogenous substrates (i.e., heme, bilirubin, steroids, leukotrienes, prostaglandins) with glutathione, resulting in the formation of a number of intermediate products. In the case of drug detoxication, the primary excretable products are usually more water soluble than the parent compounds.

Table I.
Nomenclature Comparisons for GST Isozyme Subunits

Subunit		Gene Family	
Old	New	Human	Rat
Ya	1	α	I
Yc	2	α	I
Yb_1	3	μ	II
Yb_2	4	μ	II
	5	μ	II
Yn	6	μ	II
Yf or Yp	7	π	III
Yk	8	α	I

Several isoenzymic forms of GST exist (Table I) that differ in their expression between tissues in the same animal and between species (81-86). Because of the potential for inter-tissue differences in the quantitative and qualitative expression of individual isoenzyme subunits, these isoenzymes may help to explain the differential sensitivity of tumor cells to the toxic effects of chemotherapeutic drugs.

A number of anticancer drugs are substrates for GST isoenzymes. Among these, the alkylating agents predominate (41,87-90). The GST isoenzymes catalyze the conjugation of these agents with GSH, a process which increases both the rate and extent of drug detoxication (42). Chlorambucil and melphalan can induce GST activity in target tumor cells (90) and this effect could be a causal link between acquired resistance and prior exposure to chemotherapy. A

number of other cell lines which elicit resistance to chemotherapeutic agents have been shown to have elevated GST activity (Table II).

The increase in GST activity in the rat breast carcinoma was attributable to an increase in the Yc subunit (91), a basic subunit which is a product of the same gene family as Ya (101). Subcellular distribution studies have suggested that a microsomal GST subunit similar to the Yc in molecular mass and immunoreactivity, but distinct with respect to pI, is also present in the rat breast carcinoma line resistant to chlorambucil (102). Since microsomal metabolism of chlorambucil occurs, this subunit may also be important in the detoxication of the drug. The Ya/Yc family of subunits appears to be specifically overexpressed in alkylating agent resistant cells (nitrogen mustards and nitrosoureas), suggesting that control of expression of the I (rat) or α (human) gene products may be affected by these drugs or their metabolites (90-93,95).

Table II. Elevation in Glutathione-S-transferase Activity in Resistant Cell Lines

DRUG	CELL LINE/TYPE	GST ACTIVITY	REFERENCE
Chlorambucil	Walker 256	x5	90,91
	CHO	[x2-4]	92,93
Cyclophosphamide	Normal murine bone marrow	[x2-8]	94
	rat Yoshida sarcoma	x6	95
Adriamycin	MCF7 human breast carcinoma	x45	96
Mitomycin C	human colon ca. HCT 11	x2	97
Chlorambucil/5FU/cis Pt	human ovarian carcinoma biopsy	x6	98
BCNU	rat 9L brain tumor	x2	99
Cisplatin	human lung cancer cell lines		100

The Ya/Yc family of isoenzymes is also involved in the catalytic peroxidation of certain prostaglandins in the synthetic pathways of these eicosanoids (103-105). Prostaglandins are involved in many processes related to cell division in tumor cells (106), thus the perturbations of prostaglandin synthetic pathways in drug resistant cells may provide an avenue for drug therapy. Synergistic cell kill has been achieved by combining nitrogen mustards such as chlorambucil or melphalan with prostaglandin synthesis inhibitors such as indomethacin or flurbiprofen (107, 108). Specific inhibitors of GST isoenzymes such as ethacrynic acid or piriprost (6,9-deepoxy-6,9[phenylimino]-$\Delta^{6,8}$ prostaglandin I_1) have also been shown to give enhanced cytotoxicity when given in combination with chlorambucil or melphalan (109), suggesting that other agents with GST inhibitory activities may enhance the cytotoxicity of alkylating agents (110-113).

One of the most ubiquitous GST isoenzyme subunits is Yf (rat) or π (human). This subunit is highly expressed in most tumor cell lines and is the predominant form found in many human tissues, including tumors (41). It is known to be present in large amounts in placental tissue (116) and is also elevated in a number of preneoplastic lesions suggesting that it may be a tumor marker (114-119). Although constitutively highly expressed, GST π activity is significantly overexpressed in cells with the multidrug resistant phenotype, MDR, (96,120). This isoenzyme

has selenium-independent glutathione peroxidase activity, a property which may be of relevance in detoxifying lipophilic hydroperoxides and fatty acid hydroperoxides formed following treatment with anthracyclines such as adriamycin (121,122). This isoenzyme is also involved in eicosanoid synthesis, thus the high levels found in tumor tissue may have dual functions (103).

One of the major issues which requires clarification is how prior exposure to sublethal concentrations of anticancer agents induces the increased expression of GST isoenzyme subunits. To date, no definitive evidence for gene amplification has been found and indications are that increased transcription is responsible (41). If a second messenger system has a role in this induction process, it has yet to be identified.

Conclusions - Evidence suggests that tumor cells can quantitatively or qualitatively alter their expression of enzymes which interact with anticancer drugs. Exposure of cells to such drugs provides a selection pressure favoring the establishment of cell populations which have successfully adapted to survival in the presence of the drug. Improved understanding of these events is leading to new strategies for more effective

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Section VI — Topics in Chemistry and Drug Design

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Chapter 28. Applications of NMR Spectroscopy to Protein Structure Determination

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Introduction — Until recently, single crystal X-ray crystallography represented the only practical experimental technique for the detailed study of protein structure and conformation. Although extremely useful, it is limited to the study of proteins for which a suitable single crystal is available. With the advent of high magnetic field spectrometers and two dimensional techniques, nuclear magnetic resonance spectroscopy (NMR) now compliments crystallography as a means of determining macromolecular conformations. NMR provides the opportunity to study the conformation of proteins in non-crystalline environments, most notably in aqueous solution or in micelles. A number of reviews on the subject have recently appeared (1-4).

The key to using NMR to elucidate the three-dimensional structure of macromolecules lies in the information which can be derived from the interactions amongst large numbers of protons. It is first essential to assign all of the individual resonances to specific protons (5). Until recently, this process was rate limiting due to the highly complex nature of the proton spectra of large polypeptides. The emergence of two dimensional NMR methods has greatly simplified this exercise. Two experiments, the first (COSY; **co**rrelated **s**pectroscopy) yielding information about through-bond interactions between protons, and the second (NOESY; **n**uclear **O**verhauser **e**nhancement **s**pectroscopy) about through space-interactions, make it possible to ascertain the amino acid composition, establish the sequence of these amino acids (5), and fully assign the proton spectrum (6-8). The NMR data can further be used to define the secondary structure of the protein (9). Finally, a three-dimensional structure can be determined, using constraints on interatomic distances (10,11) and backbone torsional angles derived from the NMR data (12). Several computational methods are available to accelerate the process of sampling the conformational space allowed by the experimentally derived constraints (13-19).

This chapter will review the experimental procedures and computational methods used in the determination of the spatial conformation of protein molecules. Since these subjects have been reviewed elsewhere (4,20,21), their utility will be illustrated by a survey of reported results with emphasis on a small number of representative cases. Other topics such as conformational states and protein dynamics, receptor-ligand interactions, use of NMR and site-directed mutagenesis, and future directions will also be discussed.

The Strategy for Structure Determination

Of the various magnetically susceptible nuclei in a protein (e.g., ^{1}H, ^{13}C, ^{15}N), the proton provides the greatest quantity and diversity of information due to its ubiquitous nature and high sensitivity (22). However, these very assets present practical limitations to the process

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of structure determination of even the smallest of proteins. Elucidation of the three-dimensional conformation of a protein requires a quantitative treatment of interproton coupling constants and nuclear Overhauser enhancements (4) . Extraction of this information from the one-dimensional proton spectrum of even a small protein is daunting due to the severe overlap of resonances. Two-dimensional (2D) NMR methods achieve the necessary simplification, and their use has allowed the three-dimensional structure of proteins up to approximately 10 kDa in size to be solved (3).

2D-NMR Techniques — A detailed review of the principles underlying two-dimensional NMR methods has recently been published (23). In summary, a 2D-NMR spectrum is a function of two frequency variables, S(w1,w2), which result from the Fourier transformation of an NMR signal dependent on two time variables, F(t1,t2). Quite a number of different 2D experiments have been developed for the study of through-bond couplings, or J-connectivities, between atoms separated by small numbers (e.g., 3 or 4) of bonds (4,20,23-27). Of these experiments, the H-H COSY technique is the most generally useful. A COSY spectrum is a two-dimensional map with two types of signals. Those which lie on the diagonal (w1 = w2) contain the same information as the proton spectrum itself. Those off of the diagonal indicate connectivities arising from coupling between protons and are of most interest (4). The net result is that the COSY spectrum affords all of the conventional spin-spin decoupling information with a single experiment. Using the J-connectivities derived from the COSY spectrum, all of the spin systems in a protein molecule can generally be identified, although signal overlap can still present problems. In such cases, other related experiments such as double quantum spectroscopy, relayed COSY and total quantum spectroscopy may be employed to complete the spin system identification (4). To a good approximation, the protons contained within a given amino acid residue can be treated as an isolated spin system. Thus, identification of all the spin systems will define the amino acid composition of a protein. Figure 1 illustrates two randomly chosen amino acids as they might appear in a polypeptide chain. The arrows marked J indicate the inter-proton interactions of interest within one spin system.

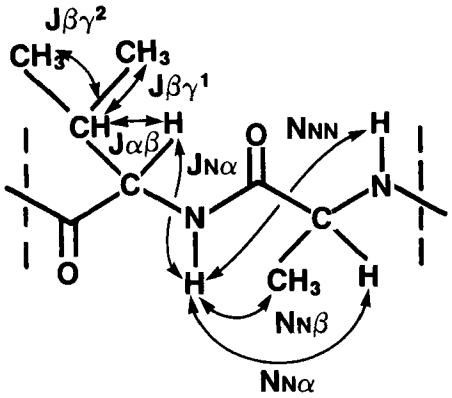


Figure 1 — Important Spin-Spin (J) and Through Space (N) Interactions Used in the Structure Determination of Proteins.

To complete the spectrum assignment, an understanding of how these various spin systems might interact with one another is required. Thus, if the position of a given amino acid (or spin system) in a sequence of residues can be defined on the basis of some observed interaction with its neighbors, then an unambiguous assignment of the proton spectrum should be possible by combining this information with the J-connectivities previously established (5). Again, 2D-NMR offers a solution to the problem. Nuclear Overhauser enhancements (NOE's) are through-space magnetization transfers from one nucleus to other nearby nuclei via dipole-dipole relaxation. In the study of small molecules, steady state NOE's

have been shown to be a sensitive measure of the interatomic distance between a perturbed and an observed nucleus (28). However, due to spin diffusion, these steady state NOE's are much less useful in the study of large molecules. Instead, the fact that the buildup of an NOE is a time dependent process can be exploited. The initial buildup rates of NOE's in macromolecular systems are inversely proportional to the sixth power of the distance between the perturbed and the observed nuclei (28). A 2D experiment (NOESY) allows for the observation of all of the proton-proton NOE's in a protein simultaneously, despite the severe overlap of signals in the proton spectrum. Additionally, if one runs a series of NOESY experiments in which a short mixing time is varied, then a semi-quantitative estimate of the distance between any two nuclei for which an NOE is detected can be made (6). In practice, this distance lies in the range of 2.0 to 4.5 Angstroms. Figure 1 illustrates the interactions (N) which are of greatest importance in the use of NOE to determine amino acid sequence. In virtually all instances, one of these three distances will be sufficiently small for a strong NOE to be observed. Other NOE's between nuclei in amino acids that are not adjacent to one another are useful for sequencing regions of a protein where there is a high degree of secondary structure (9). Even with the use of NOESY spectra, signal overlap can at times be so great that complete sequencing by this method is not possible. In these cases, other experiments such as homonuclear and heteronuclear long-range correlations can be useful (4). A very recent report describes a computer-assisted alternative to the sequential approach just described (17). This strategy, known as the main chain directed approach, relies on NOE pattern recognition and has a much decreased dependence on J-correlated spectroscopy. No report of a spectrum assignment for an uncharacterized protein using this method has yet appeared, although it is predicted to offer increased efficiency relative to the sequential approach.

In order to gain some insight into the conformational aspects of the structure of the protein, elements of secondary structure are generally the first to be considered. It has been shown that proton-proton NOE's can be used for the identification of helical, β-sheet, and tight turn secondary structures in small globular proteins (9). Additionally, *cis* peptide bonds can be readily identified using these NOE's. The NOE data is used quantitatively to determine interatomic distances. From measurements of the magnitude of the coupling constant between the amide and C-α protons derived from a phase sensitive COSY spectrum, the value of one of two key back bone dihedral angles, ϕ, (see Figure 2) can be determined using a modified version of the Karplus equation (12). The experimentally measured value of ϕ can be used as an independent means of identifying helical secondary structure, and as supporting evidence for the characterization of β-sheets (12). Finally, it is possible to make use of the exchange rates of amide hydrogens with deuterium when D_2O is used as a solvent to assess qualitatively the presence of helical or β-sheet secondary structure (3).

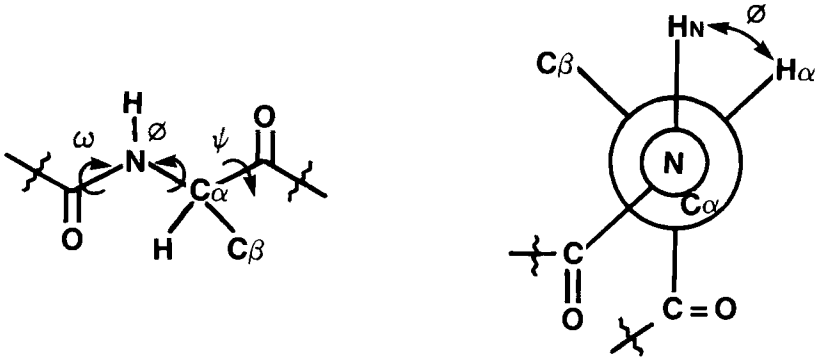


Figure 2 — Important Backbone Dihedral Angles in Polypeptides

Extensive computational treatment of proton-proton NOE's allows for the direct determination of tertiary protein structure (10,13,30,31). In this process NOE's between nuclei in amino acids far apart in the primary sequence are considered along with those used previously to define the sequence and secondary structure. These longer range interactions are crucial to the analysis of tertiary structure, as they are indicative of how the protein folds in three dimensions (3). In practice, the internuclear distances are defined as a range of allowed separations based on the magnitude of the NOE's between the nuclei in question. Other distance constraints can be added to those available from NOE's. These include distances defined by standard bond lengths and angles for atoms one or two bonds apart, those derived from known disulfide bridges between cysteine residues (32-34), and those obtained by conversion of the known backbone dihedral angles to distances for atoms separated by three bonds (3).

Computational Methods — Of the various algorithms used to calculate a structure from the experimental data distance geometry and molecular dynamics methods are the most common (13-19). Distance geometry methods are well suited to the task since they require data to be in the form of distances between points (21). Using a computation based on matrix mathematics, distance geometry methods calculate a structure with Cartesian coordinates that best accommodates the distance constraints. Since the constraints derived from the NMR data are not exact values but rather a range of allowed separations for each atom pair, the calculations are generally repeated numerous times with different starting positions selected for the atoms within the allowed ranges. The global structures which emerge from this process are then subjected to an energy minimization process (3). In the molecular dynamics approach, the atoms are supplied with kinetic energy and allowed to move according to some user-defined motion function to which terms have been added to produce an energetically unfavorable situation when the NMR-derived distance constraints have been violated . The time period of allowed motion is on the order of picoseconds. As with distance geometry methods, numerous calculations are done from different starting structures, and convergence to a similar final structure is sought (3). In practice, a combination of both of these procedures is often used (32-36).

Validity of the Structures — Proteins are capable of significant conformational motion about some average structure, and care must be taken in depicting this structural average (37). In general, structures calculated by the procedures described above characterize the conformational space of all structures compatible with the set of imposed constraints (21). Results are often reported as a number of individually-converged structures, along with the average atomic root mean square difference between them, and as a mean structure derived by averaging the coordinates from the individually-converged ones (21). Experimentally-derived NOE' s correlate to a high degree with those calculated from internuclear distances derived from the crystal structures of rigid, globular proteins such as lysozyme, basic pancreatic trypsin inhibitor, and cytochrome C (37). This observation would suggest that for proteins such as these, the average, global structure is a valuable concept. Another check on the solution structures obtained using NMR methods relies on a direct comparison of such structures with X-ray analysis results. The agreement between these independently derived structures seems generally quite good (33,38-42) . It should be pointed out, however, that although the global fold of a small protein seems to be determined reliably using NMR methods, local structures are often poor (21). This is especially true for regions of the molecule where the number of NOE's are few, such as in extended arms or in side chains on the surface. A superimposition of the individually converged structures offers a good way of visualizing regions of poorly defined local structure (see Figure 3) (21). A method of more accurately defining the side chain conformations of peptides has recently appeared (43).

The Method in Practice

Using the methods outlined in the previous section, the tertiary solution structures of more than a dozen whole proteins, subunits, and domains have been described (see Table 1). Included in this group are enzyme inhibitors (19,33,34,39,44), regulators of DNA (36,45), growth factors (32,46), a hormone (30), plant toxins (35,47), and a heavy metal detoxifying protein (48). With the exception of the hormone, glucagon, all of these structures were determined in aqueous media. Elements of secondary structure have been reported for a number of other proteins including the membrane-associated ATP synthase (49) and coliphage M13 coat protein (50,51), the extracellular cytolytic peptide, δ-haemolysin (52), human β-endorphin (53), and acyl carrier protein in both its acylated and sulfhydryl forms (54). Several specific examples are discussed in detail in the following sections.

Basic Pancreatic Trypsin Inhibitor (BPTI) — This globular protein, which inhibits the function of trypsin and other proteases, has played a significant role in the development of NMR methods for the studies of large biomolecules. Early investigations, many of which relied on information derived from the crystal structure of BPTI, reported the assignment of a number of individual resonances in the proton spectrum which were then used to study the locations of such internal motions as aromatic ring flips and amide proton exchanges. Later, as methods for sequential resonance assignments were developed, BPTI was one of the first proteins for which such results were reported (7). Newly-developed algorithms for the computation of tertiary structure from internuclear distance constraints were tested using crystal structure data (15,16). An assessment of the relative contributions of random coil and conformation dependent shifts to the observed chemical shifts was reported as part of an effort to understand the effect of protein conformation on the chemical shifts of peptide backbone protons (55). Sequence-specific resonance assignments were reported for several chemically modified analogs of BPTI; comparisons with the native protein revealed only small and localized changes in conformation and mobility (56-58). Finally, the direct determination of the polypeptide fold of BPTI in aqueous solution was reported (19). Significantly, it was observed that the global structure determined in solution using NMR methods was very similar to that from X-ray analysis, and all elements of secondary structure were conserved. Only localized differences were observed between the X-ray and solution structures, predominantly at the exterior of the protein. These differences probably reflect the fact that the external environments of the protein are not the same in the two experiments. Also, the number of NOE distance constraints are minimal in surface residues, resulting in some loss in resolution for such regions with the NMR method. This experiment shows that long distances in proteins can be determined accurately despite the relatively poor precision in short distance constraints. Very similar results were obtained when two fundamentally different distance geometry programs, DISMAN (15) and DISGEO (59) were used to compute structures indicating that calculated structures are influenced more by experimental data than by computational algorithms.

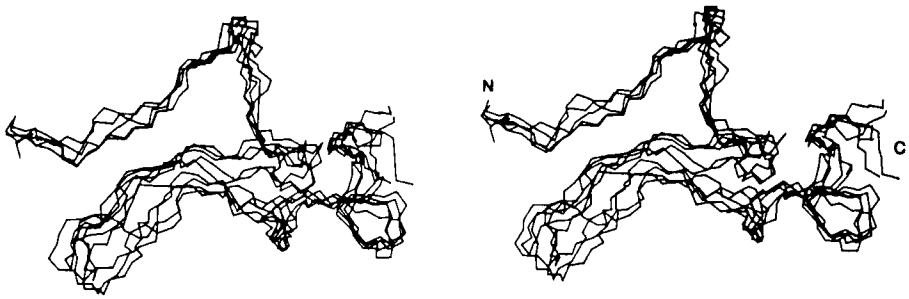


Fig. 3. A stereo view of five distance geometry structures of hEGF. Only bonds between main chain atoms (N, Cα, C') are shown. The structures are overlaid to minimize the differences for residues 3-23 and 28-46. (Reprinted by permission from *Nature*, Vol. 327, No. 6120, pp. 339-341 (1987). Copyright© 19-Macmillan Journals Limited.)

Human Epidermal Growth Factor (hEGF) — The epidermal growth factors are a group of closely related proteins believed to play an important role in the growth and differentiation of cells (60). Human epidermal growth factor is a protein of 53 amino acid residues. A derivative comprising residues 1-48 which retains complete biological activity *in vivo* has been studied by NMR. Elements of secondary structure include a major antiparallel β-sheet pairing residues 18-23 with 28-34, a type II β-turn comprising residues 34-37, and a minor β-sheet pairing residues 37-38 with 44-45 (61). The reported tertiary structure, based on 186 NOE-derived distance constraints, shows the hEGF derivative to exist as two separate domains comprising residues 1-32 and 33-48 (see Figure 3). The N-terminal domain consists of a mobile tail leading into a large loop which in turn leads into the major β-sheet. The orientation of the loop and tail are poorly defined relative to the rest of the molecule. The C-terminal domain is comprised primarily of the minor β-sheet and a short terminal tail. NMR spectra of native hEGF indicate that its solution conformation is comparable to that of the more extensively studied derivative; a very similar tertiary structure has also been reported for mouse EGF (3,46).

Histone H5 Globular Domain (GH5) — Histone H5 is a globular, lysine-rich protein derived from chicken erythrocytes that is involved in the manipulation of higher order chromatin structure (62). The native protein consists of a globular central domain along with disordered C- and N-terminal regions. The globular domain studied by NMR is composed of 79 amino acid residues and is derived from the native protein by trypsin treatment (36). The secondary and tertiary structure of GH5, based on 307 separate interproton distance constraints, has been reported to consist of a central core region composed primarily of three tightly packed-helical segments and two extended loop regions. The core and loop regions are locally well defined, but their orientation with respect to one another is less well known. From the proposed solution structure of GH5, it can be seen that the exposed face of helix 1 contains many hydrophobic residues and that it is located on the opposite side of the core region to the exposed faces of helices 2 and 3 which contain many polar residues (see Figure 4). It was suggested that this is ideal geometry for the dual protein-DNA and protein-protein interactions required of GH5 (36). A similarity between the polypeptide fold of GH5 and the DNA binding domain of the cAMP receptor protein has been noted, and the suggestion was put forth that these structural homologs may have evolved separately to fulfill like functions (36).

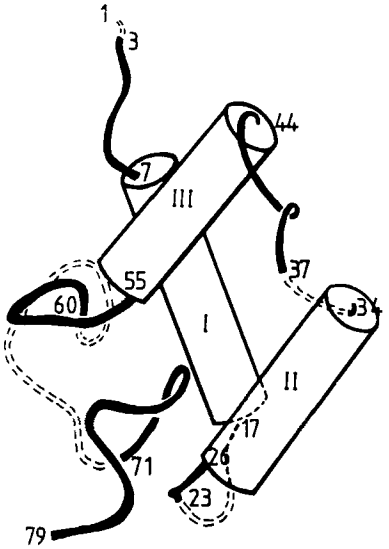


Fig. 4. Diagrammatic representation of the (RDDG)m structure of GH5. The helices are represented as cylinders and the other residues as thick lines. The two loops (residues 19-22 and 61-70) and the two ill-defined regions (residues 2-1 and 35-36) are shown as dashed lines. (From reference 36 with permission.)

Table 1
Protein Tertiary Structures Determined in Solution Using NMR Methods.

Protein	*# of Amino Acids (MW)*	*Computational Algorithm*	*Environment*	*X-Ray Structure*	*Reference*
BPTI	58 (6500)	DG	H_2O/D_2O	Yes	19
Proteinase inhibitor IIA	57 (6000)	DG	H_2O/D_2O	No	44
hEGF	53	DG/RMD	H_2O/D_2O	No	32
mEGF	53	DG	H_2O/D_2O	No	46
Metallo-thionein 2	62	DG	H_2O/D_2O	Yes	48
Tendamistat	74 (8000)	DG	H_2O/D_2O	Yes	39
lac repressor headpiece	51	RMD	H_2O/D_2O	No	45
potato carboxy-peptidase inhibitor	39	DG/RMD	H_2O/D_2O	Yes	33
Glucagon	29 (3000)	DG	DPC	No	30
Hirudin	65	DG/RMD	H_2O/D_2O	No	34
Histone H5 globular domain	79	DG/RMD	H_2O/D_2O	No	36
Phoratoxin	46	DG/RMD	H_2O/D_2O	No	35
α1 Purothionin	45	DG/RMD	H_2O/D_2O	No	47

Abbreviations: DG = Distance Geometry; RMD = Restrained Molecular Dynamics
DPC = Perdeuterated Dodecylphosphocholine Micelles

Protein Dynamics — As discussed earlier, proteins are capable of motion, and any description of structure must take into account the fluctuation of atom positions about some mean values. Early studies of internal motion relied on a minimum number of resonance assignments from 1D-NMR experiments and were limited in their utility. Along with the ability to more fully assign the resonances in the proton spectrum using 2D-NMR has come the means to study global dynamic events such as conformational changes associated with ligand binding, changes in oxidation state, and protein unfolding. For example, the binding of a series of methotrexate analogs to dihydrofolate reductase was used to study conformational changes of specific residues and of the entire enzyme (63). An NMR study of cytochrome C indicated that one side of the heme group of this protein is quite rigid, while the other is mobile and appears to undergo a conformational change during redox reactions. This observed motility may allow the rapid conformational change needed for the electron transfer process (64). Techniques of magnetization transfer have been employed to study the rate of interconversion between the folded and unfolded state of lysozyme, the cooperativity of this process, and the conformation of the unfolded state (65,66).

Modified Proteins — Recent advances in the technique of site-directed mutagenesis have made possible the modification of the gene for a protein such that mutants resulting from single amino acid substitutions can readily be produced. The use of NMR in conjunction with this technique should allow for the correlation of structure with function and stability in solution environments. Although the elucidation of the solution structure of a native protein can take quite some time, the analysis of related structures should proceed much more rapidly, provided no major conformational changes occur as a result of residue substitutions (3). Several applications of 1D-NMR to the study of such systems have appeared recently. Different spectra have been used to identify aromatic residues resonances, and this information has facilitated the characterization of the DNA binding site of the gene 32 protein, a single stranded DNA binding protein from T4 (67). Other workers have investigated the effect of two different residue substitutions on the conformation of tryptophan synthase α-subunits. In the case of a methionine for glutamic acid substitution, substantial conformational changes were seen at alkaline pH, while only local changes were observed at acidic pH (68). Finally, the existence of two distinct folded conformations of staphylococcal nuclease was ascribed to the *cis/trans* isomerism of a single proline residue by comparison of the proton NMR spectra of the native protein and a mutant protein in which the key proline residue was replaced by glycine. The process of interconversion between these two conformational states was probed using magnetization transfer techniques, and it was shown that the two isomers can interconvert directly without passing through an unfolded conformer (69). 2D-NMR methods were used to explore the effects of amino acid substitution on the structure of turkey ovomucoid third domain, a protein in which over 70% of the proton spectrum has been assigned (70). Only local effects were seen for those residue substitutions studied, based on a comparison of the NOESY spectra of the native and mutant proteins. It was suggested, however, that more global conformational changes might also result from single residue exchanges, as was observed in a study of staphylococcal nuclease (70).

Future Directions — At the present time, it is possible to accurately determine the global polypeptide fold in solution of proteins up to 10 kDa in size using a combination of NMR spectroscopy and computational methods. Two significant limitations of this approach are its lack of accuracy in the determination of localized structure and the size limitation. It has been noted that currently used computational methods give structures with precise local structures when quantitative distance constraints taken from crystal structures are employed (42). Thus, more accurate measurements of coupling constants and more quantitative analysis of NOE intensities should improve the local accuracy of protein structures determined using NMR. Elucidation of the structures of larger molecules requires further refinements in the types of spectral simplification provided by 2D techniques. The use of proton-detected heteronuclear edited and correlated NMR for proteins selectively enriched with ^{13}C and ^{15}N appears to offer great promise (22). Preliminary results from work with the 18.7 kDA bacteriophage T4 lysozyme using this approach have been reported, and a general strategy for solving the structures of proteins up to 40 kDa in size was proposed (71). This strategy utilizes proton-observe heteronucleus-perturb NMR to detect only those protons attached to an isotopic label in a uniformly ^{13}C- or ^{15}N-labeled protein (22,72). Resonances from individual amino acids are then identified using a sample labeled in one amino acid only. Specific resonance assignments can then be made using techniques such as site-directed mutagenesis, multiple isotope labeling, and isotope-directed or -detected NOE (71). Although it has been suggested that such techniques will not be routinely used to define the complete solution structure of large proteins, studies of this type for selected regions of such proteins should facilitate the investigation of structure and function. Finally, the feasibility of ^{13}C and ^{15}N natural abundance 2D heteronuclear correlation spectroscopy in small proteins has been reported and used to assign the ^{15}N NMR signals of BPTI (73,74). The development of this technique should augment the strategy outlined above. Given such promise, it appears that NMR determination of protein tertiary structure in solution will become quite generally applicable in the near future.

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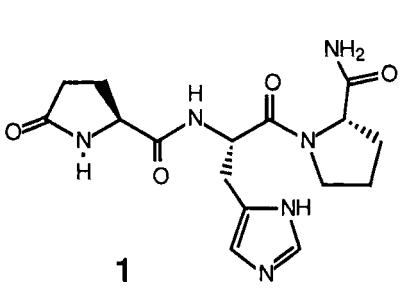
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Chapter 29. Search Strategies for Determining Bioactive Conformers of Peptides and Small Molecules

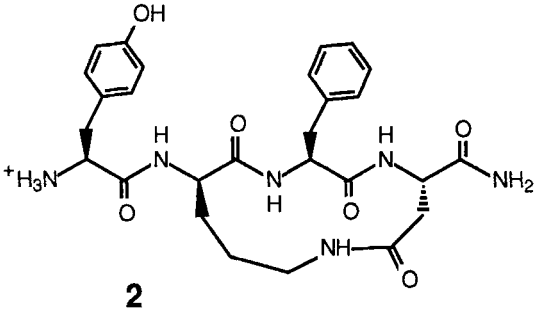
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Introduction – It is well appreciated that flexible biological molecules can exist in numerous low-energy states and are probably altered conformationally when interacting with their specific receptors. Selection of the bioactive conformation from these many possibilities is thus difficult, especially for peptides and proteins. First of all, there is the well-known multiple-minima problem. Energy minimization techniques will find the nearest local minimum, but not necessarily the global minimum, and these minima may even be relatively high in energy (1). Minimization procedures also will not jump over the barriers between minima and thus only the nearest local minimum encountered will be identified (1). Secondly, there is no guarantee that the effector molecule, when bound to the receptor, is actually at an energy minimum, although clearly it cannot lie very much above (2). There have been numerous articles about computer modeling (3-8) and a very recent review (9). In this chapter, we shall therefore focus only on methodology, the different strategies for finding low-energy conformations and bioactive conformers, with emphasis on peptides.

Strategies Using Conformational Searching – One of the most common strategies for finding peptide conformations utilizes a systematic grid search of conformational space in which only the torsional angles of the molecule are allowed to vary. Each rotatable bond is varied sequentially by a fixed angle rotation. The problem with such a search is that as the length of the peptide increases, the number of conformations that must be considered increases exponentially. For small peptides, many studies continue to be performed by brute force systematic searching. For example, the tripeptide thyrotropin releasing hormone (TRH) **1**, which has only eight variables, is small enough so that a full systematic search is feasible (10, 11). Similarly, a peptide segment of gastrin, Ac-Trp-Met-NHMe, was searched and its conformations compared to a small molecule antigastrin 5,1-benzothiazocine (12). The conformation of the μ-receptor selective opioid peptide analog, **2**, with a 13-membered ring, was examined by a grid search (13) as were several μ-selective met-enkephalinamide analogs (14). Cyclic tripeptides (15) and hexapeptides (16), which are also small enough to study systematically, have been searched to see how many different types of turns are generated during the course of the conformational analysis. However, as the length of the peptide increases, the problem rapidly outstrips the computer resources available. It is critical to prune the search tree as early and as rapidly as possible to reduce the number of conformations to be examined (17, 18).



1



2

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One strategy developed to treat larger peptides involves the use of build-up procedures (19). The starting geometries of a peptide are constructed by using combinations of minimum-energy conformations of single amino acids (20) and dipeptides (21). This method has been employed to calculate the conformations of collagen (22), gramicidin (23), met-enkephalin (24), and synthetic peptide models of antigenic sites on cytochrome c (25). It is assumed that short-range interactions are dominant. Efficiency is achieved by initially examining the much smaller conformational space of individual residues or dipeptides and eliminating early in the analysis high-energy minima that are above a chosen cut-off value due to bad intra-residue or dipeptide interactions. The small fragments are then assembled and reminimized (26). There are two caveats to the build-up method. Atomic overlap can occur when two fragments are joined in an arbitrary manner to produce a high energy conformer, since the side chains are not necessarily in a low-energy state for the resulting combined backbone structure. For a reasonable value of the cut-off energy, many minima are possible and the number of minima still increases exponentially. Algorithms to deal with these problems have been proposed and have been used to predict conformations of a 13- and a 50-residue peptide (26). The build-up should be started in regions that are unlikely to have many low-lying energy minima, e.g. near non-polar amino acids, which have fewer single residue low-lying energy minima (27).

Another strategy limits exploration to those regions of conformation space which are sterically acceptable. One begins by placing each residue in each of the four possible main chain conformations corresponding to the energetically acceptable regions of a Ramachandran plot. Each side chain torsion angle is allowed three possible 120° ranges. All the combinations for 5- to 6-residue fragments are compared energetically and larger molecules are assembled from these fragments in stages by combining overlapping segments. The 18-residue β-melanocyte-stimulating hormone (β-MSH) has been studied by this method (28).

Conformational Searches on Cyclic and End-Constrained Molecules – A complete systematic search may be feasible for larger cyclic peptides, macrocyclic molecules, and peptide loops that occur in the middle of proteins. Closure criteria or end constraints are used to limit the regions of conformational space that must be examined. One procedure uniformly samples conformation space with parameters introduced to limit the space to be explored using an empirical energy function (29, 30). In another procedure, called "random tweak", a large number of peptide conformations are generated randomly and then the geometric closure constraints are enforced using an iterated linearized Lagrange multiplier technique which introduces minimal conformational perturbation to achieve end closure. The virtue of this method is that it is linear in fragment length (31). It has since been applied to prediction of the conformations of hypervariable regions of antibody molecules with some success (32). A further method was proposed for protein loops where the conformational search tree is rapidly pruned using rules and filters that include permitting only standard bond lengths and angles (which enforces loop closure). Torsional angles must be in well-defined ranges, short nonbonded contacts are not permitted and the conformation with the lowest electrostatic energy (including solvent effects) is preferred. This method was used to construct loops in the protein *Streptomyces griseus* trypsin (33). Another technique iterates cycles which consist of alternately moving each nonhydrogen atom independently in the molecule a small random distance in a random direction, followed by energy minimization using molecular mechanics. This method, which it is claimed explores conformational space more efficiently than grid search, Monte Carlo, or molecular dynamics techniques, has been used to discover the low energy conformations of saturated hydrocarbon rings containing from 7 through 15 carbons. All previously reported and many new conformations were found for the larger ring compounds, some corresponding to new lowest energy minima (34).

Conformational Searching and Receptor Mapping – In many receptor systems, a variety of structurally diverse series of ligands have been synthesized that bind competitively to the

same receptor site. Most of these molecules are flexible, yet because of their different structures, the various series may explore somewhat different portions of conformational space. Receptor mapping (also called pharmacophore mapping) techniques have been developed to combine the information in these structures to define the bioactive conformation of the ligands and the topography of the receptor binding site (35-37).

A small number of critical functional groups on the ligands are identified as essential for binding to the receptor. A conformational search is performed on one or more representative active molecules from each series. The low energy structures of each molecule are mapped in a common space. These maps are then compared and only those respective conformations which allow the critical pharmacophore groups of the different series to coincide are retained. As a new structurally distinct series is added to the analysis, the possible conformations for the ensemble which overlap the pharmacophore decrease. In some cases, dummy atoms that represent functional groups on the receptor that interact with the pharmacophore atoms are superimposed rather than the pharmacophore atoms themselves (36, 37). Inactive molecules can also be included in the analysis and provide valuable information. Either they should not be able to assume an energetically acceptable conformation that allows overlap of critical pharmacophore groups with the active molecules or they should violate space that is occupied by the receptor itself (36). The technique can be very powerful when the structures of the different series are adequately diverse or when some of the analogs are sufficiently constrained conformationally. Then the possible conformations for the ligand series can be reduced to a small enough number of conformers to permit their use in drug design and prediction.

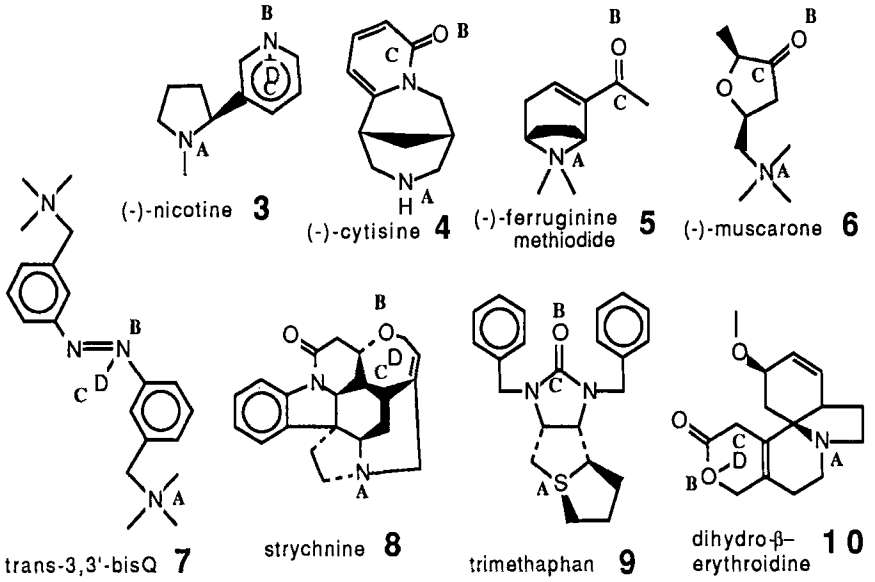
These techniques have been usefully applied to a variety of receptor systems. Various dopamine receptor sites have been mapped (35, 38), as have adrenergic receptors (35), opiate analgesics (37), and non-steroidal cardiotonics (39). Enzyme active sites have also been studied with this technique, when the three dimensional structure of the enzyme was not available from experimental sources, as in the case of angiotensin converting enzyme (ACE) inhibitors (36).

Strategies Using Distance Geometry – The distance geometry method can also be used for determining bioactive conformations. The general precepts of this method have been reviewed and will not be discussed in detail (40). Distance geometry has been widely used in protein structure determination (41). The algorithm behind this method can be described as follows. One is given a set of points (usually atoms). Two matrices of upper and lower bounds define possible distances between these points. It is then possible to find sets of three dimensional coordinates for the points that satisfy the prescribed distance constraints. In other words, the conformation space searched can be limited to the range of conformations that satisfies the distance criteria.

A more powerful application lies in the determination of common pharmacophores or in mapping of receptors. For example, distance geometry has been applied to a set of dihydrofolate reductase (DHFR) inhibitors, one at a time, to define a receptor model (42). The correlation between the predicted receptor model and x-ray data was very good, but the number of parameters that had to be introduced into the modeling was limiting. The results were later improved by the introduction of physico-chemical data (43). A similar approach was used to study the turkey erythrocyte β_1 and β_2 adrenergic receptors (44, 45).

The ensemble approach to distance geometry simultaneously treats several molecules as a single ensemble (46). This method generates a common pharmacophore from a set of bioactive compounds and was applied to a group of four nicotinic agonists (**3-6**) which are not rigid enough so that any one alone could define a pharmacophore. Three groups in each molecule were chosen for matching: a cationic center (A), an electronegative atom (B), and a dipole formed between an atom C and B ("D" defines a dummy atom which forms one end of the dipole in some of the molecules). A common pharmacophore, similar

to others found by different means, was identified using the distance geometry ensemble approach, and used to explain the agonism or antagonism of compounds not included in the original set (7-10). The full potential of this method remains to be explored.



Use of Monte Carlo Techniques – Monte Carlo techniques have been primarily used to simulate water structure around proteins and peptides (47-50), and nucleic acids (51). Applications to conformational analysis of peptides and polypeptides are not numerous (52-54) because sampling is very slow compared to molecular dynamics. A new Monte Carlo procedure, while still computationally intensive, has the advantage that the average conformation over the whole ensemble can be determined. This end is achieved by combining statistical mechanical techniques with an adaptive, importance sampling technique given the name SMAPPS (Statistical Mechanical Algorithm for Predicting Protein Structure) (55, 56). This Monte Carlo algorithm procedure differs from the standard Metropolis algorithm in that, while the sampling is over all space, the procedure adjusts to concentrate sampling in regions where the probability is highest (importance sampling). SMAPPS was applied to the pentapeptide Met-enkephalin, and in the initial study, only backbone torsion angles were allowed to vary (55, 56). Three low-energy, probable structures were found, one of which was similar to a previously predicted conformer (24), while the other two were even lower in energy. The SMAPPS procedure was also used to calculate the average backbone structure of met-enkephalin (56). In this approach, shorter independent Monte Carlo runs were carried out and an average conformation produced for each run. Since this approach does not necessarily produce low energy structures, the structures were then minimized. Ten average structures were computed, energy minimized, and found to compare favorably with the three original SMAPPS-derived probable structures. Both the calculation of the probable and the average conformers found structures lower in energy than those previously discovered by minimization alone. In a further study, in which all angles of met-enkephalin were allowed to vary, the thermodynamically preferred conformer was found to contain a γ turn, distinct from the β II' turn previously found by energy minimization (57). The γ turn structure had a conformational energy about 5 kcal/mol higher than the type II' β structure; when entropy was included in the SMAPPS procedure, the γ turn structure was lowest in energy by about 9 kcal/mol.

The advantages of the SMAPPS method are that only the sequence of the peptide is required and that both low energy, probable conformations and average conformations can

be determined. The average conformer should be comparable to that observed experimentally.

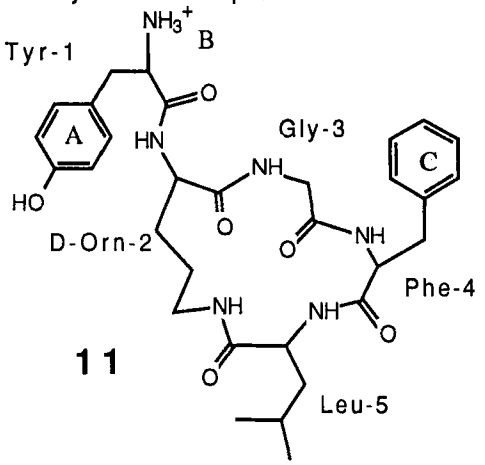
Constrained Molecular Dynamics and Energy Minimization – The use of NMR spectroscopy to determine protein structure is a rapidly expanding field (58). Numerous applications of molecular dynamics coupled with NMR-generated NOE data have been reported for the structure determination of proteins, such as crambin (59-61), potato carboxypeptidase inhibitor (62), histone H5 (63), and a thrombin inhibitor hirudin (64), enzymes (65), lac-repressor (66, 67), and oligonucleotides (68). These techniques have also been applied to smaller systems such as human growth hormone releasing factor (69), renin inhibitors (70), cyclic enkephalins (71), glycopeptide antibiotics (72), and phoratoxin (73), a protein toxin. In these examples, an extra term is added to the potential function to constrain the calculation so that interproton distances from NMR determinations are fitted as closely as possible. Distance geometry could also be used for this purpose; however, with constrained molecular dynamics and energy minimization, a structure is produced which is usually lower in energy than that found from distance geometry alone. The choice of the force constants used in the constraining potential can significantly affect the results (70). An appropriate combination of higher temperatures and lower force constants appears to offer maximal efficiency. Also, constrained molecular dynamics is better than constrained energy minimization (70). An interesting study was performed in which the absence of NOE data between protons was used as an additional constraint (67). These methods should be of great benefit because the results include experimental data and can be compared directly with experiment.

Technique of Forced Molecular Dynamics and Simulated Annealing – A major problem in peptide conformational searching, already referred to above, is how to search conformational space adequately in a reasonable time frame. The Monte Carlo method samples space well and can be used for small systems (74), but, for larger molecules, extremely long simulations are required. Molecular dynamics at 300 K can only overcome thermal barriers slowly and thus samples conformational space slowly. Torsional forcing, i.e., inclusion of an additional term in the energy function that forces particular torsional angles to change their values during the course of the dynamics trajectory, has been employed to overcome energy barriers in studying various cyclic enkephalins (75). By varying the angles to be modified and the strength of the forcing constant, very different conformations can be explored much more rapidly.

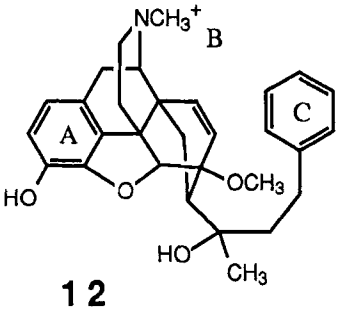
Another approach to overcoming thermal barriers involves increasing the temperature of the dynamics simulation. Unfortunately, this can lead to distorted geometries and, upon minimization, leave the molecule in high energy local minima. The technique of simulated annealing controls the negative features of high temperature dynamics while increasing the sampling efficiency. Simulations are first carried out at high temperatures to aid in crossing energy barriers. The system is then cooled and equilibrated with short dynamics runs at lower temperatures so that the molecule is not trapped in high energy local minima (76). Simulated annealing was performed on the 28 amino acid fragment of atrial naturietic factor (ANF) (77). Starting with an all-extended conformation, dynamics were run for 100 ps at 900 K and velocities were re-randomized every 5-10 ps. Conformations were taken every ps and minimized.

With so many structures generated, truly dissimilar conformations must be distinguished. Root mean square (rms) superposition of the 100 minimized structures produced a 100 x 100 matrix which, by appropriate grouping, allowed the identification of 11 distinct families of structures. A structure from each of these families was allowed to reequilibrate with short dynamics runs at 300 K, and then reminimized. The resulting structures, although still similar in conformation, were significantly lower in energy than the original high temperature dynamics minimized structures of that family. The 11 families could then be further differentiated by use of SAR data, NMR, or introduction of further molecular constraints. This technique may be very useful for large peptides.

Template Forcing – Template forcing can be used to provide an estimate of how closely relative functional moieties in molecules can overlap in space and what energy is required for "forcing" the overlap. In template forcing, the energy function is augmented by a penalty term proportional to the rms distance between forced atoms. This technique was used to show that a linear analog of GnRH (gonadotropin releasing hormone) could adopt a similar conformation to a cyclic antagonist at a relatively modest cost in energy (78). With template forcing, the balance between rms fit and cost in energy can be altered by adjusting the penalty function force constant. A similar idea was used on met-enkephalin where the tyrosine ring was restrained to be in a similar position to that of 7-[1-phenyl-3-hydroxybutyl-3-]endothenotetrahydrothebaine (PET) (79) and has recently been repeated using a cyclic enkephalin analog **11** and the rigid opioid 7-α-[(1R)-1-hydroxy-1-methyl-3-phenylpropyl]-6,14-endo-ethenotetrahydrooripavine (PEO) **12** (80). Groups that correspond in these two molecules have been labeled A, B, and C. The advantage of template forcing is that a direct comparison of common structures can be made without having to consider only the minimum energy structures. Also, two structures can be simultaneously forced onto each other and minimized together, or dynamics can be run concurrently with several positions tethered and then subsequently relaxed.



11

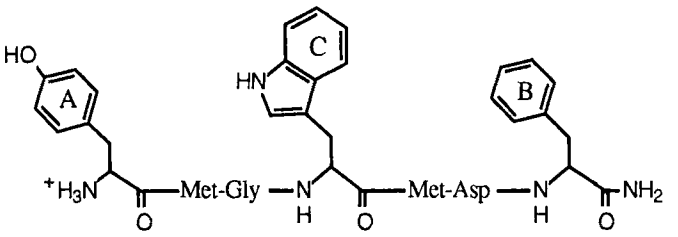


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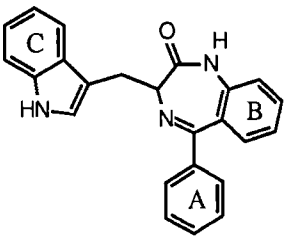
Selection of Bioactive Conformer Using Additional SAR Data – Conformational calculations on peptides, because of the multiple minima problem, are usually unable to resolve the question of the bioactive conformation. Thus, selection requires additional information, such as extensive SAR data, or a fairly rigid analog. This search strategy is illustrated by the following example.

CCK-7, cerulein, little gastrin and CCK-4 are related peptides that bind at peripheral CCK and gastrin receptors. CCK-7 and cerulein interact with the peripheral CCK receptor whereas CCK-4 and little gastrin interact with the gastrin receptor exclusively. Thus, low energy conformations common to all four peptides should not define the bioactive conformer for the peripheral CCK receptor. However, common conformers for CCK-7 and cerulein, which exclude the CCK-4 and little gastrin common conformers, should define the bioactive conformation of CCK at its peripheral receptor. The contrasting situation should define the bioactive conformer for the gastrin receptor (81). Conformations for all four peptides were constructed using a build-up procedure and energy minimized. Then common conformers from one class of compounds were compared with conformers from the other class. It was found that CCK-7 and cerulein have β turn structures while CCK-4 and little gastrin are more helical. The global minimum for CCK-7 is the lowest energy structure common to CCK-7 and cerulein. The global minimum for cerulein has no counterpart for CCK-7 (**13**), but the lowest common structure for both is superimposable on an indolyl benzodiazepine (**14**), a highly constrained molecule that interacts at the peripheral CCK receptor as an antagonist; the phenolic side chain of Tyr-1 approximates the aromatic ring of the antagonist (A), Phe-7 overlays the fused aromatic

ring of the benzodiazepine (B), and the indole ring of Trp-4 is near the 3-indolyl moiety (C). The essential feature of the model is that the two phenyl groups of CCK-7 and cerulein are proximal and point away from the indole ring. Since met-enkephalin also antagonizes the action of CCK-8, a comparison was made between an already computed low energy form of this opiate peptide and the CCK-7 model. It was found that met-enkephalin could resemble the first 5 residues of the CCK-7 lowest energy structure.



13



14

Another technique for the selection of the bioactive conformation similar to the above involves the comparison of active and inactive conformers (82). Conformations are calculated for all analogs, both active and inactive, and low energy conformers common to the two sets are excluded as the possible bioactive conformer. A low energy conformer of an agonist should be a high energy conformer for an inactive compound, and vice versa. Using this strategy, energy tables with an arbitrary cut-off are set up and cross comparisons are made. Thus, the final selection is not necessarily the lowest energy conformer, but one that is sufficiently low in energy and still meets the other criteria. This procedure has been applied to met-enkephalin and μ-receptor agonists that are morphiceptin analogs (82). The candidate selected was similar to a previously determined conformer for met-enkephalin and contains a type-II β turn like that found in a recent crystal study (82).

Conformational Constraints in Peptides – The many searching methods outlined above are being used to derive the various possible bioactive conformations for flexible small molecules and peptides. However, because of the flexible nature of these molecules, a large number of reasonably low energy conformations are usually discovered. A major challenge to the modeling medicinal chemist remains to distinguish between these possible conformations to select a very small number that can then be used as the structural basis for further drug design and chemical synthesis. The major way in which this selection is effected is by the addition of structurally modified analogs of the small molecule or peptide to the conformational analysis. The modeling chemist can help accelerate this process by suggesting which modifications would be most efficacious for zeroing in on the bioactive conformation. These considerations, with emphasis on peptide modifications, have been described in several reviews (83, 84).

Introduction of conformational constraints into peptides or substitution of amino acids can be beneficial in determining molecular conformation and receptor interactions of peptides. Changes of L- to D-amino acids (82, 85, 86) or incorporation of conformationally restricting side chain substitutions, such as α,α-dimethylglycine (α-aminoisobutyric acid or Aib) (87) or dehydroamino acids (88) can indicate the relative contribution of an individual residue or of a conformational restriction to receptor binding and biological activity. Metabolic stability may also be enhanced. For example, if both D- and L-amino acids are similarly accommodated, then the conformation of the peptide is such that the side chain in either position can reach the same receptor site or, alternatively, the side chain in question is not important in binding. However, if the potency is greatly altered, then either the conformation has been changed by the modification or the side chain interacts directly with the receptor site (82, 86). Backbone modifications such as ketomethylene, hydroxyethylene, ester, reduced amide, thioamide or trans-substituted olefin replacements can be used to explore hydrogen bonding, but it should be kept in mind

that these changes may also alter the backbone geometry (89). These two effects must thus be differentiated. A local or regional macrocycle will significantly limit the conformational space that that portion of the peptide can explore (90-93). Larger cycles, such as end to end or side chain to more distant side chain, also limit the possible conformations; however, they do not greatly rigidify the peptide and many local minima may still be found. Thus, even with macrocycles, determination of a unique bioactive conformation may still be extremely difficult.

Summary and Conclusions - Many methods have been described to derive energetically acceptable conformations for flexible small molecules and short peptides. In all but the most restrictive cases, the search is severely computationally intensive. A large number of conformations are usually found and it may not even be possible to determine if all the relevant low energy conformations have been discovered. In almost all cases, additional information from structure-activity data and from chemically modified analogs that partially rigidify and constrain the conformation is essential. Two major challenges face the modeling medicinal chemist. One is to combine the information from all these strategies and analogs to define one or a small number of conformers that approximate the bioactive conformation of the mediator of interest. The second, even more exciting, challenge is to develop the knowledge base and tools to use these models of the bioactive conformation to design useful new therapeutic agents (94, 95).

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Chapter 30. Oligonucleotide Inhibitors of Gene Expression in Living Cells: New Opportunities in Drug Design

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Introduction - A number of drugs currently in use today function by directly interacting with DNA. Many of these compounds intercalate or bind in the minor groove of DNA (1). However, these agents, with their limited ability to recognize specific nucleic sequences, cannot take full advantage of the sequence information available in DNA, and thus are unable to inhibit expression of specific genes.

The control of gene expression can, in theory, be achieved by single-stranded nucleic acids, which can specifically read complementary mRNA sequences via Watson-Crick base-pairing interactions. By binding to the appropriate target sequence, these nucleic acids could inhibit processing or translation of mRNA. Such nucleic acids are often termed "antisense" because they are complementary to the sense or coding strand of the gene.

The preparation, introduction and expression of antisense RNAs require rather sophisticated molecular biology techniques. They can be prepared in the laboratory and then introduced into cells by microinjection or expressed in cells after transfection with plasmids carrying an antisense gene (2-5). Antisense RNAs have been used to selectively inhibit expression of viral and cellular mRNAs, including those encoded by oncogenes (6-12). Interestingly, naturally occurring anti-sense RNAs have been found in both bacterial and mammalian cells (13-16).

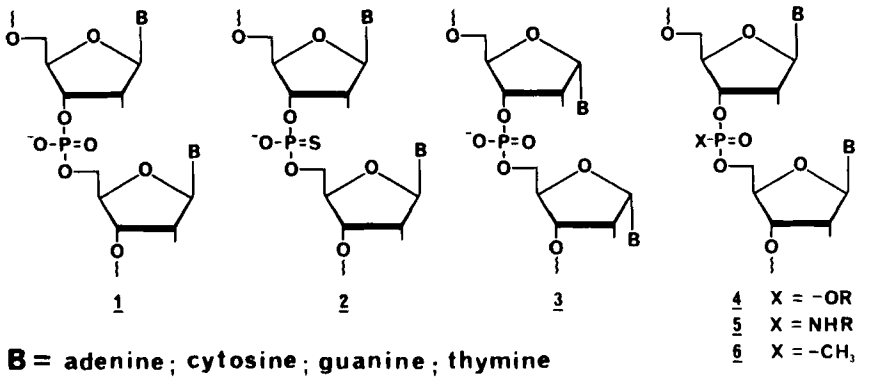
A simpler approach involves the use of short antisense oligonucleotides or oligonucleotide analogs prepared by chemical synthesis. This review will describe some of these oligomers and will focus on experiments in which they have been used to control gene expression at the mRNA level, particularly in living cells. It appears that this concept could be exploited to develop clinically useful drugs to control the growth of viruses or malignant cells.

Antisense Oligonucleotides and Analogs

Essentially three types of oligonucleotides have been studied: oligodeoxyribonucleotides; oligomers having modified sugar-phosphate backbones; and oligomers derivatized with specific functional groups.

Oligonucleotides - Oligodeoxyribonucleotides (1) contain β-2'-deoxyribonucleosides linked via 3'-5' phosphodiester internucleotide bonds and are readily prepared by modern DNA synthesizers using phosphoramidite or H-phosphonate chemistry (17,18). Most studies have employed oligomers containing 10 - 30 nucleotides. Oligoribonucleotides have not been considered because the ribose-phosphate backbone is diffucult to synthesize and readily hydrolyzed by ubiquitous ribonucleases (17).

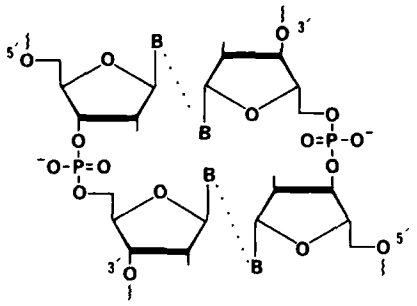
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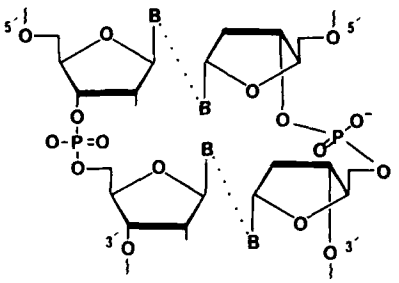
Backbone-modified Oligonucleotides - Although oligodeoxyribonucleotides form very stable duplexes with complementary nucleic acids, the sugar-phosphate backbone is susceptible to hydrolysis by the exo- and endonucleases found in mammalian serum and in the cytoplasm and nucleus of cells (19). Furthermore, the negatively charged phosphates may limit the ability of the oligomers to pass through the phospholipid cell membrane. Therefore, efforts have been made to synthesize oligonucleotide analogs which have increased resistance to nuclease hydrolysis and/or are more lipophilic than naturally occurring oligonucleotides. The modifications have been directed mainly towards the sugar-phosphate backbone and usually involve a slight change in the phosphate group in order to minimize changes in the geometry of the internucleotide bond and thus maintain the stability and fidelity of oligomer binding. However, more radical changes of internucleoside linkages such as carbonate or carbamate linkages have also been explored (20-22).

Oligomers containing phosphorothioate linkages (2) represent perhaps the simplest modification tested. These analogs are readily prepared by oxidation of phosphite or H-phosphonate intermediates with elemental sulfur (23,24). The negatively charged phosphorothioate linkage is chiral. Thus, an oligomer containing n phosphorothioate bonds consists of 2^n diastereoisomers. The phosphorothioate linkage is considerably more resistant than the phosphodiester linkage to hydrolysis by exo- and endonucleases commonly found in the intercellular environment, although both the Rp and Sp isomer can be cleaved slowly by these enzymes (23,25). The configuration of the phosphorothioate group affects the stability of duplex formation as evidenced by studies on self complementary oligomers containing a single phosphorothioate bond in each strand (26).

α-Anomeric oligodeoxyribonucleotides (3) represent another class of oligomers with a negatively charged backbone (27). These analogs are hydrolyzed approximately 30 times more slowly by snake venom phosphodiesterase than are the corresponding β-anomeric oligomers (28). In contrast to β-oligomers, which form duplexes with their sugar-phosphate backbones oriented in an antiparallel manner (7), the α-oligomers form stable, parallel duplexes (8) with complementary single-stranded DNA and RNA (29-31). Duplexes formed by α-oligomers and complementary single-stranded RNA are more stable than duplexes formed with single-stranded DNA, a property which may be useful in designing antisense molecules targeted against cellular mRNAs (32).



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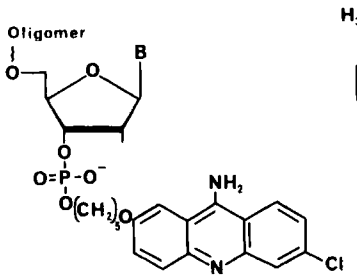


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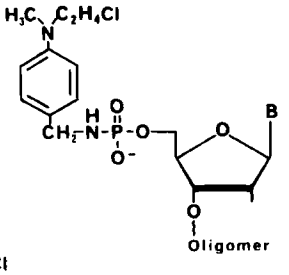
A number of "nonionic" oligonucleotide analogs have been prepared, including oligodeoxyribonucleotide alkyl phosphotriesters (4), oligonucleotide phosphoramidates (5) and oligodeoxyribonucleoside methylphosphonates (6) (24,33,34). Of the three, the methylphosphonates have been studied most extensively (35,36). These analogs are readily synthesized on insoluble polymer supports using protected nucleoside 3'-methylphosphonate imidazolide or nucleoside 3'-methylphosphonamidite synthons (37,38). The noncharged methylphosphonate group is chiral. The oligomers form stable duplexes with both single stranded DNA and RNA, with the stability of the duplexes apparently dependent in some cases upon the configuration of the methylphosphonate linkage (39,40). This dependence may be due to stabilizing hydrophobic interactions between Sp methyl groups and the base pairs of the duplex and to unfavorable solvation of Rp methyl groups (39). Methylphosphonate oligomers are completely resistant to hydrolysis by nucleases and unlike oligonucleotides or phosphorothioate analogs, are very lipophilic (33). They are taken up intact by mammalian cells in culture and survive for long periods of time under such conditions (33,35,36). Oligomers greater than four nucleotides in length are not taken up by bacteria, presumably due to their inability to pass through the rigid cell wall (33).

Derivatized Oligonucleotides - Derivatization of oligonucleotides with a variety of functional groups has been carried out in an effort to increase their resistance to nucleases as well as the stability of duplex formation and biological activity. Oligodeoxyribonucleotides linked at their 3'-end with derivatives of acridine (9) form duplexes of increased thermal stability compared to underivatized oligomers (41-44). The acridine stabilizes the duplex by intercalating between the base pairs formed by the oligomer and its complementary sequence (44). Acridine-derivatized oligomers show increased resistance to degradation by exonucleases (41).

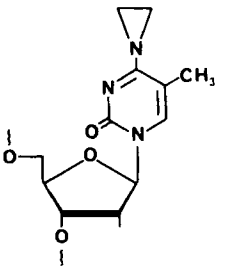
Irreversible binding to complementary nucleic acids can be achieved by derivatizing oligonucleotides with groups capable of crosslinking with the targeted nucleic acid. The simplest examples are oligonucleotides which incorporate alkylating agents such as aromatic 2-chloroethyl amine analogs (10) similar to the antitumor drug, chlorambucil (45,46). While effective as crosslinking agents, the highly reactive alkylating groups can cause binding to sites other than that targeted by the oligomer (47). In an attempt to avoid such problems, oligonucleotides carrying a 5-methyl-N^4,N^4-ethanocytosine base (11) were synthesized (48). These oligomers are capable of reacting only with complementary guanine residues when the oligomers are hydrogen-bonded to their target sequences. However, the long half-life of the crosslinking reaction, 30 hrs., makes it unlikely that such derivatives will have practical utility.



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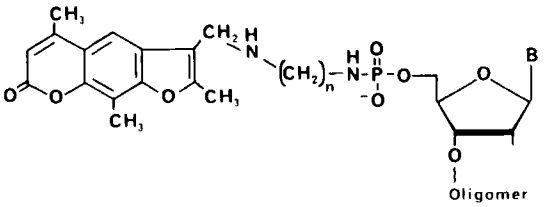


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Oligonucleoside methylphosphonates have been derivatized with 4-aminoalkyl-4,5'8-trimethylpsoralens (12). Upon irradiation at 365 nm, a wavelength above the absorption range of nucleic acid bases, the psoralen group undergoes a photocycloaddition reaction with thymine or uracil residues when the oligomer is hydrogen bonded to single stranded DNA or RNA (49). Up to 80% crosslinking has been achieved at low (1 to 10 μM) concentrations of oligomer. The oligomers crosslink selectively with their target sites, and crosslinking is dramatically reduced or eliminated upon introduction of mispaired bases. This demonstrates that crosslinking is strictly dependent upon sequence-specific hydrogen bonding interactions between the oligomer and the target and not upon non-specific intercalation by the psoralen group.



12

Oligonucleotides have also been derivatized with groups designed to hydrolyze the backbone of the targeted nucleic acid. Ethylenediaminetetracetate (EDTA) has been attached *via* a methylene linker arm to either a 5'-phosphate or through the 5-position of a deoxyuridine residue of the oligomer (50-52). In the presence of Fe(II) and a reducing agent such as dithiothreitol, hydroxyl radicals are produced which can degrade the sugar-phosphate backbone. Oligomers have also been derivatized with 1,10 phenanthroline/Cu complexes and with porphyrins (53-55). The latter derivatives produce oxygen radicals and hydroxyl radicals upon photoirradiation. The oligomer guides the radical generating group to the desired site in order to achieve selective cleavage of the target sequence. It appears that these derivatized oligomers cleave single stranded DNA more efficiently than RNA. A recent report has demonstrated that an EDTA-derivatized oligomer capable of forming triple-stranded complexes with polypurine tracts of double-stranded DNA, effectively cleaves both strands of the DNA in a site-specific manner (56). To date oligomers derivatized with radical generating groups have been used only in *in vitro* cleaving experiments.

Since these oligomers undergo rather rapid, extensive autodegradation and produce highly reactive oxygen or hydroxyl radicals their utility in living cells will probably be limited.

Control of Gene Expression by Antisense Oligonucleotides

The ability of antisense oligonucleotides to inhibit nucleic acid function in a specific manner has been demonstrated in cell free systems as well as in living cells.

Targets for Antisense Oligonucleotides - Most antisense oligonucleotides and oligonucleotide analogs have been targeted against single-stranded RNA molecules such as mRNA or viral genomic RNA. Specific binding sites include: the 5'-cap site, the initiation codon region or the coding regions of mRNA; the splice junctions of nuclear precursor mRNA; and the primer binding sites of retrovirus RNA. In principle, it should be possible to target DNA when it is in a single-stranded form during transcription or replication. It should also be possible to target polypurine tracts in duplex DNA by forming triple-stranded complexes with oligomers containing only pyrimidine nucleosides (56).

Control in Cell-free Systems - Inhibition of mRNA translation by antisense oligomers has been studied extensively in cell-free systems including rabbit reticulocyte lysates and wheat germ extracts. Oligonucleotides, acridine derivatized oligonucleotides and oligonucleoside methylphosphonates complementary to the 5'-cap site, the initiation codon region or the coding regions of mRNA, have been tested (57-63). Oligodeoxyribonucletides complementary to the common 5'-leader sequence found on all trypanosome mRNAs have also been studied (64,65). In most cases these agents effectively inhibit mRNA translation in a selective manner at low (0.1 to 10 μM) oligomer concentrations whereas the methylphosphonate oligomers function only at higher concentrations (50 to 200 μM). Oligomers which are complementary to the 5'-cap or the initiation codon region generally appear to be more effective inhibitors than those targeted against the coding region or the 3'-untranslated region of the message (57,66,67). In one case oligomers complementary to the coding region produced truncated proteins whose amino acid sequence terminated at a position corresponding to the binding site of the oligomers (66). The effectiveness with which the oligomers inhibit translation may be due in part to RNA secondary structure which can interfere with oligomer binding (67).

Antisense oligomers can inhibit mRNA translation by essentially two different mechanisms. They can prevent initiation of protein synthesis by blocking ribosome binding at the 5'-end or initiation codon region or by blocking polypeptide elongation when bound to the coding region of the mRNA. Alternatively, the oligomers can cause degradation of the message by activating ribonculease H, an enzyme which cleaves the RNA strand of DNA/RNA duplexes (68). It appears that ribonuclease H or an equivalent enzymatic activity exists in frog oocytes, wheat germ extract, most mammalian cells and to varying extents in rabbit reticulocyte lysates (69). The ability of oligodeoxyribonucleotides to cause substantial inhibition of translation at low oligomer concentrations is probably due to this activity. Methylphosphonate oligomers probably do not activate ribonuclease H and thus, rather high concentrations of these analogs are required to block ribosome binding.

Control in Living Cells - It has been demonstrated that a tridecadeoxyribonucleotide complementary to the 3'- and 5'-terminal redundant sequence of Rous sarcoma virus RNA inhibited virus growth in RSV-infected chick embryo fibroblasts (70). The oligomer was active in the concentration range 2 to 20 μM and was not cytotoxic. It also inhibited translation of RSV 35S RNA in a cell-free system (71). This activity and the potential for preventing circularization of the virus could account for the observed inhibition.

Oligodeoxyribonucleotides complementary to the initiation codon region or the coding region of mRNA inhibit mRNA translation in Xenopus oocytes and transformed cells. Oligonucleotides 18 to 23 nucleotides long were targeted against sequences in various parts of the coding region of Interleukin-2 mRNA (72). The oligomers (1 to 10 μM) specifically inhibited mRNA translation 95-99% when a mixture of the mRNA and oligomer was injected into the oocyte. Prehybridization was not required for activity. The observed inhibition is probably due to the presence of ribonuclease H activity in the oocytes. Injected β globin mRNA or endogenous calmodulin mRNA was shown to be degraded in oocytes which had been injected with complementary oligodeoxyribonucleotides (73). Noncomplementary oligomers did not cause degradation, and degradation of the β globin mRNA increased with increasing oligomer concentration. Oligomers targeted to different regions of the mRNA were not equally effective in causing degradation. This observation may reflect the presence of secondary structure at the oligomer binding site which would decrease or prevent binding.

A pentadecadeoxyribonucleotide complementary to the initiation codon region of the cellular oncogene, c-myc, inhibited the rate of growth of HL-60 cells, a human leukemic cell line (74). The oligomer entered the cells and was protected from S_1 nuclease hydrolysis, evidence that it was bound to the c-myc mRNA. It is not known if the oligomer caused degradation of the mRNA. The oligomer also inhibited expression of the c-myc protein in mitogen-stimulated T-lymphocytes and prevented their entry into S-phase (75). A non-complementary "sense" oligomer had little or no effect on the growth of the cells or c-myc protein synthesis. These studies suggest that antisense oligonucleotides should be useful for studying the role of cellular oncogenes in cell growth and transformation.

The effect of oligodeoxyribonucleotides on the expression and replication of the AIDS virus, HIV, has also been studied in virus-infected T-lymphocytes (76). An oligomer complementary to the acceptor splice site of precursor mRNA coded by the tat-III gene inhibited reverse transcriptase activity 65% in H9 cells. This same oligomer inhibited virus replication approximately 90% as measured by the reduction of the viral proteins, p15 and p24. Oligomers complementary to a site adjacent to the $tRNA^{Lys}$ primer binding site also inhibited reverse transcriptase activity and virus replication, although to a lesser extent than the splice junction specific oligomer.

The uptake of the anti-HIV oligomers by HeLa cells was also measured (76). Incubation of HeLa cells with 20 μM [^{32}P]-labeled 20-mer in serum-free medium showed that counts corresponding to intact oligomer were rapidly associated with the membrane/cytosol fraction of the cells and that the final concentration of the oligomer was approximately 1.5 μM. It was not possible to determine if the oligomer was in the interior of the cell or tightly associated with the cell membrane.

The effectiveness of inhibition by oligodeoxyribonucleotides can be increased by derivatizing the oligomer with poly-L-lysine (77,78). A 15-mer whose sequence is complementary to the initiation codon region of vesicular stomatitis virus (VSV) N-protein mRNA was derivatized through its 3'-end with the ∈-amino groups of poly-L-lysine. This oligomer inhibited synthesis of the five VSV proteins 95% in virus-infected cells but had no detectable effect on the synthesis of cellular proteins or on the replication of encephalomyocarditis virus. It is not clear how the poly-L-lysine conjugation increases biological activity. It may enhance compound uptake into cells, increase resistance to degradation by nucleases or facilitate binding to mRNA.

Oligodeoxyribonucleotide phosphorothioates show potent antiviral activity against HIV in HIV-immortalized human T cells (79). However, the inhibitory activity was not sequence specific. Thus, antisense oligomers complementary to the coding region of the art/trs mRNA, oligomers with the same sequence as the coding region of the mRNA and random noncomplementary oligomers all exhibited inhibitory activity. A homooligomer consisting of 28 deoxycytidine residues completely protected cells against virus infection. The protective effect diminished as the length of the homooliogmer was reduced. Although the antiviral effect is essentially independent of the oligomer sequence, an oligomer in which the N^3 position of thymine residues was blocked by methylation gave no inhibition. This result suggests that the antiviral activity of these analogs is due at least in part to their ability to hydrogen bond to complementary sequences. Since noncomplementary oligomers display activity, such phosphorothioate analogs must function by a mechanism other than strict antisense recognition, possibly by binding to cellular or viral enzymes like reverse transcriptase.

Nonionic oligonucleoside methylphosphonates have antiviral activity against herpes simplex virus (HSV) types 1 and 2, and against VSV in virus-infected cells in culture. An octanucleoside methylphosphonate complementary to the acceptor splice junction of HSV-1 immediate early precursor mRNA 4 and 5 selectively inhibited virus replication in HSV-1 infected Vero cells (80). Virus DNA synthesis but not cellular DNA synthesis was inhibited 70-75% and specific inhibition of viral protein synthesis was also observed. The growth of uninfected Vero cells was not affected. Prolonged treatment with the oligomer produced no apparent effect on cell growth. Inhibition of HSV replication was dependent upon the sequence of the methylphosphonate oligomer. A "mutated" methylphosphonate octamer in which the sequence of the middle two nucleosides was reversed gave essentially no inhibition of HSV replication. This result is consistent with the proposal that the octamer binds to the targeted splice junction sequence.

Oligonucleoside methylphsophonates complementary to VSV N-, NS- and G-protein mRNAs inhibit VSV mRNA translation in a rabbit reticulocyte lysate and inhibit VSV protein synthesis in VSV-infected mouse L-cells (81). Between 60 to 99% inhibition of VSV protein synthesis but not mouse L-cell protein synthesis was observed. Each oligomer, although specifically targeted to one mRNA species, inhibited synthesis of all five VSV proteins. A similar result was obtained for a poly-L-lysine derivatized oligodeoxyribonucleotide complementary to N-protein mRNA (78). In addition to their interaction with mRNA, it is possible that the oligomers interact with complementary sequences on the plus strand viral RNA. This interaction could prevent transcription and result in reduced levels of viral mRNAs and viral proteins. As expected, the

oligomers reduced virus replication by a factor of ten. No effect on mouse L-cell growth or cloning efficiency could be detected.

The effects of antisense oligonucleotides and oligonucleotide analogs (15-mers) targeted against a coding sequence of chloramphenicol acetyl transferase (CAT) mRNA on expression of the CAT gene in plasmid-transfected CV-1 cells were compared (82). Gene expression was inhibited 84% by the phosphorothioate oligomer, 65% by the methylphosphonate oligomer and 34% by the unmodified oligodeoxyribonucleotide. Introduction of N^3-methylthymidine into the phosphorothioate oligomer significantly reduced the inhibitory effect.

Antisense Oligonucleotides as Potential Drugs

Antisense oligonucleotides provide an attractive strategy for rationally designing antiviral and chemotherapeutic agents. Unlike conventional drugs which are often targeted against enzyme active sites of unknown structure or drugs which bind to DNA but have limited base specificity, antisense oligomers interact only with specific nucleic acid sequences. In theory an oligomer 12 nucleotides long should specifically interact with a single mRNA species in mammalian cells and thus selectively inhibit a single gene product (36).

Opportunities and Limitations - While oligonucleotides can be studied in cell culture, their hydrolytic instability makes experiments in animals most difficult. Thus, the use of these oligomers as antiviral or chemotherapeutic agents would appear to be quite limited.

In contrast, phosphorothioate oligomers show increased resistance to nuclease hydrolysis and are apparently taken up by cells in culture (79). The ability of these analogs to activate ribonuclease H has not been reported. Phosphorothioate analogs do, however, inhibit at low concentrations. Because this inhibitory activity is not strictly related to the sequence of the oligomer, it is unclear if it will be possible to use these analogs to inhibit selectively the expression of specific genes in a predetermined manner.

Methylphosphonate oligomers are completely resistant to nuclease hydrolysis, readily taken up by cells in culture and relatively lipophilic, and thus are attractive candidates for use in animal experiments (36). It appears that methylphosphonates inhibit gene expression by directly blocking the target sequence and consequently, rather high concentrations are required to achieve inhibition. It may be possible to overcome this disadvantage with derivatives which bind irreversibly to their targeted nucleic acid sequences.

Conclusions - To date there have been no published reports on the use of antisense oligonucleotides or oligonucleotide analogs in animals. Thus, little is known about the stability, distribution, acute and/or chronic toxicity or immunogenicity of this class of compounds in vivo. Also, methods must be developed for synthesizing large quantities of these rather complex, high molecular weight molecules. It is unclear if current procedures can be used in a cost effective manner to synthesize the gram or kilogram quantities of oligomers which will undoubtedly be required for any drug development program. Despite these unknowns, the promising results obtained in cell culture studies suggest that antisense oligonucleotide analogs offer a unique approach for the rational design of drugs with highly specific antiviral or antitumor activity.

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Chapter 31. Enzyme Assisted Transformations in Organic Media

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Introduction - Enzymes have become recognized as routine catalysts in organic synthesis because of their unique properties of inducing high reaction rates under mild conditions and high regio- and stereo-selectivity. Various reviews have described the enzyme classes used in all aqueous systems to prepare amino acids, hydroxy acids, chiral alcohols and natural products (1-7). Methods to identify and immobilize biocatalysts have also been surveyed (8,9). Since enzyme biocatalysis was last reviewed in this series (10), there has been an increased interest in transformations assisted by organic solvents. This review will focus on enzymatic and whole cell biocatalysis in which a significant portion of the reaction medium contains an organic component. Three major solvent systems will be considered: all-organic, incorporating small amounts of dissolved water; aqueous/organic two-phase mixtures; and aqueous/miscible organic co-solvent systems.

General Principles Governing Biocatalyzed Reactions in Organic Solvents - Rules for using enzymes in organic solvents have been proposed (11). The hydration shell around the protein must be maintained by dissolving small amounts of water in the solvent. The enzyme should be prepared at its pH optimum prior to use (12). Reaction rates in organic media are strongly affected by solvent type and are sometimes slower than in water. Enzyme stability can be enhanced, even at high temperatures, due to the low water content of the reaction system (13). For whole cell biocatalysis, highly non-polar solvents lower toxicity and minimize disruption of cell membranes. Solvent polarity and properties affect cell viability (14,15). For free and immobilized enzyme reactions, the hydrophobicity index of the solvent does not apply, as was demonstrated for enzyme catalyzed glucose isomerization (16) and amino acid esterification (17) in 90 % ethanol, glycohydrolase reactions in 78 % DMSO (18) and in anhydrous DMF (19), and other transformations (20-22).

Manipulation of water content and activity in organic solvent systems significantly increases the synthetic utility of enzymes. The equilibrium position in reversible hydrolytic/esterification (amidation) reactions, the degree of ionization of enzyme and reactants for both hydrolytic and redox reactions, and partition coefficients in multi-phase systems can be quantitated and controlled by the amount of water present (23-27). Substrate partitioning between enzyme binding pockets and organic solvents is altered relative to all-aqueous media, resulting in reaction medium-dependent variations in K_m values. The extent of medium solvation has even been shown to reverse enzyme-substrate preference (28,29). Also, use of a second phase or co-solvent can alter the enantioselectivity of enzymes: methyl-2-chloropropionate was kinetically resolved by Candida lipase-catalyzed hydrolysis in water/carbon tetrachloride, but low enantioselectivity resulted when the compound was substantially dissolved in the aqueous phase (30). The enantioselectivity of liver esterase hydrolyses can be enhanced by addition of DMSO or methanol (31,32). A striking

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example involves the hydrolysis of cis-N-benzyl-2,5-bismethoxycarbonylpyrrolidine in which a 17 % e.e. (aqueous reaction) was increased to 100 % e.e. in a 25 % DMSO co-solvent (33). These enhancements may be a result of differential activity modulation of various enzymes present in the preparation (34).

In two-phase systems, the organic phase serves as a reservoir for poorly water soluble substrates, minimizing reaction volume. For more polar substrates, the second phase serves to regulate the aqueous phase concentrations of reactants and products. If either substrate or product is an inhibitor to the enzyme, removal by partitioning into the organic phase lessens its effect. This property is useful for kinetic hydrolytic resolutions where the aqueous concentration of the more slowly hydrolyzed isomer should be minimized to achieve a maximal conversion rate. Also, the degree of ionization and partition of reactants can be used to control pH during reaction (35). Transport and partition phenomena may become rate limiting parameters, especially when an additional phase in the form of an immobilized biocatalyst is added (36,37).

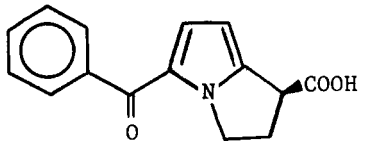
Biocatalyst immobilization for two-phase and co-solvent media use is treated as for all-aqueous solvents. All-organic reaction solvents offer the significant advantage of ease, since enzyme/cells can be entrapped within, dried onto or precipitated on insoluble particles (38-40). No covalent chemistry is required and the catalyst support is recovered by washing to remove inactivated enzyme. As an alternative to fixed bed and stirred tank operation, biocatalysts have been entrapped within hollow fiber and sheet membranes. Multi-phasic media have been used in continuous and batch mode processes involving concentrated reactant and product streams without emulsion problems (41-43). Surfactant encapsulation of enzymes together with small amounts of water (20 to 100 molecules) forms reverse micelles in solvents. Reverse micelles reduce protein denaturation and degradation (44).The internal ordered structure decreases water activity without the need for a co-solvent, providing the thermodynamic impetus to carry out synthetic reactions such as protease- and lipase-catalyzed peptide and ester synthesis (normally,the hydrolysis reaction is strongly favored). Enzymatic reactions in reverse micelles have been recently reviewed (44). Covalent modification of enzyme surfaces to permit organic solvent dissolution has been most successful using polyethylene glycol attachment. Modified enzymes (lipases, proteases, catalase and peroxidase) are soluble in benzene, toluene, acetone, ethanol, dimethyl formamide and chlorinated hydrocarbons and have been used to catalyze ester and peptide bond formation, oxidations and peroxide decomposition (45,46)

Enzyme - catalyzed organic transformations have been conducted in two non-traditional solvent systems, supercritical fluids and aqueous two-phase solutions. Two-phase aqueous/immiscible aqueous polymer solutions are useful for partitioning hydrophilic compounds between phases. Applications, although limited, include purification of proteins from fermentation broths (47) and the extraction of acetone, butanol (48) or reaction products such as 6-amino penicillanic acid away from enzymatic benzyl penicillin hydrolyses (49). Lastly, enzyme reactions have been carried out in super-critical fluids. Higher diffusivities and the ability to differentially alter solubility by changing temperature and pressure may offer advantages (50).

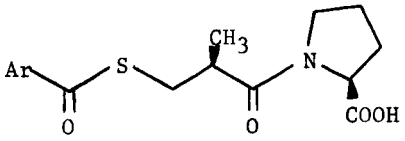
Examples of Biotransformations

Hydrolyses and Esterifications - Hydrolases are presently the most synthetically useful proteins. These enzymes are small, robust catalysts, tolerant of many solvents over a range of reaction conditions. Substrate specificities are not absolute and a wide variety of molecules are hydrolyzed with high enantio- and regio-specificity (51). More importantly, the common use of hydrolases in the food, detergent and textile industries makes available thousand pound quantities of protein at minimal cost. Their broad substrate specificity requires that a number of enzyme or cell culture preparations be screened for activity and specificity for a given target compound (8). Most applications to date have been kinetic resolutions of chiral compounds; structure activity relations have been required to achieve desired reaction rates and optical purities (52,53). For homogeneous reaction systems, useful mathematical approximations have been derived to determine product optical purity as a function of reaction extent and catalyst selectivity (54,55). Optical purity enhancement is achieved using chiral bifunctional synthons, as shown for resolution of several β,δ diacetoxy-cyclopentyl compounds (56). These methods were extended to biphasic systems and validated using resolutions of menthol and 2-(chlorophenoxy)propionate by lipase-catalyzed esterification (57).

Resolution of α-substituted acids in organic media has been used to prepare important pharmaceutical and agricultural intermediates. Various 2-substituted propionic acids were resolved by Candida lipase with greater than 95 % e.e. to generate S-2-halopropionic acids (30,53) and substituted R-2-phenoxy propionates (58) for use in aryloxy phenoxy propionate herbicides or anti inflammatory drugs such as naproxen (59). Ketorolac, **1**, similarly was resolved in 92 % yield using S.griseus protease (60). Substituted cyclopropane carboxylic esters were resolved both enantio- and diastereo-selectively in 65 to 90 % yield by porcine liver esterase (61). Recently, 3-aroylthio-2-methylpropionic acids, **2**, intermediates in the synthesis of the ACE inhibitor, captopril, were prepared using various lipases. Enantiomeric excesses were strongly influenced by both the enzyme and the aroylthio moiety (62). Resolutions of amino acids have been extensively carried out on an industrial scale using various ester, amide or hydantoin derivatives in suspension reactions (4,9). Biphasic reactions have been used to advantage for phenylalanine resolution (63) and D-arylglycine antibiotic side chain production (64), with optical purity and isolated yields greater than 90 %.



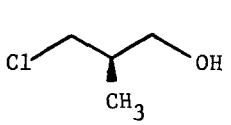
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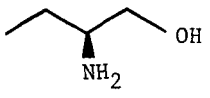
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Resolution of primary and secondary alcohols using suspensions, where the substrate acts as the second phase, have provided intermediates for a number of important compounds. For example, resolved halo alcohols were used to prepare chiral epoxides (65,66), and optically-resolved glycidyl butyrate, commonly used in β-blocker synthesis, was obtained by a pancreatic lipase resolution procedure (67). Other methods for β-blocker

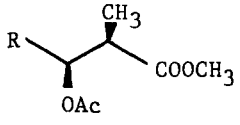
resolution have included stereo- specific hydrolyses of 1-acetoxy-2-aryloxypropionitriles (79 % yield) (68) and 3-alkyl-5-alkoxymethyl-2-oxazolidinones by Pseudomonas lipase (69). Optically active propargylic alcohols, intermediates in the synthesis of pyrethroid insecticides, were made in 20 to 60 % yield using B.subtilis lipase (70,71). Intermediates **3-5** for the synthesis of the psychotropic drug, levomepromazine (72), antitubercular drug, ethambutol (73), and oudemansin A (74) were also kinetically resolved using lipase-catalyzed hydrolyses or transesterifications. Finally, terpene alcohols have been resolved by direct transesterification (75) in water-saturated hexane (76) and heptane (77); α-substituted cyclohexanols have been similarly resolved in organic media (78).



3



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The earliest usage of organic co-solvents for enzyme reactions was in the area of peptide synthesis. The equilibrium constant for peptide hydrolysis is near one, but due to ionization, the apparent equilibrium constant in water, pH 7, lies strongly on the side of hydrolysis. Co-solvent addition to reduce ionization, or extraction of organic soluble derivatives in two-phase media drives the enzyme-catalyzed reaction toward peptide bond formation (79,80). This technology has been applied to the synthesis of the dipeptide sweetener, aspartame, in 93 % yield from precursor amino acids with minimum protecting groups (81). Racemic mixtures of amino acids were also used with simultaneous enzymatic coupling and kinetic resolution (82). Other protease-catalyzed peptide syntheses have been used to prepare an L-aspartyl-D-alanine sweetener and leucine enkephalin (83). The acyl intermediate formed by lipases during ester hydrolysis can also be used to introduce a variety of nucleophiles other than water. Lipases were shown to catalyze peptide bond formation in ether, ethyl acetate or pyridine, using a variety of D- and L- amino acids and glycinal dimethyl acetal as acyl donors and acceptors (84). Although reaction rates were slower with lipases than with proteases their wider specificity allows for the synthesis of peptides with unusual acyl moieties. This methodology has been used to prepare penicillin G peptide precursors suitable for enzymatic β-lactam ring closure (85). Subtilisin protease has also been shown to catalyze the formation of D-amino acid peptides in organic solvents (29).

Increasing attention is being paid to selective enzymatic protection and deprotection of carbohydrates, a difficult task with traditional chemical methods. Acylation of the primary hydroxyls in free sugars has been achieved by transesterification with lipases and proteases in pyridine or dimethyl formamide (19,86,87). Examples of enzymatically prepared intermediates include 6-deoxy-6-fluoro-D-glucose, an anti tumor agent (86), riboflavin-5'-monobutyrate, nucleoside base monobutyrates and various amino acid acylated sugars (19). Selectivity between secondary hydroxyls can also be achieved (87).

Enzymatic transformations of prochiral and meso difunctional substrates in organic solvents have been utilized to solve the "undesired isomer problem" inherent in classical resolutions in which 50 % of the starting material is discarded. Liver esterase

and pancreatic lipase enantiospecific hydrolyses to afford half esters have been performed on disubstituted malonates and glutarates, and cis difunctional cycloalkenyl and cyclopropane compounds. These chiral building blocks have in turn been used to prepare mevalonolactones, verrucarinic acid, pyrethroids, brefeldin A and cyclopentanoid natural products (1,88,89). Dimethyl (N-benzyloxycarbonyl) β-aminoglutarate has been hydrolyzed to its half ester (93 % yield; 96 % e.e.) and subsequently converted to the corresponding monocyclic β-lactam (90). Cis-diacetoxy cyclopentenes have been converted with liver esterase, using substrate suspensions, to half esters, and then modified to give prostaglandin intermediates (88,91). Transesterification of 2- substituted 1,3-propane diols by porcine pancreatic lipase in methyl acetate gave optical purities between 10 and 95 % depending on the 2-substituent and the degree of enzyme purity. Notably, the enzyme hydrolyzes or esterifies the same enantiomer, and therefore the stereo configuration of the half-ester product is determined by the solvent used (i.e. water or organic) (92). Esterase and chymotrypsin have been used in 25 % DMSO to generate optically active dialkylated malonate half esters. Subsequent chemical conversions afforded α-substituted amino acids in high yields and optical purity (93). Chiral 2-fluoro-2-substituted malonate half esters have also been investigated (94).

Oxidations and Reductions - Oxido-reductases catalyze a variety of stereo-specific reactions (1). Their usage in organic media has been limited, partly due to the required use of expensive cofactors for isolated enzymes or the instability of whole cell systems to solvents. As noted, progress has been made in stabilizing whole cells toward organic media (14,15). Reaction conditions for regio- and stereo-specific oxidoreductions of hydroxyl/keto groups on steroids using various hydroxysteroid dehydrogenases in two-phase solvents has been recently reported (95). Oxidation, hydroxylation and dehydrogenation of steroids has been reviewed for transformations in biphasic (36), cosolvent and benzene/n-heptane systems (37) using immobilized and free whole cells or enzymes. However, conversion rates and catalyst life were less than optimal. Other non-steroid oxidations have been reported (96).

For isolated enzyme reactions, cofactor recycle systems have been devised to permit practical use (97). These systems fall into two categories: one enzyme used with co-solvent as regenerant, or two enzyme, coupled reactions. The single enzyme system makes use of the reversibility of the enzyme reaction. Substrate is reduced (oxidized) in the forward reaction and a co-solvent in excess is oxidized (reduced) by the same enzyme in the reverse reaction by mass action. The cofactor consumed in the forward reaction is regenerated by the reverse solvent reaction. For example, a series of ketone analogues was asymmetrically reduced on a preparative scale by thermostable enzymes from Thermoanaerobium brockii (98) and Sulfolobus solfataricus (99) in the presence of 8 to 10 % isopropanol cosolvent as a cofactor regenerant. Recycle numbers of 10^4-10^6 per NAD(P)H molecule have been achieved (100). Similar recycle schemes have been used for reversed micelle entrapped horse liver dehydrogenase acting on cyclohexanone, ethanol and the steroid eticholan-3β-ol-17-one, in cyclohexane (101).

The two enzyme system relies on the forward enzyme reaction of two different enzymes acting as an oxidase and a reductase on two different substrates. Each uses the cofactor product of the other reaction. Inherently, two products are produced, requiring separation. Various coupled alcohol dehydrogenases have been used in biphasic media to

generate ∝-trifluoromethyl benzyl alcohol, S-methyl 4-hydroxy hexanoate (pheromone intermediate), alkyl furan carbinols (L-sugars) and 3- ∝-,20-hydroxy steroids, all in greater than 92 % e.e. (102). The organic phase serves to deliver reactant and extract product, assuring reaction completion. Oxidation of meso 6 generates optically active 7 and 8, (79% yield; 100% e.e.). Cofactor is regenerated by reductive amination of 9 (Figure 1). The two-phase system serves to extract the product lactone, an inhibitor of the enzyme, as it is formed (103). Also, numerous organic media have been used in the regio- and enantio-selective preparation of bicyclo[3.2.0] hept-2-ene-6-ols (104). Oxidation/reduction with cofactor recycle in all organic media is possible, but enzymatic rates are strongly dependent on solvent composition and the amount of dissolved water (22).

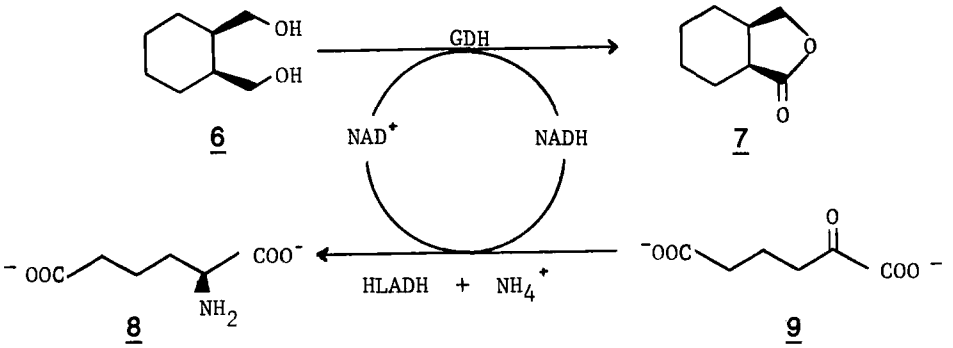


Figure 1. Cofactor recycle system using two enzymes: Glutamate Dehydrogenase (GDH), Horse Liver Alcohol Dehydrogenase (HLADH).

Hydrolase and reductase reactions are useful for generating ∝-halo alcohols from which chiral epoxides can be synthesized. Several microbial organisms directly and stereo specifically epoxidized alkenes. Non-growing Pseudomonas cells mono-epoxidized 1,7-octadiene in a two-phase solvent system (105). The cyclohexane present served to prevent inhibition and inactivation by the epoxide. Mycobacterium cells entrapped in calcium alginate gels epoxidized propene in n-hexadecane (106). Organisms such as Pseudomonas stereospecifically epoxidized various para-substituted phenylallyl ethers which were converted into the β-blockers,metoprolol and atenolol, with optical purities up to 99% (107).

Useful enzymatic oxidations may not occur in the presence of excess water. Peroxidase depolymerized lignin in dioxane, dimethyl formamide or methyl formate with 5 % buffer but not in aqueous solution (108). L-DOPA was prepared in 71 % yield in chloroform, whereas the reaction is negligible in water due to quinone polymerization and enzyme inactivation (109). Horseradish peroxidase catalyzed the free radical polymerization of substituted phenols; polymer molecular weights generated in 85 % dioxane were up to 50 times higher than those in water (110). In an opposite sense, benzoquinone, acting as an electron acceptor for glucose oxidase oxidation, was converted to hydroquinone in nearly 100 % yield. The reaction was strongly dependent on the cosolvent used (111).

Conclusions- Enzyme-assisted organic synthesis is no longer only of academic interest. Increased enzyme availability augmented by genetic engineering and the use of organic reaction solvents makes biocatalysis practical. Enzymatic transformations should be considered as complementary to chemical methods for achieving a high degree of stereospecificity, each technology offering clear advantages for certain applications.

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Chapter 32. Mechanistic Aspects of Xenobiotic Metabolism as Related to Drug Design

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Introduction - The essence of rational drug design is the reasoned extrapolation of our understanding of the complex relationships between the physicochemical properties of a substance and biological activity. These relationships include not only the interaction between the drug and its biochemical target, but also the physical and metabolic disposition of the substance (1). Unfortunately, predictions of the metabolic fates of drugs are difficult in the best of circumstances, due not only to the numerous metabolic opportunities in complex organisms, but also to the relatively crude nature of our understanding of the enzymology of drug metabolism.

Metabolic transformations of drugs and xenobiotics are catalyzed largely, though certainly not exclusively, by a group of detoxication enzymes (2). Examples of such enzymes include the cytochromes P-450 and flavin containing monooxygenases, reductases and dehydrogenases, hydrolases including esterases and epoxide hydrolase, and a variety of transferases such as glutathione S-transferase, UDP-glucuronyltransferase, and sulfotransferases. These enzymes usually exhibit rather broad substrate selectivity but manifest marked kinetic, stereochemical and regiochemical discrimination toward structurally different substances. Often the metabolism of a drug proceeds by multiple serial or parallel reactions. The choice of pathway can be dictated by the chemical and/or stereochemical outcomes of a number of enzymic reactions occuring early in metabolism (2-4). A recent discussion of the general catalytic properties of detoxication enzymes has appeared (3). In addition, earlier work on the properties of a large number of individual enzymes can be found in an excellent series of monographs edited by Jakoby *et. al.* (2,4). The participation of enzymes other than detoxication enzymes in drug metabolism is also common,since many therapeutic agents are structural mimics of endogenous compounds and are, therefore, substrates for specific enzymes normally associated with "natural" substances. The field of study is broad, indeed.

The collation of kinetic and structural information about a group of detoxication enzymes into a useful guide to drug design is fraught with uncertainties. Firstly, many detoxication enzymes occur in groups of isoenzymic species with unique but overlapping substrate specificities (5-8). Secondly, the concentrations of various isozymes are often dependent on genetic control mechanisms which respond to environmental changes such as diet and toxic stress (9-11). Thirdly, most detailed enzymological investigations have been conducted on enzymes isolated from species other than humans. Species to species differences in drug metabolism and xenobiotic metabolizing enzymes are well documented (12). Genetic polymorphism of, for example, human detoxication enzymes can give rise to tremendous differences in metabolism (12,13). The latter two problems are coming under increased scrutiny with recent advances in purification and cataloguing of enzymes from humans (5, 13-16).

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The remainder of this chapter will discuss the development of methods by which catalytic profiles of detoxication enzymes can be determined, recent progress in mechanistic investigations and finally, the application of metabolic considerations to drug design.

Determination of Catalytic Profiles

Methods for the investigation of catalysis by detoxication enzymes are fundamentally the same as those used for any enzyme catalyzed reaction. Any differences lie in the rather broad substrate selectivity of this class of enzyme which allows more leeway in the design of mechanistic probes. The most common approaches are kinetic analysis of substrate selectivity, mapping of the topology of the active site using probe molecules and ultimately, the determination of the three dimensional structure of the protein.

Kinetic Analysis - Kinetic analysis is the single most useful tool for establishing the catalytic behavior of detoxication enzymes. For multiple substrate reactions, the order of addition of substrates and release of products can give a vague notion of the mutual orientation of the substrates or products in the active site. Conventional initial velocity steady-state kinetic measurements, along with substrate, product, and dead-end inhibition can be used to determine the kinetic mechanism (17). Recently, a more efficient alternative substrate diagnostic has been proposed in which the kinetic mechanism of two substrate enzyme catalyzed reactions can be deduced from initial velocity patterns obtained from varying the identity of alternative substrates vs the concentration of the second substrate (18). The technique has been demonstrated to work well with kanamycin acetyltransferase (19) and glutathione transferase (20), leading to the conclusion that both enzymes exhibit random sequential mechanisms. The success of the method requires alternative substrates for both the xenobiotic substrate and the physiological co-substrate. Although finding the former is not generally a problem, a distinct disadvantage of the method can be the necessity to synthesize alternative physiological co-substrates. Although a collection of the catalytic rate constants k_c and k_c/K_m for various substrates is helpful, the quality and extent to which such data can be interpreted is considerably enhanced if it is obtained in a systematic manner. For example, linear-free energy relationships have been used to examine the transition state for glutathione transferase (20) and cytochrome P-450 catalyzed reactions (21). In the latter case differential substituent effects on heme alkylation by phenylacetylenes and substrate turnover requires that the two pathways diverge early in the catalytic process and suggests that radical cation intermediates are not formed (21). More general quantitative structure-activity relationships can be used to predict substrate recognition (K_m values) as recently demonstrated with the sulfotransferases (22) and UDP-glucuronosyltransferases (23). Isotope effects on kinetic constants are also finding increasing usefulness in studies of the mechanisms of detoxication reactions as exemplified in studies of cytochrome P-450 and epoxide hydrolase (24-26).

Active Site Mapping - Topological maps of the active sites of detoxication enzymes are particularly useful in predicting metabolism since such information can be used to define substrate orientations. Crude active site topologies can be inferred from analysis of the stereoselectivity and regioselectivity of catalysis or from specific reversible or irreversible inhibition (3). A single substrate molecule, which has several loci where reaction can occur, can be used to deduce preferred orientations of the substrate in the active site. This approach has been suc-

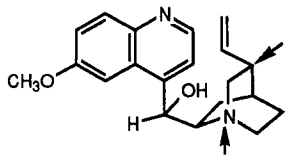
cessful with the cytochromes P-450 (27-30), and in one favorable case has led to the proposal of a minimal steric boundary for a substrate binding site (29). A similar method has been used with transferases and hydrolases where the stereo- or regiochemical discrimination between two reaction sites in a series of substrates is determined. This technique has been applied recently to epoxide hydrolase (31,32), glutathione transferase (33) and UDP-glucuronosyltransferase (34,35). Intermolecular kinetic discrimination between regioisomeric phenols (36) and conformationally isomeric dihydrodiols of polycyclic aromatic hydrocarbons (34) has been a useful method for studying the active site topology of UDP-glucuronosyltransferase.

The dependence of specific inhibition (either type of inhibition or K_i) or spectral properties on the structure or stereochemistry of a bound inhibitor can be used to infer geometric preferences in a binding site. For instance, nitroxide spin-labeled inhibitors have been utilized to probe the microenvironment of cytochrome $P\text{-}450_{scc}$ leading to the suggestion that the substrate cholesterol disrupts a histidine nitrogen coordination with the heme iron (37). Stereospecific inhibition of glutathione transferase by the diastereomers of 9,10-dihydro-9-(S-glutathionyl-)-10-hydroxyphenanthrene has been observed (38). However, the relationship between inhibition (K_i) and the spatial organization of the active site is unclear in this instance. Stereospecific inhibition of aromatase by 10-oxirane- and 10-thiirane-substituted androgens has led to a model for the geometric organization of the substrate binding site with respect to the heme moiety (39). Competitive inhibition of bilirubin conjugation by triphenylcarboxylates was recently used to probe UDP-glucuronosyltransferase (40).

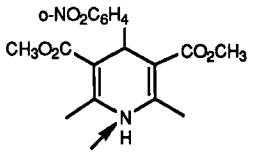
Three Dimensional Structure Determination - Very little is known about the three dimensional structures of detoxication enzymes. Structures of a few, such as alcohol dehydrogenase, rhodanese and glutathione peroxidase, have been determined by X-ray crystallography (41-43). The most important recent advance has been the determination of the crystal structures of substrate-free (44), substrate-bound (45) and inhibited (46) cytochrome $P\text{-}450_{cam}$. These studies have provided, for the first time, a detailed look at intermolecular interactions responsible for the regioselective and stereoselective behavior of a cytochrome P-450. Large X-ray-quality single crystals have been obtained for glutathione transferase (47).

Progress in Mechanistic Investigations

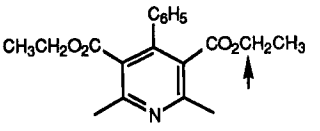
Oxidations - The study of oxidative transformations dominates the field of drug and xenobiotic metabolism. The central role and versatile catalytic nature of the cytochromes P-450 continues to attract enormous interest. Increased attention is being paid to human isoenzymes. For example, oxidation of the anti-arrhythmic quinidine, **1**, has been shown to occur through cytochrome $P\text{-}450_{nf}$, even though the drug binds much more tightly to (and inhibits) the $P\text{-}450_{db}$ isoenzyme (48). The regioselective oxidations of a number of substrates for these two isoenzymes have been summarized (48,49). Oxidation of the dihydropyridine class of Ca^{2+} channel blockers including nifedipine, **2**, has also been described (50). Regiochemistry of the oxidation of **1** and **2** by cytochrome $P\text{-}450_{nf}$ is indicated by the arrows. A novel oxidative cleavage of a carboxylic ester **3**, by rat liver P-450's was reported, suggesting that ester cleavages need not occur exclusively by hydrolytic routes (51). The stereoselective loss of the 5'-*pro*-E proton in the cytochrome P-450-catalyzed oxidation of (S)-nicotine to the corresponding iminium ion suggests that this reaction,



1



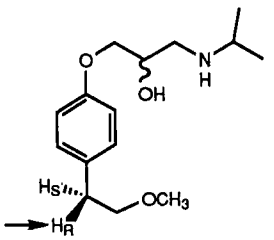
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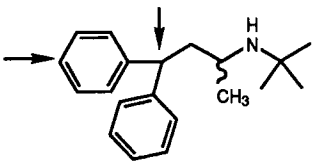
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like many others, is under steric control of the enzyme (52). A model for the active site of human placental aromatase cytochrome P-450 has been presented based on the sensitivity of the enzyme to cyanide inhibition of the metabolism of 19-norandrostenedione and androstenedione (53).

Interesting and informative observations on the stereochemistry of cytochrome P-450-catalyzed oxidations continue to be made with enzymes from species other than man. The rat liver enzymes have been found to hydroxylate both the 2R and 2S enantiomers of the β_1-selective adrenergic antagonist metoprolol, 4, at the H_R-position of the prochiral 1'-methylene group to give predominately the (1'R,2R) and (1'R,2S)-diastereomeric products (54). In contrast, the regioselectivity of hydroxylation of terodiline, 5, differs considerably between the two substrate enantiomers. Aromatic *p*- hydroxylation predominates with the R-antipode whereas benzylic hydroxylation is prefered with the S-isomer (55). The diastereomeric composition of the aromatic hydroxylation product also depends on the chirality of 5. Stereoselective epoxidation of the polycyclic aromatic hydrocarbon triphenylene *via* the rapidly racemizing 1,2-oxide has been recently reported (56).



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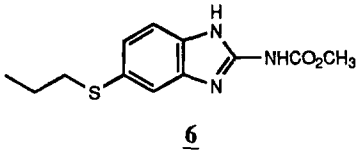


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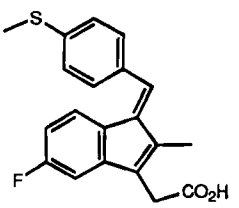
The flavin containing monooxygenases (FMO) are thought to contribute substantially to the oxidation of a number of drugs, particularly those containing nucleophilic sulfur. Sulfoxidations have been reviewed recently (57). The mechanistic differences between monooxygenation of sulfur by cytochrome P-450 and FMO are quite distinct (58). Oxygen transfer by FMO involves nucleophilic attack of the substrate on the 4a-flavin hydroperoxide ,whereas the P-450 catalyzed reaction probably involves single electron transfer from sulfur followed by oxygen rebound from the heme. The relative contribution of the two pathways has been examined in the sulfoxidation of the anthelmintic, albendazole, 6 , by pig liver microsomes and is found to depend on the concentration of 6 (59). Chiral sulfoxidation of sulindac sulfide, 7 , (the active anti-arthritic drug derived from the sulfoxide prodrug) has been shown to be catalyzed predominately by FMO and may be important in the redox cycling of this long-lasting drug (60,61). The substrate specificity of hog-liver FMO toward divalent sulfur compounds with anionic groups suggests that the position of the charged group relative to the sulfur is critical for substrate recognition (62).

Oxidations of aldehydes to carboxylates catalyzed by liver aldehyde dehydrogenases have also been examined. The enzyme from rat hepatoma cells has been shown to exhibit *pro*-R-specific hydride transfer (63).

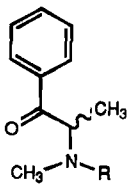
Reductions - A number of drugs are known to be metabolized or metabolically activated by reduction (*e.g.* sulindac, *vida supra*). Stereoselective carbonyl reduction of dimethylpropion, **8**, and monomethylpropion, **9**, was recently reported (64). Only the 1S;2S and 1R;2R isomers of the methylephedrine product are reported from 2S-**8** and 2R-**8**, respectively. The reduction of quinones to hydroquinones catalyzed by two electron transfer from DT-diaphorase or by single-electron transfer from NADPH-cytochrome P-450 reductase followed by dismutation is thought to be the obligatory first-step in the formation of quinol mono- and diglucuronides (65-67).



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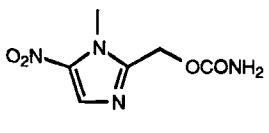


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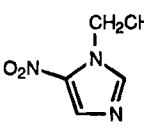


8 R = CH_3
9 R = H

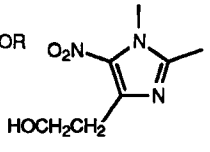
The reduction of 5-nitroimidazoles, many of which are effective antitrichomonal agents, has long been thought to be necessary for expression of their therapeutic activity as well as their undesired mutagenic activity. Recent investigation into the mechanism of metabolism and formation of protein-bound metabolites appears to suggest that the metabolism of ronidazole, **10**, follows different pathways in mammals and *T. foetus* (68). More importantly, these results were used to design molecules in which the antitricomonal and mutagenic activities were partially separated (69). Thus, placing a gallyl group in the molecule (e.g. **11**, where R = 3,4,5-triacetoxygallyl), to offset possible production of hydroxyl radicals from the one-electron-reduced radical anion and oxygen, and introduction of a substituent at the 4-position as in **12** to prevent nucleophilic attack on the four-electron reduced species were found to reduce mutagenic activity while showing more modest diminutions of antitrichomonal activity (69).



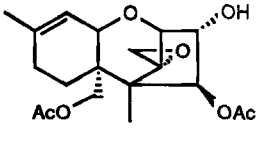
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11



12



13

A new glutathione peroxidase has been isolated from human plasma. It is suggested that the enzyme may be responsible for the regulation of plasma hydroperoxide levels (70).

Hydrolysis - Five microsomal carboxylesterase isoenzymes have been isolated from mouse liver (71). The enzymes were found to exhibit different regioselectivities toward the mycotoxin,diacetoxyscripenol, **13**.

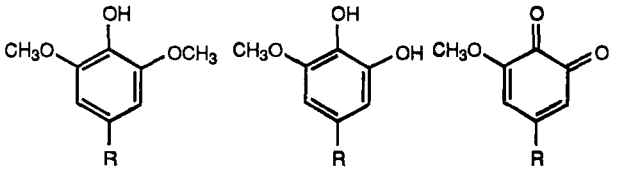
Transfer Reactions - Recent progress in the enzymology of glutathione transferases (3,5), sulfotransferase (3) and UDP-glucuronosyltransferases (3,72) has been summarized in review articles. The effect of microsomal membrane dynamics on UDP-glucuronosyltransferase kinetics was recently examined (73). Increased cholesterol lowered the K_m value for both substrates (p-nitrophenol and UDP-glucuronate) and increased V_{max}.

Phenylethanolamine N-methyltransferase has been extensively probed with both substrates and competitive inhibitors (74-77). These investigations have revealed a flat hydrophobic pocket beyond that which interacts with the aromatic ring of phenylethanolamine. In addition, the substrate requirements for enzyme activity suggest that tight, simultaneous interaction with the hydroxyl and amino groups requires an extended conformation of the aminoethyl side chain.

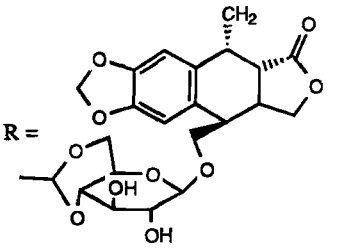
Metabolic Aspects of Drug Design

Knowledge of metabolism has contributed significantly to the discovery of new drugs (as biologically active metabolites), to the design of drugs with altered metabolic and pharmacokinetic profiles and particularly to the development of prodrugs.

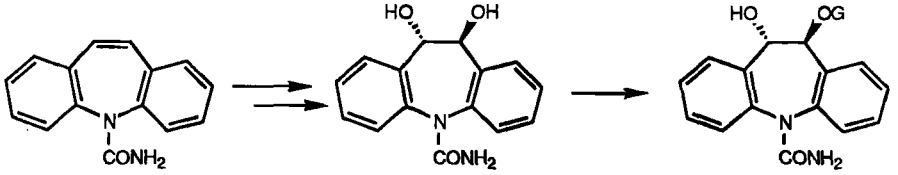
Active Metabolite Studies - A number of potential new drugs have been discovered as the result of the characterization of biologically active metabolites (1). A recent example is found in the study of the *in vitro* metabolism of the anti-neoplastic agent etoposide, **14**, which suggests that the 3',4'-dihydroxy derivative **15**, formed by the cytochrome P-450 catalyzed O-demethylation of the drug, or the corresponding *o*-quinone **16** may have antitumor activity (78).



14 **15** **16**

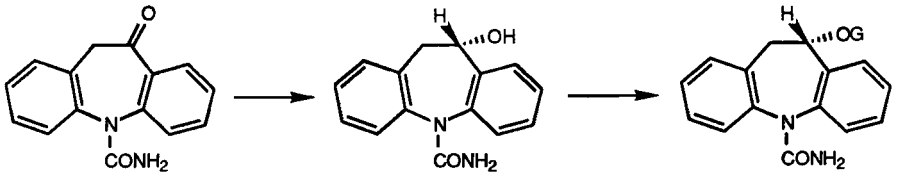


Alteration of Metabolic Profiles - An interesting example of altering the metabolic route and hence the pharmacokinetic stability of a drug is found in the anti-epileptic drug carbamazepine, **17**. Carbamazepine is stereoselectively metabolized to the (10S,11S)-dihydrodiol, **18**, and the corresponding monoglucuronide, **19**, by the serial action of cytochrome P-450, epoxide hydrolase and UDP-glucuronosyltransferase (79). Oxcarbazepine, **20**, on the other hand, is stereoselectively metabolized *via* a



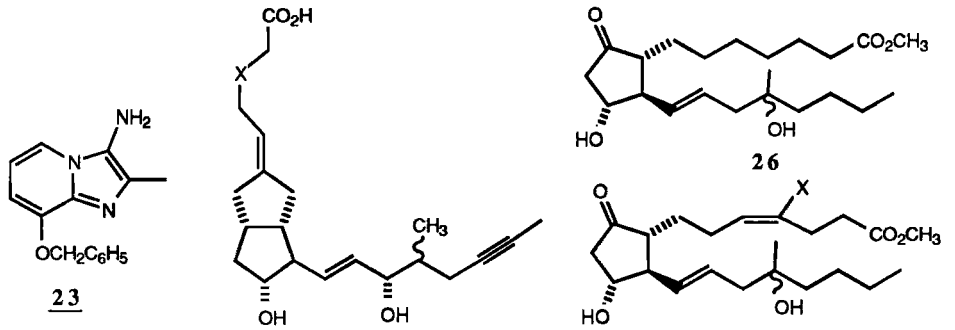
17 **18** **19**

reductive pathway to give the 10S-hydroxy- and glucuronosyl-metabolites 21 and 22 (80). The reductive pathway is apparently less susceptible to pharmacokinetic variability (1).



20 21 22

Antiulcer agents have been the target of a number of investigations aimed at increasing metabolic stability and efficacy. Metabolic studies of imidazo[1,2-a]pyridines and related imidazo[1,2-a] pyrazines have led to the identification of 23 which exhibits good antisecretory and cytoprotective activity with low toxicity (81,82). Replacement of the β-methylene group of the carboxylic side chain of iloprost, 24 with oxygen (25) has been found to increase efficacy by altering the susceptibility to β-oxidation (83). Modification of the susceptibility of misoprostol, 26, to β-oxidation has been accomplished by introduction of one (e.g. 27) or two double bonds in the α-chain (84,85). Unexpectedly, the 4-fluoro analogue 28 of the α-chain-unsaturated compound, enisoprost, 27, is much less potent than the parent (86). Other developments in this area have been reviewed recently (87).



23

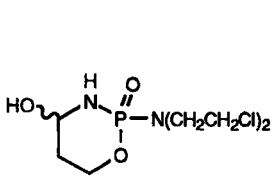
24 X = CH_2
25 X = O

27 X = H
28 X = F

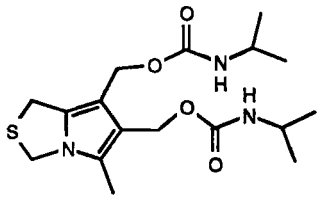
Prodrugs - A number of metabolic transformations have been exploited for the *in vivo* generation of biologically active species from essentially inactive precursors. Prodrug-drug transformations rely on either more or less ubiquitous enzymatic reactions (*e.g.* esterolysis) for systemic release of the drug or on differential metabolism which may impart tissue-selective release (88). Prodrug strategies have been recently reviewed (89).

Prodrugs are often used as cancer chemotherapeutics (88). An excellent example is the cyclophosphamide class of prodrugs. Hydroxylation of cyclophosphamide followed by metabolite decomposition converts the prodrug to the cytotoxic phosphoramide mustard. In this instance the complex solution chemistry of the 4-hydroxycyclophosphamide, 29, is as important as the initial metabolic event and has received considerable attention in the last few years (90-92). The effects of substituents on

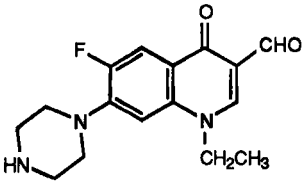
solution chemistry may offer a clue to improving the therapeutic index of this class of drugs (90). An unusual sulfone reduction has been proposed recently to explain the anti-leukemic activities of **30** and the corresponding sulfoxide and sulfone (93).



29



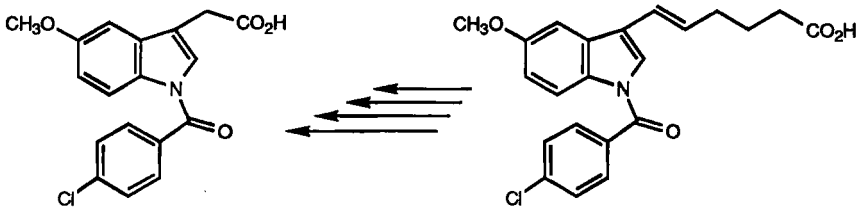
30



31

The 3-formyl derivative (**31**) of the antimicrobial, norfloxacin, has recently been shown to act as a prodrug in which the formyl group is oxidized to a carboxylate group generating the active drug, norfloxacin (94).

The formation of an active species from a precursor may involve a number of enzymic transformations. The generation of indomethacin, **32**, from an arylhexenoic acid precursor, **33** (95), a conversion that involves β-oxidations of the hexenoate side chain, is one example. Though the conversion is not particularly efficient and extrapolation of results to humans is difficult, it is an informative example of the complexities that can be encountered in the metabolic activation of a prodrug.



32 **33**

Conclusions - The use of metabolic information has profound implications for the future of drug design; the current state-of-the-art is far from representative of its ultimate potential. Increased awareness of the intricacies of xenobiotic metabolism will have a significant impact on the design of new therapeutic agents. For this reason the enzymology of drug metabolism should continue to play an influential role in the future of medicinal chemistry.

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Section VII. Special Topics

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Chapter 33. To Market, To Market - 1987

Helen H. Ong and Richard C. Allen
Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ 08876

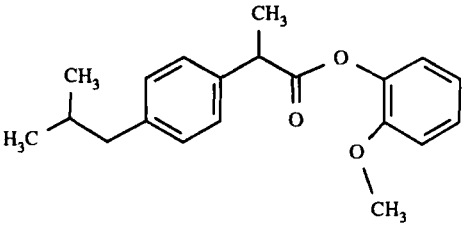
The new chemical entities (NCEs) for human therapeutic use introduced into the world marketplace for the first time during 1987 exceeded the previous year's total by more than 25% (1). This represents an even more impressive gain of 50% when compared with the yearly NCE launches occurring in 1985 or 1984 (2,3). As in previous years, Japan held the lead with 21 or one third of the total new introductions. Italy, France, Switzerland, the United States and the United Kingdom followed in close succession, accounting for one half of the total worldwide entries. It is worth noting that more than 50% of the NCEs originated in two countries: 18 in Japan and 15 in the United States, followed by Italy, West Germany, France and Denmark.

As a continuation of recent trends, antiinfectives and cardiovascular agents dominated the field. More so in 1987 than any previous years, a significant number of NCEs from these two categories could be considered as possessing major therapeutic gains. This list includes APSAC, alteplase, lovastatin and zidovudine. For a longitudinal overview of NCEs introduced to the world market in the past 5 to 15 years, the reader is referred to a number of excellent reviews which appeared last year (4-6) and the cumulative indexes beginning on page 379 of this volume.

During 1987 a record number of new NCEs were approved in the United States (7) and 17 reached the marketplace. Five of these, encainide hydrochloride, esmolol hydrochloride, lovastatin, mometasone furoate and recombinant human growth hormone, were also first worldwide launches and are included in the following compilation.

AF-2259 (antiinflammatory) (8, 9)

Country of Origin:	**Italy**
Originator:	**Angelini**
First Introduction:	**Italy**
Introduced by:	**Angelini**
Trade Name:	**Benflogin**

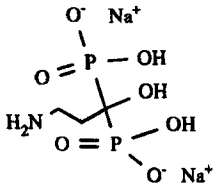


AF-2259 is a guaiacol ester derivative of ibuprofen with comparable antiinflammatory potency but greater gastrointestinal tolerance. It is also reported to be effective in ameliorating symptoms due to common colds and influenza.

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APD (calcium metabolism regulator) (10-12)

Country of Origin:	**W. Germany**
Originator:	**Henkel**
First Introduction:	**Argentina**
Introduced by:	**Labs Dr. Gador**
Trade Name:	**Aminomux**



APD is a calcium metabolism regulator useful in the treatment of Paget's disease, tumor-induced hypercalcemia and other bone disorders involving increased osteoclastic activity.

APSAC (thrombolytic) (13-15)

Country of Origin:	**United Kingdom**	Introduced by:	**Beecham**
Originator:	**Beecham**	Trade Name:	**Eminase**
First Introduction:	**W. Germany**		

APSAC is an anisoylated human plasminogen-streptokinase complex useful in the treatment of myocardial infarct. Its reported advantages over other existing thrombolytics such as alteplase and streptokinase include a longer half life, ease of administration, and a more specific local antithrombotic effect.

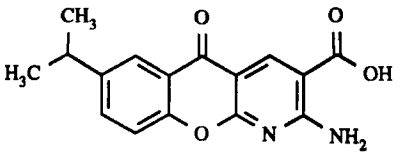
Alteplase (thrombolytic) (16-19)

Country of Origin:	**USA**	Introduced by:	**Boehringer Ingelheim**
Originator:	**Genentech**	Trade Name:	**Actilyse; Activase**
First Introduction:	**New Zealand**		

Alteplase is a recombinant single-chain tissue plasminogen activator useful in the management of thrombosis in acute myocardial infarct. It reportedly causes no allergic reactions but requires intravenous infusion due to a short half-life. Alteplase is the only agent on the U.S. market indicated for the reduction of incidence of congestive heart failure following a heart attack.

Amlexanox (antiasthmatic) (20, 21)

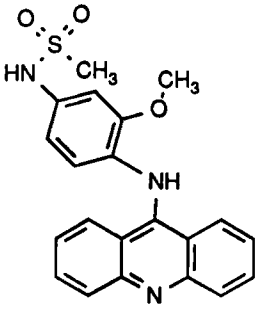
Country of Origin: **Japan**
Originator: **Takeda**
First Introduction: **Japan**
Introduced by: **Takeda**
Trade Name: **Solfa**



Amlexanox is an orally-active antiasthmatic agent useful in the treatment of bronchial asthma and allergy-related sinus disorders. The therapeutic effect of amlexanox appears to be attributed to its inhibitory actions on leukotriene D_4, PAF and histamine.

Amsacrine (antineoplastic) (22, 23)

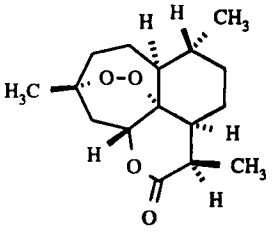
Country of Origin: **New Zealand**
Originator: **Auckland Cancer Chemotherapy Lab**
First Introduction: **Sweden**
Introduced by: **Bristol-Myers**
Trade Name: **Amsakrin**



Amsacrine is a cytostatic reported to be active against adult lymphoblastic leukemia which has failed primary treatment or become resistant. Its clinical use, however, is associated with significant neuro-, gastro- and hepatotoxicity.

Artemisinin (antimalarial) (24-26)

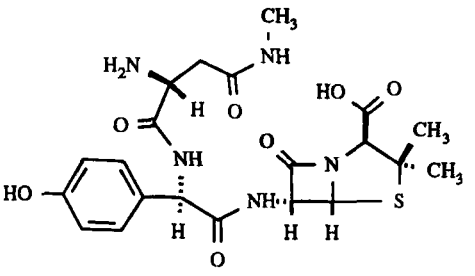
Country of Origin: **China**
Originator: **Ping Hau Sau Res. Group**
First Introduction: **China**
Introduced by: **Baiyunshan**



Artemisinin, a sesquiterpene isolated from a traditional Chinese remedy (quinghao), is useful in the treatment of *Falciparum* malaria, including infections caused by chloroquine resistant strains. It is reported to clear parasitemia quicker than i.v. quinine, and is effective in cerebral malaria.

Aspoxicillin (antibiotic) (27, 28)

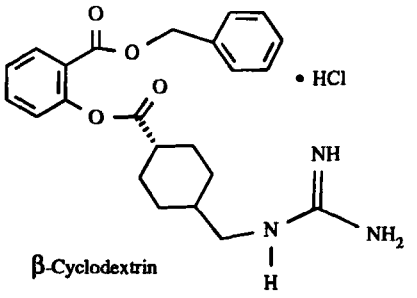
Country of Origin: **Japan**
Originator: **Tanabe**
First Introduction: **Japan**
Introduced by: **Tanabe**
Trade Name: **Doyle**



Aspoxicillin is an injectable, amino acid-type penicillin highly active against Gram-positive and Gram-negative bacteria, including the β-lactamase producing *Bacillus fragilis*. It is reportedly effective in the treatment of peritonitis, pneumonia and bronchitis.

Benexate Hydrochloride (antiulcer) (29, 30)

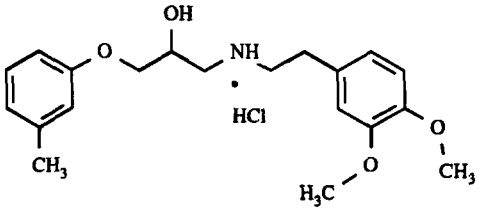
Country of Origin: **Japan**
Originator: **Teikoku Chemical**
First Introduction: **Japan**
Introduced by: **Nagase; Shionogi**
Trade Name: **Lonmiel; Ulgut**



Benexate hydrochloride is a cytoprotective agent useful in the treatment of ulcers. In experimental models benexate inhibits the decrease of PGE_2 and PGI_2 induced by indomethacin or stress. Its relatively long duration of action offers a distinct advantage over other cytoprotective drugs.

Bevantolol Hydrochloride (antihypertensive) (31-33)

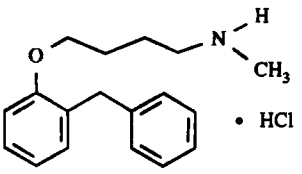
Country of Origin: **USA**
Originator: **Warner-Lambert**
First Introduction: **Philippines**
Introduced by: **Warner-Lambert**
Trade Name: **Ranestol**



Bevantolol hydrochloride is a cardioselective (β_1) adrenergic blocker, devoid of intrinsic sympathomimetic and local anesthetic activities. As with other agents of this category, it is useful in the treatment of hypertension and angina.

Bifemelane Hydrochloride (nootropic) (34, 35)

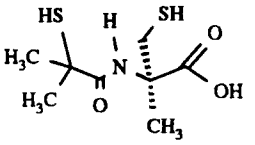
Country of Origin: **Japan**
Originator: **Mitsubishi; Eisai**
First Introduction: **Japan**
Introduced by: **Fujisawa; Eisai**
Trade Name: **Alnert; Celeport**



Bifemelane is a nootropic reported to have antianoxic, anti-ischemic and memory enhancing activities. In man, bifemelane appears to increase cerebral blood flow, while in rodents it reverses scopolamine-induced dementia. Bifemelane has an added adrenergic component reportedly arising from norepinephrine uptake inhibition.

Bucillamine (immunomodulator) (36-38)

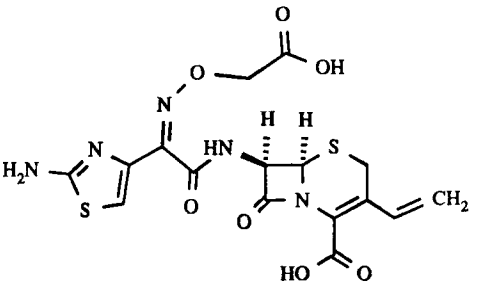
Country of Origin: **Japan**
Originator: **Santen**
First Introduction: **Japan**
Introduced by: **Santen**
Trade Name: **Rimatil**



Bucillamine is an orally active immunomodulator useful in the treatment of rheumatoid arthritis. Its potencies in inhibiting collagenase activity and inactivating rheumatoid factor far exceed those of penicillamine. In man, bucillamine reportedly brings about significant improvements over placebo in erythrocyte sedimentation, grip power, joint swelling and duration of morning stiffness.

Cefixime (antibiotic) (39-41)

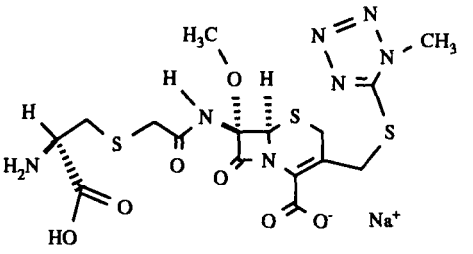
Country of Origin: **Japan**
Originator: **Fujisawa**
First Introduction: **Japan**
Introduced by: **Fujisawa**
Trade Name: **Cefspan**



Cefixime is the first orally active third-generation cephalosporin with a true broad spectrum of activity. It is reportedly more effective against Gram-negative bacteria than conventional oral cephems such as cephalexin and cefaclor.

Cefminox Sodium (antibiotic) (42, 43)

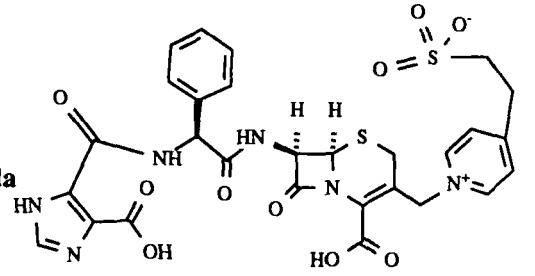
Country of Origin: **Japan**
Originator: **Meiji Seika**
First Introduction: **Japan**
Introduced by: **Meiji Seika/Bristol**
Trade Name: **Meicelin**



Cefminox sodium is a cephamycin antibiotic active against a broad spectrum of microorganisms excluding *Pseudomonas*. Its overall activity profile appears similar to that of cefotetan and cefbuperazone, and slightly/markedly superior to that of cefoxitin/cefmetazole, respectively.

Cefpimizole (antibiotic) (44, 45)

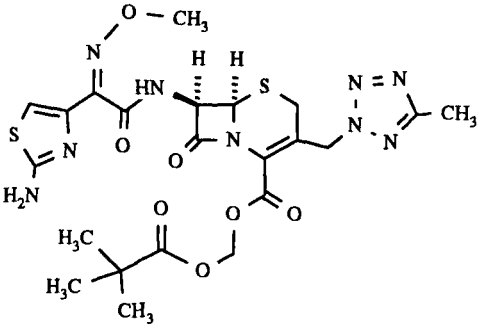
Country of Origin: **Japan**
Originator: **Ajinomoto**
First Introduction: **Japan**
Introduced by: **Ajinomoto; Mochida**
Trade Name: **Ajicef; Renilan**



Cefpimizole sodium is a third-generation cephalosporin with a spectrum of activity somewhat narrower than cefotaxime and cefoperazone.

Cefteram Pivoxil (antibiotic) (46, 47)

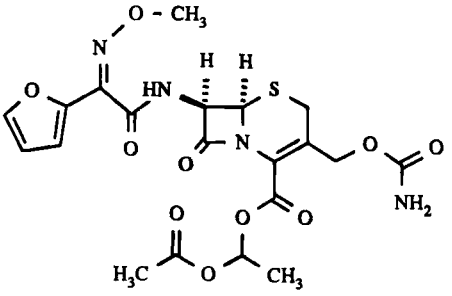
Country of Origin: **Japan**
Originator: **Toyama**
First Introduction: **Japan**
Introduced by: **Toyama**
Trade Name: **Tomiron**



Cefteram pivoxil is an orally-active cephalosporin antibiotic with a broad spectrum of activity. It is reportedly effective against *Citrobacter*, indole-positive *Proteus* and *Enterobacter* species resistant to cefaclor, cephalexin and amoxicillin. Cefteram pivoxil is indicated primarily for urinary tract infections.

Cefuroxime Axetil (antibiotic) (48-50)

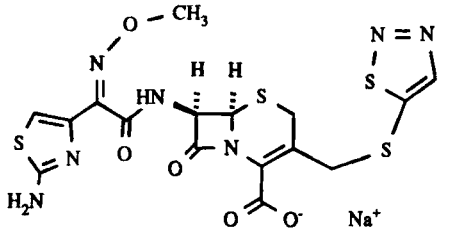
Country of Origin: **United Kingdom**
Originator: **Glaxo**
First Introduction: **United Kingdom**
Introduced by: **Glaxo**
Trade Name: **Zinnat**



Cefuroxime axetil is the acetoxyethyl ester and oral prodrug of cefuroxime, a second-generation cephalosporin. It has a broad spectrum of action and is resistant to most β-lactamases. Cefuroxime axetil is indicated for serious bacterial infections, especially where no identification of organism(s) has been made.

Cefuzonam Sodium (antibiotic) (51, 52)

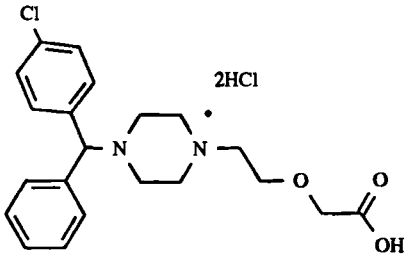
Country of Origin: **USA**
Originator: **Lederle**
First Introduction: **Japan**
Introduced by: **Lederle/Takeda**
Trade Name: **Cosmosin**



Cefuzonam is an injectable cephalosporin reported to be active against a large percentage of Gram-negative bacteria and some Gram-positive bacteria, including those resistant to other third-generation cephalosporins.

Cetirizine Hydrochloride (antihistamine) (53, 54)

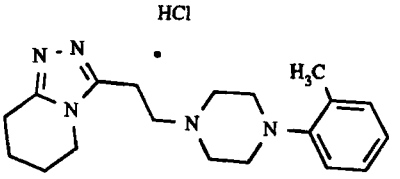
Country of Origin: **Belgium**
Originator: **UCB**
First Introduction: **Belgium**
Introduced by: **UCB**
Trade Name: **Zyrtec**



Cetirizine hydrochloride is a once-daily, non-sedating antihistamine useful in the treatment of allergic rhinitis, urticaria and conjunctivitis. It is reported to be more effective than most other agents in this category such as terfenadine and astemizole (55).

Dapiprazole Hydrochloride (antiglaucoma) (56, 57)

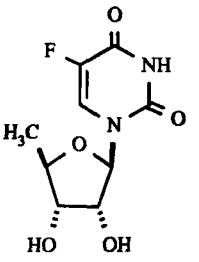
Country of Origin: **Italy**
Originator: **Angelini**
First Introduction: **Italy**
Introduced by: **Angelini**
Trade Name: **Glamidolo**



Dapiprazole is a new anti-glaucoma agent especially useful in the treatment of narrow-angle glaucoma. It is also reported to have antipsychotic activity potentially devoid of extrapyramidal symptoms.

Doxifluridine (antineoplastic) (58-60)

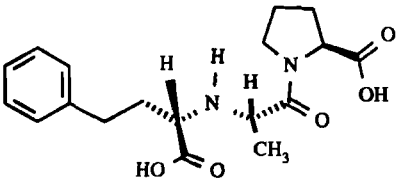
Country of Origin: **USA**
Originator: **Hoffmann-LaRoche**
First Introduction: **Japan**
Introduced by: **Hoffmann-LaRoche**
Trade Name: **Furtulon**



Doxifluridine is a prodrug derivative of 5-fluorouracil (5-FU). As an antineoplastic it is more potent than 5-FU in the treatment of breast, stomach, colon and rectal cancers.

Enalaprilat (antihypertensive) (61-63)

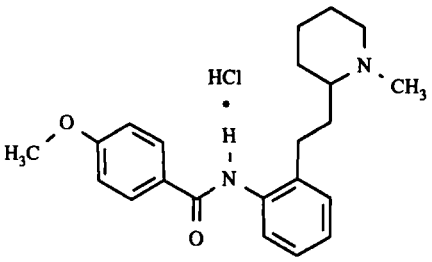
Country of Origin: **USA**
Originator: **Merck**
First Introduction: **Sweden**
Introduced by: **Merck**
Trade Name: **Renitec**



Enalaprilat is the diacid active metabolite of enalapril. As an injectable ACE inhibitor, it is reportedly useful in the treatment of uncomplicated accelerated hypertension and hypertensive emergencies. Enalaprilat is more effective in reducing blood pressure in the elderly than in the young, despite the fact that renin activity is lower in old age.

Encainide Hydrochloride (antiarrhythmic) (64-66)

Country of Origin: **USA**
Originator: **Bristol-Myers**
First Introduction: **USA**
Introduced by: **Bristol-Myers**
Trade Name: **Enkaid**



Encainide is an oral and injectable antiarrhythmic structurally related to flecainide. It is reportedly useful in the treatment of symptomatic and life-threatening ventricular arrhythmias. While it appears to have higher efficacy than quinine, the morbidity and mortality rates are comparable.

Enoxaparin (antithrombotic) (67, 68)

Country of Origin: **France**
Originator: **Pharmuka**
First Introduction: **France**
Introduced by: **Pharmuka**
Trade Name: **Lovenox**

Enoxaparin is a low molecular weight heparin fraction obtained by controlled depolymerization of pig heparin. As an anticoagulant it is reportedly useful in the treatment of venous thromboembolism, and the prevention of coagulation in the extracorporeal circulation during hemodialysis.

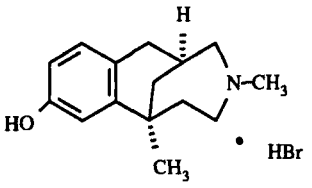
Epidermal Growth Factor, Recombinant (wound healing agent) (69)

Country of Origin: **Italy**
Originator: **Inpharzam**
First Introduction: **Switzerland**
Introduced by: **Inpharzam**
Trade Name: **Gentel**

An ophthalmic formulation of epidermal growth factor (urogastrone) has been launched for stimulation of healing of the corneal epithelium following various corneal diseases.

Eptazocine Hydrobromide (analgesic) (70-72)

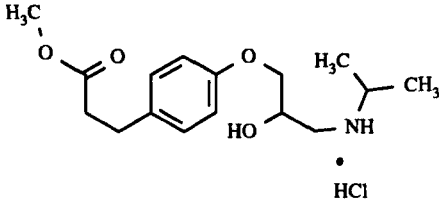
Country of Origin: **Japan**
Originator: **Nichiiko**
First Introduction: **Japan**
Introduced by: **Nichiiko; Kaken**
Trade Name: **Sedapain**



Eptazocine hydrobromide is a centrally-acting analgesic useful in the management of post-operative and cancer pain. In animal studies eptazocine exhibits a mixed agonist/antagonist profile; its analgesic activity is reported to be mediated via opiate receptors and catecholaminergic neurons.

Esmolol Hydrochloride (antiarrhythmic) (73, 74)

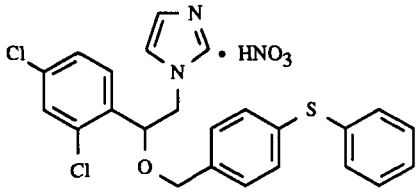
Country of Origin: **USA**
Originator: **DuPont Critical Care**
First Introduction: **USA**
Introduced by: **DuPont Critical Care**
Trade Name: **Brevibloc**



Esmolol is an ultra-short acting, cardioselective β-blocker with no intrinsic sympathomimetic activity. The capability of rapid reversal makes esmolol uniquely suitable for the acute management of supraventricular tachycardia and the control of blood pressure and heart rate during surgery or other cardiac emergencies.

Fenticonazole Nitrate (antifungal) (75, 76)

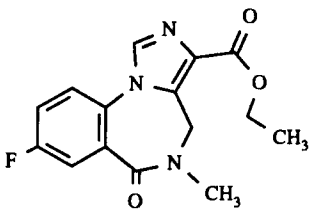
Country of Origin: **Italy**
Originator: **Recordati**
First Introduction: **Italy**
Introduced by: **Recordati**
Trade Name: **Lomexin**



Fenticonazole nitrate is an antifungal imidazole derivative reported to be more effective than other imidazole antifungals such as miconazole, clotrimazole and econazole. In addition to its potent, broad-spectrum fungicidal activity, fenticonazole is also active against some Gram-positive bacteria.

Flumazenil (benzodiazepine antagonist) (77-79)

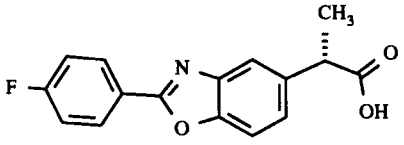
Country of Origin:	**Switzerland**
Originator:	**Hoffmann-La Roche**
First Introduction:	**Switzerland**
Introduced by:	**Hoffmann-La Roche**
Trade Name:	**Anexate**



Flumazenil is a benzodiazepine antagonist useful as a fast-acting antidote in the treatment of benzodiazepine intoxication, and in reversing the central sedative effects of benzodiazepines during anesthesia.

Flunoxaprofen (antiinflammatory) (80, 81)

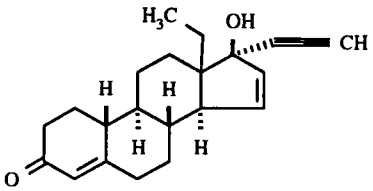
Country of Origin:	**Italy**
Originator:	**Ravizza**
First Introduction:	**Italy**
Introduced by:	**Ravizza**
Trade Name:	**Priaxim**



Flunoxaprofen is an antiinflammatory related to now withdrawn benoxaprofen, reportedly useful in the treatment of rheumatoid arthritis, myositis, synovitis and osteoarthritis. Its major mechanism of action is reported to be lipoxygenase inhibition, thus enabling the control of inflammation with less severe gastric disturbances.

Gestodene (progestogen) (82, 83)

Country of Origin:	**W. Germany**
Originator:	**Schering AG**
First Introduction:	**United Kingdom**
Introduced by:	**Schering AG**
Trade Name:	**Femodene**



Gestodene is a progestogen, recently launched in combination with ethinyl estradiol as an oral contraceptive.

Goserelin (hormone) (84-86)

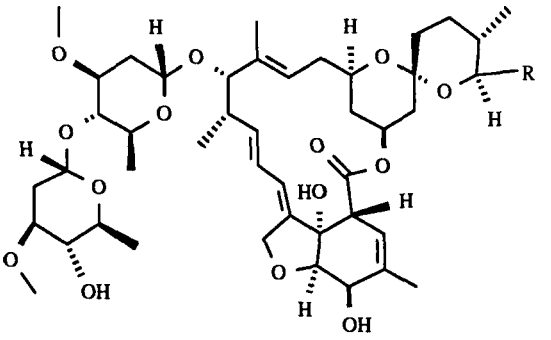
Glu-His-Trp-Ser-Tyr-D-Ser (Bu^t)-Leu-Arg-Pro-$NHNHCONH_2$

Country of Origin:	**United Kingdom**	Introduced by:	**ICI**
Originator:	**ICI**	Trade Name:	**Zoladex**
First Introduction:	**United Kingdom**		

Goserelin is an analog of luteinizing hormone-releasing hormone (LHRH) useful in the treatment of malignancies responsive to hormonal manipulation. Administered parenterally in a biodegradable depot, goserelin is reportedly as effective as orchidectomy and oophorectomy in patients with advanced prostate carcinoma and pre-menopausal breast cancer.

Ivermectin (antiparasitic) (87)

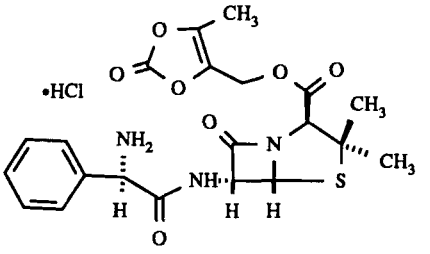
Country of Origin:	**USA**
Originator:	**Merck**
First Introduction:	**France**
Introduced by:	**Merck**
Trade Name:	**Mectizan**



Ivermectin is an antiparasitic agent effective in the treatment of onchocerciasis, or "river blindness". Since ivermectin acts to prevent the adult worm from producing microfilariae, it needs to be administered only once or twice a year.

Lenampicillin Hydrochloride (antibiotic) (88, 89)

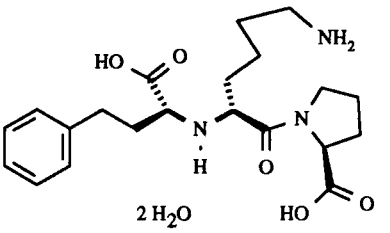
Country of Origin:	**Japan**
Originator:	**Kanebo**
First Introduction:	**Japan**
Introduced by:	**Kanebo; MECT**
Trade Name:	**Varacillin; Takacillin**



Lenampicillin hydrochloride is an orally-effective, prodrug ester of ampicillin with improved bioavailability.

Lisinopril (antihypertensive) (90, 91)

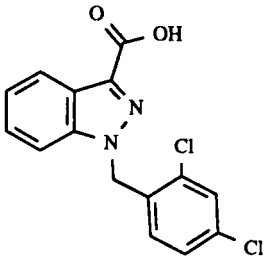
Country of Origin: **USA**
Originator: **Merck**
First Introduction: **New Zealand**
Introduced by: **Merck; ICI**
Trade Name: **Prinivil**



Lisinopril is an angiotensin converting enzyme inhibitor structurally related to enalaprilat. It is reportedly useful in the management of hypertension and congestive heart failure.

Lonidamine (antineoplastic) (92, 93)

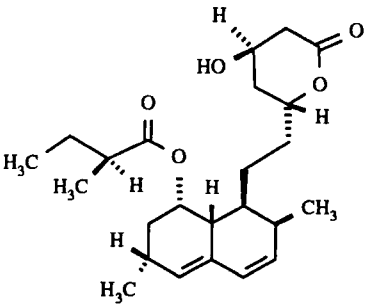
Country of Origin: **Italy**
Originator: **Angelini**
First Introduction: **Italy**
Introduced by: **Angelini**
Trade Name: **Doridamina**



Lonidamine is an antineoplastic agent reportedly effective for the treatment of various cancers, including lung, breast, prostate and brain tumors. Its clinical effect appears to be associated with changes in cellular energy metabolism.

Lovastatin (hypocholesterolemic) (94-98)

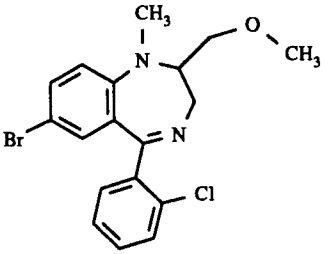
Country of Origin: **USA**
Originator: **Merck**
First Introduction: **USA**
Introduced by: **Merck**
Trade Name: **Mevacor**



Lovastatin is an orally-active hypocholesterolemic useful in the treatment of familial hypercholesterolemia. The drug acts by inhibiting the HMG-CoA reductase-catalyzed rate limiting step of cholesterol biosynthesis. While treatment with lovastatin leads to significant reductions in total and LDL cholesterol, its effect on atherosclerosis or coronary heart disease has not been established.

Metaclazepam (anxiolytic) (99)

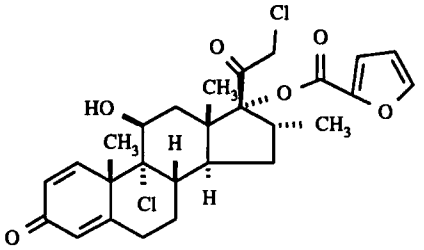
Country of Origin:	**W. Germany**
Originator:	**Kali-Chemie**
First Introduction:	**Switzerland**
Introduced by:	**Kali-Chemie**
Trade Name:	**Talis**



Metaclazepam is a benzodiazepine anxiolytic with an antidepressant component. In man, it is reported to produce only minimal sedative and muscle relaxant side-effects.

Mometasone Furoate (topical antiinflammatory) (100, 101)

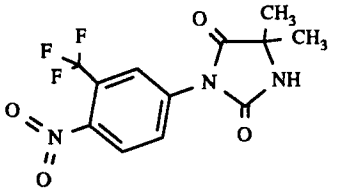
Country of Origin:	**USA**
Originator:	**Schering-Plough**
First Introduction:	**USA**
Introduced by:	**Schering-Plough**
Trade Name:	**Elocon**



Mometasone furoate is a topical steroidal antiinflammatory useful in the treatment of corticosteroid-responsive dermatoses. As a steroidal antiinflammatory of grade II potency, mometasone furoate is characterized by a once-daily dose regimen and a relatively wide safety margin.

Nilutamide (antiandrogen) (102-104)

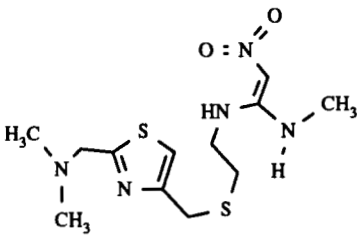
Country of Origin:	**France**
Originator:	**Roussel**
First Introduction:	**France**
Introduced by:	**Roussel (Cassenne)**
Trade Name:	**Anandron**



Nilutamide is a non-steroidal anti-androgen similar in potency to the acyclic urea flutamide (55) and useful in the management of prostatic carcinoma.

Nizatidine (antiulcer) (105, 106)

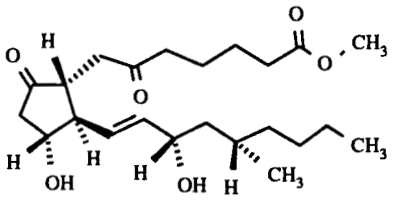
Country of Origin:	**USA**
Originator:	**Lilly**
First Introduction:	**United Kingdom**
Introduced by:	**Lilly**
Trade Name:	**Axid**



Nizatidine is the fifth H_2-antagonist introduced to the world market as an antiulcer agent. It is reported to be effective in the treatment of both duodenal and gastric ulcers or the prevention of their recurrences. Given once-daily, nizatidine's bioavailability is not diminished by the concurrent administration of an antacid.

Ornoprostil (antiulcer) (107, 108)

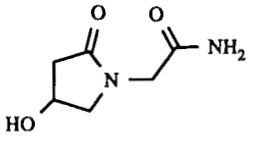
Country of Origin:	**Japan**
Originator:	**Ono**
First Introduction:	**Japan**
Introduced by:	**Upjohn; Ono**
Trade Name:	**Alloca; Ronok**



Ornoprostil is a prostaglandin based antiulcer agent structurally related to alprostadil, enprostil and misoprostol (3). It is reportedly useful in the treatment of gastric ulcer.

Oxiracetam (nootropic) (109-112)

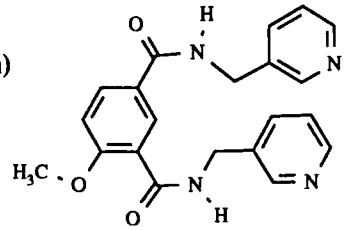
Country of Origin:	**Italy**
Originator:	**ISF**
First Introduction:	**Italy**
Introduced by:	**ISF**
Trade Name:	**Neuromet**



Oxiracetam is a 4-hydroxy analog of piracetam, reportedly useful in relieving the symptoms of cerebral insufficiency in the elderly.

Picotamide (antithrombotic) (113-115)

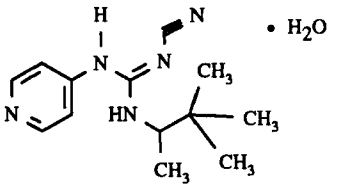
Country of Origin: **Italy**
Originator: **Manetti-Roberts (Beecham)**
First Introduction: **Italy**
Introduced by: **Samil**
Trade Name: **Plactidil**



Picotamide is an unique antithrombotic agent which acts by selectively inhibiting thromboxane synthesis and blocking platelet thromboxane receptors without affecting prostaglandin synthesis. It is reported to be useful in the treatment of myocardial infarction, cerebrovascular disorders, pulmonary embolism and atherosclerosis.

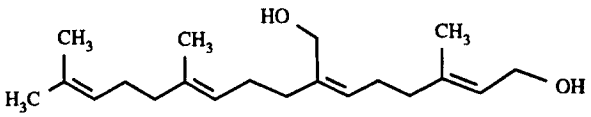
Pinacidil (antihypertensive) (116, 117)

Country of Origin: **Denmark**
Originator: **Leo**
First Introduction: **Denmark**
Introduced by: **Leo**
Trade Name: **Pindac**



Pinacidil is a peripheral vasodilator useful in the management of arterial hypertension of all degrees of severity, usually in combination with a diuretic or beta-blocker. Preclinical evidence suggests that its hypotensive effect may be attributed to potassium channel activation.

Plaunotol (antiulcer) (118, 119)

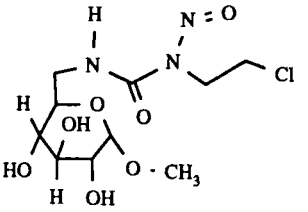


Country of Origin: **Japan**
Originator: **Sankyo**
First Introduction: **Japan**
Introduced by: **Sankyo**
Trade Name: **Kelnac**

Plaunotol is a diterpene alcohol extracted from the Thai plant, plau-noi. Related to teprenone (2), it is reportedly useful as a cytoprotective anti-ulcer agent.

Ranimustine (antineoplastic) (120, 121)

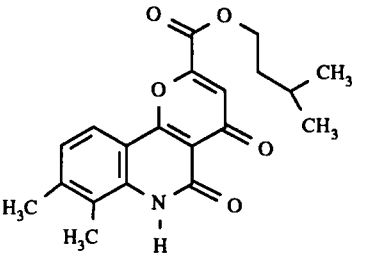
Country of Origin: **Japan**
Originator: **Tokyo Tanabe**
First Introduction: **Japan**
Introduced by: **Tokyo Tanabe**
Trade Name: **Cymerine**



Ranimustine, a new nitrosourea derivative, is a water soluble antitumor agent with low hematological toxicity and diabetogenic effect. It is reportedly effective in the treatment of hematologic malignancies, especially the myeloproliferative disorders.

Repirinast (antiallergic) (122, 123)

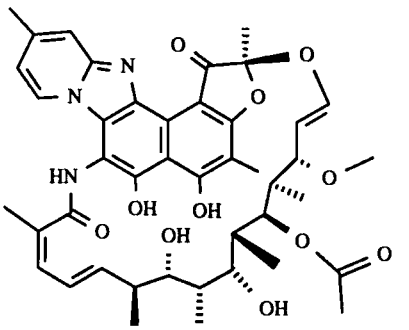
Country of Origin: **Japan**
Originator: **Mitsubishi**
First Introduction: **Japan**
Introduced by: **Nikken; Tokyo Tanabe**
Trade Name: **Romet**



Repirinast is an orally-active antiallergic agent useful in the treatment of allergic asthma. Its activity is reportedly attributed to a metabolite, the corresponding free acid (MY-1250), which is 17 times more potent than disodium cromoglycate in inhibiting histamine release due to antigen-antibody reactions.

Rifaximin (antibiotic) (124, 125)

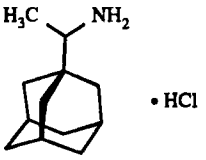
Country of Origin: **Italy**
Originator: **Alfa**
First Introduction: **Italy**
Introduced by: **Alfa**
Trade Name: **Normix**



Rifaximin is an antibiotic structurally related to rifamycin. It is reported to be efficacious in the treatment of gastrointestinal infections and hepatic encephalopathy, being highly active against Gram-positive and -negative aerobic and anaerobic bacteria. Due to poor systemic absorption, rifaximin is effective in presurgical sterilization of the GI tract.

Rimantadine Hydrochloride (antiviral) (126, 127)

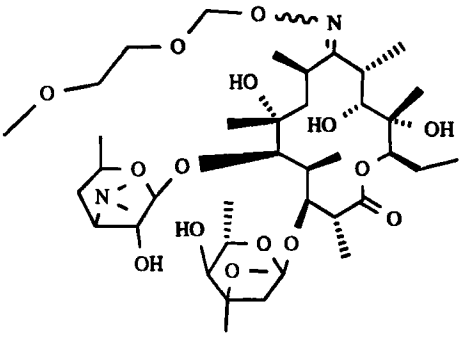
Country of Origin: **USA**
Originator: **DuPont**
First Introduction: **France**
Introduced by: **Hoffmann-La Roche**
Trade Name: **Roflual**



Rimantadine hydrochloride is an antiviral analogue of amantadine, reportedly useful in the prophylaxis and treatment of influenza A. Compared with amantadine, rimantadine appears to have a lower side-effect profile.

Roxithromycin (antibiotic) (128, 129)

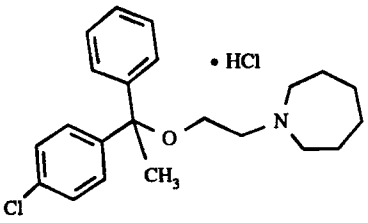
Country of Origin: **France**
Originator: **Roussel**
First Introduction: **France**
Introduced by: **Roussel**
Trade Name: **Rulid**



Roxithromycin, an oxime ether derivative of erythromycin, is a new macrolide antibiotic of high potency and long duration. Its *in vitro* activity spectrum is similar to that of erythromycin, but is superior to the latter against *Legionella, Mycoplasma* and *Chlamydia.*

Setastine Hydrochloride (antihistamine) (130)

Country of Origin: **Hungary**
Originator: **EGIS**
First Introduction: **Hungary**
Introduced by: **EGIS**
Trade Name: **Loderix**



Setastine is an H_1-antagonist antihistamine with negligible sedative side-effects. It is reported to be effective in the treatment of allergic skin and upper respiratory diseases, with an efficacy range comparable to that of clemastine.

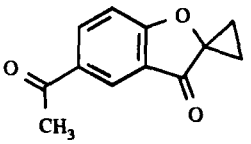
Somatropin, Recombinant (hormone) (131)

Country of Origin:	**USA**	Introduced by:	**Lilly**
Originator:	**Lilly**	Trade Name:	**Humatrope**
First Introduction:	**USA**		

Somatropin is a growth hormone useful in the treatment of hypopituitary dwarfism and other disorders resulting from growth hormone deficiencies. Lilly has used recombinant technology to produce human somatropin identical to the natural hormone. Somatrem, the N-methionyl derivative of somatropin, was launched in 1985 by Genentech for the same indications.

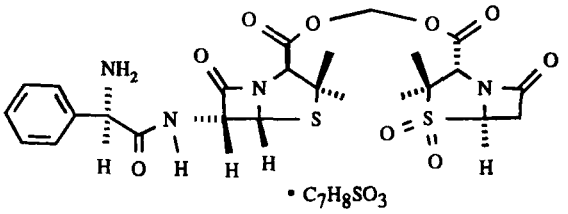
Spizofurone (antiulcer) (132, 133)

Country of Origin:	**Japan**
Originator:	**Takeda**
First Introduction:	**Japan**
Introduced by:	**Takeda**
Trade Name:	**Maon**



Spizofurone is an antiulcer agent that acts primarily by increasing mucus secretion and microcirculation in gastric mucosa and also by its inhibitory effect on acid secretion.

Sultamycillin Tosylate (antibiotic) (134-136)



Country of Origin:	**Denmark**	Introduced by:	**Pfizer**
Originator:	**Leo / Pfizer**	Trade Name:	**Unasyn**
First Introduction:	**Philippines**		

Sultamycillin tosylate is an orally-effective, mutual prodrug of β-lactamase inhibitor sulbactam with ampicillin. It is effective in the treatment of soft tissue, respiratory and urinary tract infections, even against many ampicillin-resistant strains.

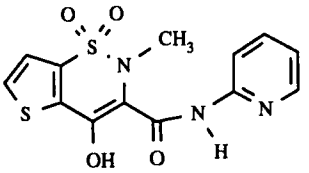
Surfactant TA (respiratory surfactant) (137, 138)

Country of Origin:	**Japan**	Introduced by:	**Tokyo Tanabe**
Originator:	**Tokyo Tanabe**	Trade Name:	**Surfacten**
First Introduction:	**Japan**		

Surfactant TA is a reconstituted surfactant containing bovine lung extracts supplemented with dipalmitoylphosphatidylcholine and other lipids. It is reported to effect significant reduction in pulmonary morbidity and mortality in premature infants with severe hyaline membrane disease through improvement in oxygenation.

Tenoxicam (antiinflammatory) (139, 140)

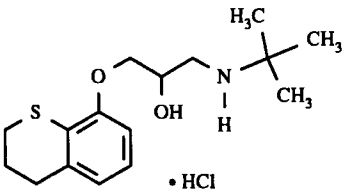
Country of Origin:	**Switzerland**
Originator:	**Hoffmann-La Roche**
First Introduction:	**Switzerland**
Introduced by:	**Hoffmann-La Roche**
Trade Name:	**Tilcotil**



Tenoxicam is a non-steroidal antiinflammatory agent with a profile similar to related piroxicam and now withdrawn isoxicam (55). It is useful in the treatment of rheumatoid arthritis, osteoarthritis and related disorders.

Tertatolol Hydrochloride (antihypertensive) (141, 142)

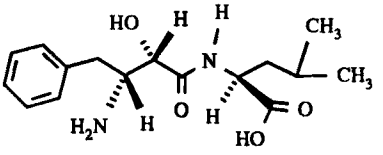
Country of Origin:	**France**
Originator:	**Servier**
First Introduction:	**France**
Introduced by:	**Servier**
Trade Name:	**Artex**



Tertatolol hydrochloride is a new beta-blocker reportedly of high potency and long duration of action. It appears relatively free of detrimental effects on renal hemodynamics and is indicated for all types of hypertension.

Ubenimex (immunostimulant) (143, 144)

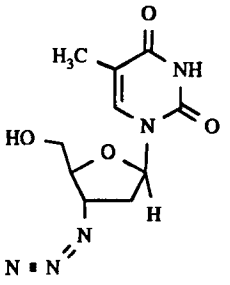
Country of Origin:	**Japan**
Originator:	**Inst. of Microbial Chem.**
First Introduction:	**Japan**
Introduced by:	**Nippon Kayaku**
Trade Name:	**Bestatin**



Ubenimex is an immunostimulant useful in the treatment of non-lymphocytic leukemia. In other types of cancers ubenimex added to radiotherapy or chemotherapy following surgery significantly increases survival time through immunopotentiation.

Zidovudine (antiviral) (145-148)

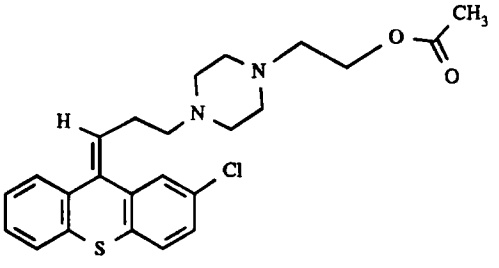
Country of Origin:	**USA**
Originator:	**Detroit Inst. Cancer Res.**
First Introduction:	**United Kingdom**
Introduced by:	**Burroughs Wellcome**
Trade Name:	**Retrovir**



Zidovudine, also known as azidothymidine (AZT), is an antiviral agent acting via reverse transcriptase inhibition. It was first launched in the U.K. and subsequently introduced in over a dozen countries for the management of severe manifestations of HIV infection. In patients with AIDS and ARC, zidovudine reduces the risk of opportunistic infections and prolongs survival time. In symptom-free patients it shows promise in halting further immunological deterioration.

Zuclopenthixol Acetate (antipsychotic) (149)

Country of Origin:	**Denmark**
Originator:	**Lundbeck**
First Introduction:	**Switzerland**
Introduced by:	**Lundbeck**
Trade Name:	**Clopixol Acutard**



Zuclopenthixol acetate is an intermediate-acting derivative of the thioxanthene neuroleptic zuclopenthixol. Clinical effects of zuclopenthixol acetate are reported to last for 3 days following a single injection with a half life of 36 hours.

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leuprolide acetate	hormone	1984	20, 319
levacecarnine HCl	nootropic	1986	22, 322
levobunolol HCl	antiglaucoma	1985	21, 328
lidamidine HCl	antiperistaltic	1984	20, 320
lisinopril	antihypertensive	1987	23, 337
lobenzarit sodium	antiinflammatory	1986	22, 322
lonidamine	antineoplastic	1987	23, 337
loprazolam mesylate	hypnotic	1983	19, 321
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
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

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nimesulide	antiinflammatory	1985	21, 330
nimodipine	cerebral vasodilator	1985	21, 330
nitrefazole *	alcohol deterrent	1983	19, 322
nitrendipine	antihypertensive	1985	21, 331
nizatidine	antiulcer	1987	23, 339
nomegestrol acetate	progestogen	1986	22, 324
norfloxacin	antibacterial	1983	19, 322
norgestimate	progestogen	1986	22, 324
ofloxacin	antibacterial	1985	21, 331
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
pefloxacin mesylate	antibacterial	1985	21, 331
picotamide	antithrombotic	1987	23, 340
piketoprofen	topical antiinflammatory	1984	20, 322
pimaprofen	topical antiinflammatory	1984	20, 322
pinacidil	antihypertensive	1987	23, 340
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
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
rifaximin	antibiotic	1985	21, 332
rifaximin	antibiotic	1987	23, 341
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
sodium cellulose PO4	hypocalciuric	1983	19, 323
sofalcone	antiulcer	1984	20, 323
somatropin	hormone	1987	23, 343
spizofurone	antiulcer	1987	23, 343
sufentanil	analgesic	1983	19, 323
sulbactam sodium	B-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344
temocillin disodium	antibiotic	1984	20, 323
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terconazole	antifungal	1983	19, 324
tertatolol HCl	antihypertensive	1987	23, 344
thymopentin	immunomodulator	1985	21, 333

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timiperone	neuroleptic	1984	20, 323
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
zidovudine	antiviral	1987	23, 345
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

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quinfamide	AMEBICIDE	1984	20, 322
mepixanox	ANALEPTIC	1984	20, 320
alfentanil HCl	ANALGESIC	1983	19, 314
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emorfazone		1984	20, 317
eptazocine HBr		1987	23, 334
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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
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ciprofloxacin	ANTIBACTERIAL	1986	22, 318
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norfloxacin		1983	19, 322
ofloxacin		1985	21, 331
pefloxacin mesylate		1985	21, 331
aspoxicillin	ANTIBIOTIC	1987	23, 328
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temocillin disodium		1984	20, 323
mupirocin	ANTIBIOTIC, TOPICAL	1985	21, 330
chenodiol	ANTICHOLELITHOGENIC	1983	19, 317
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fluoxetine HCl	ANTIDEPRESSANT	1986	22, 320
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medifoxamine fumarate		1986	22, 323
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toloxatone		1984	20, 324
fenticonazole nitrate	ANTIFUNGAL	1987	23, 334
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butoconazole nitrate	ANTIFUNGAL, TOPICAL	1986	22, 318
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befunolol HCl	ANTIGLAUCOMA	1983	19, 315
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astemizole	ANTIHISTAMINE	1983	19, 314
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loxoprofen sodium		1986	22, 322
nabumetone		1985	21, 330
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piketoprofen		1984	20, 322
pimaprofen		1984	20, 322
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artemisinin	ANTIMALARIAL	1987	23, 327
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dronabinol	ANTINAUSEANT	1986	22, 319
amsacrine	ANTINEOPLASTIC	1987	23, 327
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adamantanium bromide	ANTISEPTIC	1984	20, 315
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nizatidine		1987	23, 339
ornoprostil		1987	23, 339
plaunotol		1987	23, 340
rosaprostol		1985	21, 332
roxatidine acetate HCl		1986	22, 326
sofalcone		1984	20, 323
spizofurone		1987	23, 343
teprenone		1984	20, 323
troxipide		1986	22, 327
rimantadine HCl	ANTIVIRAL	1987	23, 342
zidovudine		1987	23, 345
buspirone HCl	ANXIOLYTIC	1985	21, 324
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flutazolam		1984	20, 318
flutoprazepam		1986	22, 320
metaclazepam		1987	23, 338
mexazolam		1984	20, 321
flumazenil	BENZODIAZEPINE ANTAG.	1987	23, 335
doxofylline	BRONCHODILATOR	1985	21, 327
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goserelin		1987	23, 336
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acetohydroxamic acid	HYPOAMMONURIC	1983	19, 313
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binifibrate		1986	22, 317
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thymopentin		1985	21, 333
lentinan	IMMUNOSTIMULANT	1986	22, 322
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oxiracetam		1987	23, 339
gestodene	PROGESTOGEN	1987	23, 335
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