25th Anniversary Volume

ANNUAL REPORTS IN MEDICINAL CHEMISTRY

Volume 25

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

Editor-in-Chief: **JAMES A. BRISTOL**

PARKE-DAVIS PHARMACEUTICAL RESEARCH DIVISION
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ANNUAL REPORTS IN MEDICINAL CHEMISTRY

Volume 25

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PREFACE

With this volume, we are proud to present the twenty-five year anniversary issue of *Annual Reports in Medicinal Chemistry*. A continuing objective of this series has been to combine regular updates of significant areas of research in medicinal chemistry with emerging areas of biological science destined to impact the future discovery and development of new medicinal agents.

Volume 25 retains the familiar format of previous volumes, this year with 35 chapters. Sections I–IV are related to specific medicinal agents, with annual updates on antipsychotics, antihypertensives, antiallergy agents, and antibacterials, where the objective is to provide the reader with the most important new results in a particular field. Also included are chapter topics accorded frequent recent updates, including cognition enhancers, serotonin, antiarrhythmics, peptic ulcer disease, and diabetes. Several chapters cover topics of high current interest, including acute myocardial ischemia, acute ischemic CNS disease, vasoactive peptides, antiretroviral therapy for AIDS, and interleukin-1. The antiinfective field is well represented with chapters on macrolides, antifungals, and mechanism-based anticancer agents. The importance of molecular biology as an enabling technology to medicinal chemical research is duly reflected in most of the chapters in these sections.

Sections V and VI emphasize important emerging areas of medicinal chemistry and biology as well as the interface between these disciplines. Traditionally, areas of research reviewed in Topics in Biology (Section V) will appear in a future chapter related to medicinal agents when sufficient time has passed to allow new compounds to be developed from a biological strategy. Chapters in Section V on CRF, neurotrophic factors, and P-glycoprotein are complementary with chapters on specific agents in Sections I and III. Also covered in Section V are ion channels and cell adhesion molecules, two topics of broad current interest.

Chapters in Section VI reflect the current focus on mechanism-directed drug discovery and newer technologies including PET, catalytic antibodies, and the interface between molecular biology and medicinal chemistry. Also included are chapters on irreversible ligands and characterization of peptide receptors.

Trends and Perspectives (Section VII) is expanded somewhat from previous volumes with chapters on NCE introductions, stereochemistry and drug development, and concluding with a special chapter which provides a perspective on how twenty-five volumes of *Annual Reports in Medicinal Chemistry* have been reflective of the evolution of the field of medicinal chemistry over that same time period.

It has been a distinct pleasure for me to complete my first year as Editor-in-Chief of *Annual Reports in Medicinal Chemistry*. In the course of this work, I have been fortunate to work with six highly professional section editors and 68 authors, whose excellent and incisive contributions constitute Volume 25. Special recognition goes to

Marc Agnew for assistance with the indexing program and to Penny Bliss, Lisa Gregory and Jeanette Johnson for their assistance in assembling the volume. Last, I wish to acknowledge Denis Bailey and Richard Allen, two former editors-in-chief with whom I had the privilege to work as a section editor in previous years and who helped to establish and maintain the high standards of *Annual Reports in Medicinal Chemistry.*

James A. Bristol
Ann Arbor, Michigan
May, 1990

Section I - CNS Agents

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

Chapter 1. Novel Antipsychotic Agents

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Introduction - Although all currently marketed antipsychotic agents are effective in ameliorating the symptoms of psychosis, they all possess unwanted side effects (1). These side effects range from akathisia, acute dystonia, Parkinsonism, anticholinergic effects, hypotension, increased prolactin levels and, eventually to tardive dyskinesia (1,2). Because of the limitations of currently available antipsychotic drugs, the search continues for compounds with fewer deleterious side effects and better efficacy. Over the past three decades, several classes of antipsychotic compounds have been developed and in spite of their considerable structural diversity, all these agents appear to share an ability to directly or indirectly modulate dopaminergic neurotransmission.

DOPAMINERGIC AGENTS

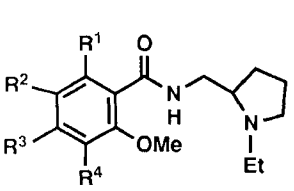
Dopaminergic compounds are currently classified into three categories based on their mechanism of action: dopamine (DA) receptor (D_1 and/or D_2) antagonists, dopamine partial agonists and selective DA autoreceptor agonists.

D_2 Receptor Antagonists - In recent years, efforts have been devoted to the development of atypical antipsychotic agents. These drugs demonstrate either D_2 receptor antagonist selectivity (e.g. benzamides, such as remoxipride) or regional specificity by acting preferentially on mesolimbic and mesocortical DA pathways (e.g. clozapine).

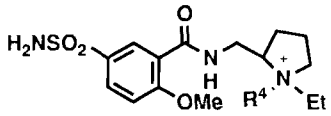
Raclopride (**1**) and remoxipride (**2**) are benzamide derivatives with highly selective D_2 antagonist properties. These compounds are currently under clinical evaluation as atypical antipsychotic agents. Raclopride was well tolerated and proved effective in 7/10 schizophrenic patients and was accompanied by a prolactin increase of only short duration (3). The D_2 receptor occupancy measured using Positron Emission Tomography (PET) (See Chapter 28), was comparable to that found in patients treated with classical antipsychotics (4). This may account for the high level of extrapyramidal symptoms (EPS) observed with raclopride. Remoxipride, currently in development, was orally well tolerated up to 20 mg without noticeable adverse effects in all subjects (5). Like raclopride, a transient increase in plasma prolactin was noted. However, unlike raclopride, a low incidence of EPS was observed. Several studies have shown that sulpiride (**3**), a substituted benzamide D_2 antagonist, weakly inhibits dopamine mediated behavior; while its coadministration with a small ineffective dose of the D_1 antagonist SCH 23390 enables sulpiride to fully express a wider range of antidopaminergic activity in rats (6). Quarternary analogs **4** and **5** of sulpiride blocked the inhibitory effect of apomorphine on the potassium-evoked release of [^{3}H] acetylcholine from striatal slices at higher concentrations than sulpiride (7). The lower activity of these compounds was attributed to the absence of a hydrogen atom at the charged center (7). Amisulpiride (**6**), also a benzamide derivative, produced stimulatory effects at low doses. At higher doses, this compound was effective against both the positive and negative symptoms of schizophrenia, while producing few EPS effects (8).

The role of dopamine-serotonin interactions has been emphasized among various proposed mechanisms for offsetting antipsychotic-induced EPS. Recent studies with

haloperidol and clozapine in the amfonelic acid (AFA) test, have demonstrated that both the D_2 and 5-HT_2 antagonist activity of clozapine may contribute to its atypical antipsychotic profile (9).



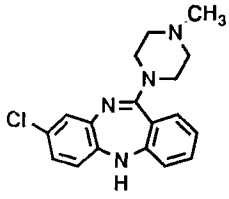
	R^1	R^2	R^3	R^4
1	OH	Cl	H	Cl
2	OMe	H	H	Br
3	H	SO_2NH_2	H	H
6	OMe	H	NH_2	SO_2Et



4, R^4 = Me
5, R^4 = Et

Clozapine (7), a dibenzodiazepine antipsychotic agent developed in Europe in the 1970s, is now being marketed in the United States for the treatment of resistant schizophrenia. It is rapidly absorbed after both single or repeated doses and its two metabolites, clozapine N-oxide and N-desmethylclozapine are less active than the parent drug (10). Its neurochemical profile has been established (11,12) and recently its preclinical and clinical profiles were extensively reviewed (13-36). Unlike most classical antipsychotics, it has a lower affinity for D_2 receptors and equally binds to D_1 receptors (37,38). Clozapine is an atypical neuroleptic with superior antipsychotic efficacy and favorable extrapyramidal side effect profile (39). Although the agranulocytosis associated with clozapine therapy is fully reversible upon discontinuation of the drug, patient white cell counts must be frequently monitored because of the gravity of this side effect (40).

Risperidone (8) and gevotroline (9) are other antipsychotics under development which also demonstrate some serotonergic activity. Compound 8 has potent D_2 and also serotonin-2 (5-HT_2) receptor antagonist properties (41,42). Initial results of clinical studies with 17 patients suggested that treatment with risperidone, in doses up to 25 mg/day, results in significant improvement of the negative and positive symptoms of schizophrenia without cardiovascular or hematological side effects with the exception of a slight increase of aspartate aminotransferase and alanine aminotransferase that was noted in one patient (43). Gevotroline (Wy-47,384) (9), a new atypical antipsychotic agent with D_2 antagonist properties, was well tolerated and has demonstrated some clinical efficacy in early Phase II studies (44). Gevotroline demonstrated modest, but equal affinity for D_2 and 5-HT_2 receptors and potent activity at sigma receptors, a neurochemical profile predictive of antipsychotic activity and low potential for EPS liability (45).



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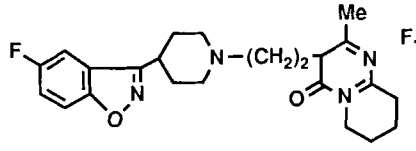

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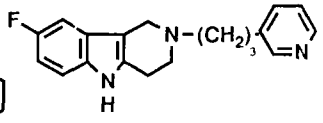


(9)

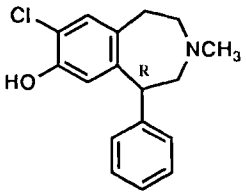
D1 Receptor Antagonists -The benzazepines are the only class of compounds providing both D_1 agonists and antagonists. SKF 38393 and SKF 77434 were among the first D_1 agonists discovered (46-51) with SKF 77434 being the most potent and selective central D_1 agonist known to date (52). Extensive SAR studies have led to the discovery of several D_1 antagonists (53, 54) such as SKF 8366 and SCH 23390 (10). SCH 23390 is currently the prototype D_1 receptor antagonist and its pharmacological profile has been fully characterized (55-59).

Injection of 10 into the nucleus accumbens inhibited methamphetamine-induced hyperlocomotion markedly (60), while its injection into the dorsal striatum was less effective. Administration of 10 into the dorsal striatum did not alter the gnawing induced by either methamphetamine or apomorphine. However, its injection into the nucleus accumbens significantly reduced methamphetamine-induced gnawing without altering the effects of apomorphine. Compound 10 decreased striatal neuronal activity by more than 50% and

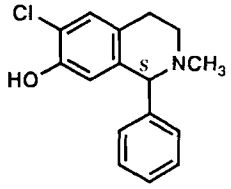
effectively blocked the effect of dextroamphetamine. These studies suggest that **10** preferentially affects D_1 as opposed to D_2 mediated behaviors (61).

Compound **10** also blocked conditioned avoidance responding (CAR) in rats at low doses. This blockade was similar to that produced by D_2 antagonists which might suggest the possible existence of an interaction between D_1 and D_2 receptors in CAR (62). Further studies have indicated that CAR blockade by either **10** or haloperidol can also be differentiated by the anticholinergics atropine and scopolamine. Low doses of atropine or scopolamine potentiated CAR blockade produced by **10** and blocked CAR blockade produced by haloperidol. These results suggest a possible involvement of reciprocal cholinergic/dopaminergic mechanisms in modifying CAR. Like other atypical antipsychotic agents, subchronic administration of **10** produced a selective depolarization blockade of A10 neurons (40). Whether this blockade is indicative of desirable antipsychotic action, or is associated with D_1 or 5-HT_2 antagonism, is not known.

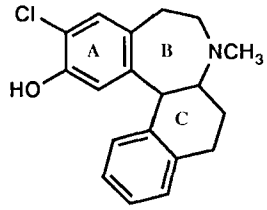
SAR studies with conformationally ring-contracted 1-phenyl, 4-phenyl and 1-benzyl derivatives of SCH 23390 (**11**-**13**) showed that, unlike the active enantiomer of **10** which possesses the (R) configuration, the most active enantiomer of **11** is the (S) enantiomer (64). In vitro binding studies of the conformationally restricted analogs of **10** (65,66) having the B/C-trans (**14**) and B/C-Cis (**15**) ring junctions showed that compound (-)**14** has significantly greater D_1 affinity and selectivity than **15** and was very potent in blocking CAR (MED of 0.1 mg/kg) (65,66).



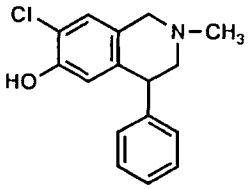
(**10**)



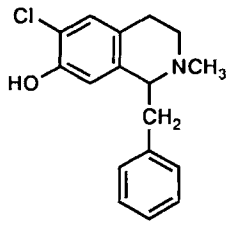
(**11**)



(**14**), B/C trans
(**15**), B/C cis



(**12**)



(**13**)

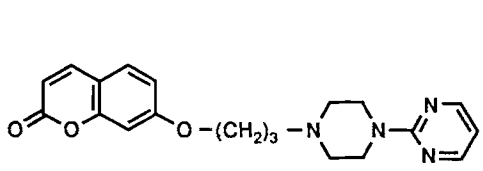
SCH-39166 (**14**), displayed high selectivity being 270 and 75 times more potent at D_1 receptors than at D_2 and 5-HT_2 receptors, respectively (44). This compound blocked CAR with a long duration of action following p.o. administration in both rats and squirrel monkeys (65). The relative lack of ability of SCH 39166 to modify acetylcholine release in the striatum (in comparison with D_2 antagonists) could be interpreted as an indication of a lower potential for EPS (65). Furthermore, it was reported that SCH 39166 did not produce catalepsy. Because of its favorable profile, this compound is currently under development as a potential antipsychotic agent (67).

DA Autoreceptor Agonists - The development of drugs with greater autoreceptor specificity, which will preferentially stimulate presynaptic autoreceptors and reduce dopaminergic neurotransmission, has become a major challenge in recent years. PD 118,717 (**16**), a pyrimidinylpiperazine, demonstrates a good separation in pre- versus postsynaptic dopaminergic effects. Most notably, it did not antagonize apomorphine-induced climbing nor produce significant apomorphine-like hyperactivity with large i.p. doses up to 300 mg/kg. Compound **16** had greater affinity for 5-HT_{1A} than D_2 receptors, increased locomotion in rats with supersensitive

DA receptors (6-OHDA lesions) and was more potent than (-)3PPP in reducing dopaminergic neuronal firing in the substantia nigra (68-70).

U-66444B (17), a dihydrophenelene, has been identified as a DA autoreceptor agonist based on neuropharmacological data. SAR studies in this series have shown that the (+) enantiomer (U-68553B) is by far the most potent enantiomer. (+)17 was 10 times more potent than (-)17 in inhibiting the release of dopamine and inhibiting amphetamine stimulated locomotor activity. The differences in potency of enantiomers was not related to pharmacokinetics as both produced comparable brain levels of 17 (71,72). CGS-15873A (18) is a benzopyranopiperidine with DA autoreceptor activity. It reversed γ-butyrolactone (GBL)-induced DOPA accumulation (ED_{50} = 0.52 mg/kg) and increased motor activity in reserpinized rats (73).

PD 125530 (19), a benzopyranoxazine derivative, closely related in structure to CGS 15855A (20), has demonstrated DA autoreceptor activity (50), both racemic 19 and (+)19 (PD 128907; R,R) have modest affinity for D_2 receptors. (+)19 potently inhibited DOPA accumulation and an oral dose of 5 mg/kg produced 100% reversal of GBL-induced DOPA accumulation in rats. This compound was potent in inhibiting spontaneous locomotor activity in naive rats, but also produced stereotyped behavior at low doses (74).



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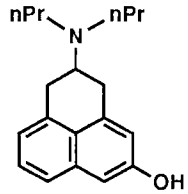

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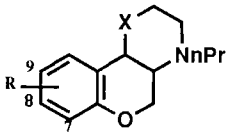
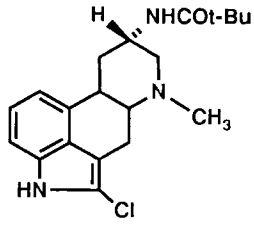


18, X = CH_2, R = 7-OH
19, X = O, R = 9-OH
20, X = CH_2, R = 9-OH



(21)

SDZ 208-912 (21), an ergoline derivative with partial D_2 receptor agonist activity, is also currently under development as an antipsychotic agent (75).

PD 118440 (22) and PD 120697 (23), are tetrahydropyridinyl derivatives of 3-PPP with an aminothiazolyl moiety as a bioisosteric replacement for the m-hydroxyphenyl. Both 22 and 23 demonstrated DA agonist properties following oral administration. These compounds exhibited selectivity for the DA autoreceptor at low doses; and at higher doses, they stimulated postsynaptic DA receptors (76).

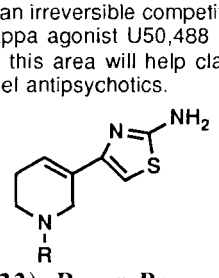
Many compounds with DA autoreceptor agonist activity also influence other neurotransmitter systems. They may interact with the noradrenergic system and produce alpha-1 antagonist effects. The behavioral consequences of this mixed activity were determined by examining the effect of the alpha-1 antagonist, prazosin on the stereotyped behavior of rats induced by either the selective D_2 agonist, quinpirole, or the combination of quinpirole and the selective D_1 agonist SKF 38393. Prazosin potentiated the locomotor suppression produced by quinpirole in rats. However, sniffing and licking, which are produced by D_1 (quinpirole) and D_2 (SKF 38393) agonist effects were attenuated by prazosin without any effects on locomotor activity. These data suggest that D_1 and D_2 agonist effects at postsynaptic receptors could be masked by alpha-1 antagonist activity (77).

NEW APPROACHES TO ANTIPSYCHOTIC DRUG DEVELOPMENT

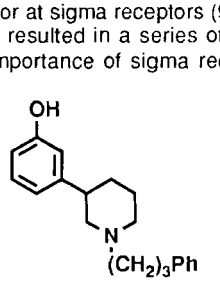
Sigma Receptor - The sigma binding site remains an active area of preclinical investigation in identifying novel antipsychotics. Both typical and atypical antipsychotics bind to the sigma receptor, including some agents that also bind to the D_2 receptor (78). Potential antispychotic agents identified as having sigma activity are rimcazole, remoxipride, BMY-14802, tiosperone, cinuperone and gevotroline (79). The rationale for this approach has been recently reviewed (78). The sigma binding site is complex; however, there has been recent progress in the characterization, localization and identification of ligands for this site (80-85). In addition, experiments have been conducted to address the agonist/antagonist functionality of the sigma receptor site (86, 87).

In rat spinal cord, receptor binding and autoradiographic techniques have shown that phencyclidine (PCP) and sigma sites are two distinct populations which are differentially localized (80). PCP and sigma sites have also been characterized in a cloned cell line (81). In irradiation studies (82), a multiple-site model for the sigma receptor has been proposed. However, the potential for multiple sigma receptor types remains a possibility. Additional autoradiographic studies have localized haloperidol-displaceable ^{3}H-DTG binding sites in the substantia nigra pars compacta and this specificity was associated with an increase in D_2, but not D_1, related ligand binding (83). This suggests an interaction between dopaminergic and sigma systems. This interaction is supported by recent experiments which demonstrated that the sigma site is linked to a guanine nucleotide regulatory (G) protein and that subchronic treatment with rimcazole produced an up-regulation and decrease in affinity for the sigma site accompanied by an increase in dopamine turnover (84). Chronic administration of haloperidol also reduced sigma receptors and this was accompanied by an up-regulation of D_2 receptors (85). Neurophysiological studies have also demonstrated a relationship between sigma receptors and the dopaminergic neuronal system. These studies may serve to distinguish between sigma agonist and antagonist activity (86), and suggest that sigma receptors may be localized on dopamine neurons in the substantia nigra (87). These studies suggest the potential for modulation of dopaminergic systems by sigma ligands. However, not all of these interactions may relate to antipsychotic activity as recent behavioral experiments illustrate the possibility that sigma receptors may be associated with EPS liability (88). Drug discrimination procedures have been used to further characterize the sigma receptor and provide a behavioral correlate to sigma binding (89).

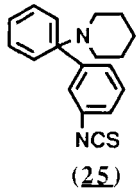
The identification of more specific and selective sigma ligands will aid in the characterization of the sigma receptor and its possible role in antipsychotic drug action. Progress has recently been described in this area. Dextropentazocine has been identified as a potent and selective ligand for sigma receptors (90) and 3-PPPP(**24**), the N-phenylpropyl derivative of norpropyl-3-PPP, shows potent sigma affinity in vitro and in vivo (91). It is interesting that sertraline, a 5-HT uptake inhibitor antidepressant, displaces 3-PPP binding at sigma sites (92). Metaphit (**25**), an isothiocyanate derivative of PCP, was found to be an irreversible, non-competitive inhibitor at PCP receptors and an irreversible competitive inhibitor at sigma receptors (93). Lastly, synthetic variations of the kappa agonist U50,488 (**26**) have resulted in a series of sigma ligands (94). Further research in this area will help clarify the importance of sigma receptor activity in the development of novel antipsychotics.



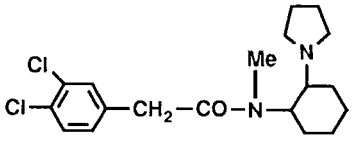
(**22**), R = n-Pr
(**23**), R = allyl



(**24**)



(**25**)



(**26**)

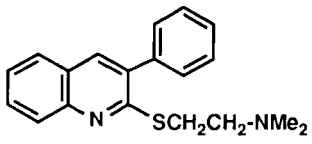
Serotonergic Agents - There is a growing interest in novel antipsychotics which have non-dopaminergic antipsychotic activity or agents which indirectly modulate dopaminergic neuronal systems. Experimental efforts have recently focused on serotonergic agents (see Chapter 5) in this regard. In addition to clozapine (**7**) and risperidone (**8**) discussed above, several other compounds with effects on serotonergic neuronal systems are being investigated in clinical studies as potential antipsychotic agents. These include amperozide and ICI 169,369 (**27**) which are compounds with postulated 5-HT_2 antagonist activity (95-97). These compounds may have other pharmacological effects and their psychotropic action may extend beyond antipsychotic activity. However, recent experimental results with (**27**), in neurophysiological tests following acute and repeated administration, predict antipsychotic efficacy with a low EPS liability for this compound (98). Serotonin-3 antagonists have also been proposed as novel antipsychotic agents. The rationale for this approach has been recently reviewed (99, 100) and ondansetron and zacopride remain in clinical trials for this indication. These compounds are very potent in reversing the behavioral effects resulting from excess mesolimbic dopamine (101). As such, they should produce antipsychotic effects and have a low EPS liability (99, 100). However, 5-HT_3 antagonists are not generally active in classical antipsychotic models and definitive efficacy in clinical trials is needed to demonstrate their antipsychotic potential. 5-HT_3 antagonists are also characterized as having a wide spectum of psychotropic activity (99, 100).

Preclinical experiments have examined the potential interaction between serotonergic and dopaminergic neuronal systems. Atypical antipsychotics such as clozapine, melperone, and setoperone blocked the hyperthermic response to the 5-HT agonist MK 212, but not the hypothermic response to 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)tetraline (8-OH-DPAT) suggesting a relationship between 5-HT_2 activity and atypical antipsychotic effects (102). The 5-HT_2 antagonist ritanserin increased the activity of midbrain dopamine neurons showing that 5-HT exerts an inhibitory control of midbrain dopaminergic cell activity through a 5-HT_2 mechanism (103).

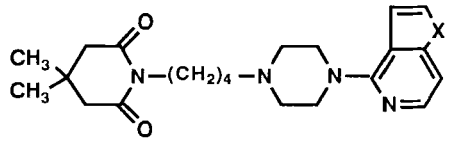
Interactions between 5-HT_{1A} and dopaminergic function have also been reported. The 5-HT_{1A} agonist 8-OH-DPAT selectively inhibits limbic forebrain dopamine synthesis and this effect may be mediated through direct stimulation of dopamine autoreceptors (104). 8-OH-DPAT also selectively decreases limbic versus striatal dopamine synthesis and suppresses conditioned avoidance responding and rearing in the rat. This suggests the potential for antipsychotic activity (105). In addition, phenylpiperazines, such as MCPP (m-chlorophenyl-piperazine), and the antidepressants trazodone and etoperidone, have been reported to reduce conditioned avoidance responding, while not blocking amphetamine-induced stereotypy or producing catalepsy (106). The conditioned avoidance activity of these agents was blocked by metergoline which suggests a serotonergic mechanism for novel antipsychotic activity with a low potential for EPS side effects.

In spite of these positive results, others (107) have failed to demonstrate a consistent effect of 8-OH-DPAT on reduction of conditioned avoidance responding. In addition, in a sensitive primate model of antipsychotic-induced extrapyramidal symptoms, 5-HT_2 antagonists and 5-HT_{1A} partial agonists induced, rather than reduced, dyskinesia (108). These later studies emphasize the complexities of cooperative or reciprocal interactions between serotonergic and dopaminergic systems. Further research is needed to characterize the relationship of serotonergic systems to both antipsychotic effect and EPS symptomatology.

Recent synthetic efforts in this area have concentrated on the identification of novel serotonergic agents with weak or no dopaminergic activity. A series of phenylpiperazines have been described which are effective in attenuating conditioned avoidance responding, yet have low affinity for both D_1 and D_2 sites (107). In addition, two arylpiperazine derivatives, thieno- (**28**) and furano[3,2-c]pyridine (**29**) have demonstrated preclinical antipsychotic potential by blocking apomorphine-induced stereotyped and climbing behavior as well as reducing Sidman and conditioned avoidance behavior (109). These derivatives have potent affinity for 5-HT_{1A} and 5-HT_2 receptors with only weak affinity for D_2 sites. A series of N-(4-phenyl-and 4-pyridyl-1-piperazinylethyl) and N-(4-phenyl-1-piperidinylethyl)-phthalimides were found to suppress spontaneous and pergolide-induced locomotor activity and to antagonize apomorphine-induced climbing behavior, while lacking cataleptogenic activity (110). The interesting pharmacological profile of these compounds indicates that it may be possible to develop novel antipsychotic agents which lack potent dopaminergic activity. Such compounds would likely lack or have a low potential for EPS side effects.

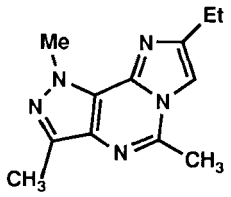


(27)



(28), X = S
(29), X = O

Other agents - Several novel compounds which cannot be easily categorized are in various stages of development as novel antipsychotic agents. The most advanced of these compounds is CI-943 (**30**), a novel antipsychotic agent whose preclinical pharmacology has recently been described (111-113]. Compound **30** did not demonstrate affinity for dopamine receptors either



(30)

in vitro or in vivo, yet it accelerated dopamine turnover. Following repeated treatment, **30** did not alter the affinity or number of striatal D_2 receptors. A series of neurophysiological studies supported a lack of dopamine antagonist activity. In behavioral tests, **30** reduced spontaneous locomotor activity and antagonized apomorphine-induced climbing behavior. This compound also reduced avoidance responding in rats and primates. This pharmacological profile predicts antipsychotic activity with a low EPS liability. The results of on-going clinical trials may shed light on the mechanism of action of this compound.

Peptide modulation of dopaminergic activity remains an area of research in the search for atypical antipsychotic agents. Cholecystokinin (CCK) modulates mesolimbic dopamine activity by attenuating the postsynaptic effects of dopamine in the nucleus accumbens (114), while it also inhibits dopamine release in the striatum (115). Neurotensin has been shown to potentiate the stimulation-induced release of dopamine (116) and a series of hexapeptide neurotensin analogs have been synthesized (117) which may have utility as antipsychotics agents.

This brief review points out the multiple biochemical substrates that may play a role in antipsychotic drug action. The future holds promise for molecular biology to aid in the discovery of unique, selective and effective antipsychotic compounds. The recent identification of a second molecular form of the D_2 receptor (118-121) should pave the way for exciting developments in this area.

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Chapter 2. Analgesics

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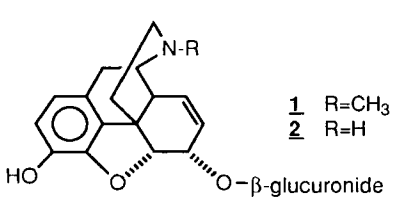
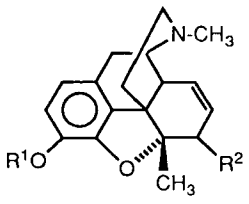
Introduction - In the interim since the last review, two new volumes covering the opiates, differing somewhat in perspective, have appeared (1,2). A narrative of the discovery of the opiate receptor and the enkephalins has been published (3), as well as a NIDA monograph on the problems of drug dependence (4). Reviews have described the anatomy of CNS opioid receptors (5), methods used in their study (6), and opioids and sexual behavior (7). Among the most interesting findings is the ability of buprenorphine to control cocaine addiction in monkeys (8), suggesting its use in treating combined heroin-cocaine addicts.

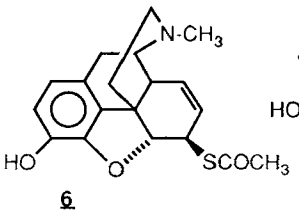
Opiates - A major metabolite of morphine, its 6-glucuronide (**1**), is a μ-selective ligand and is twenty fold more potent than morphine after i.c.v. administration (9). A recent clinical study has suggested that **1** provides most of the analgesic effect of morphine in cancer patients (10). The nor-compound (**2**) is also a more potent analgesic than its parent (11). The 5-methyl derivatives of codeine (**3**), morphine (**4**) (12), and morphinone (**5**) (13) are less potent analgesics than morphine, but this substitution usually confers an improved pharmacological profile. The 6ß-acetylthiomorphine (**6**), epimeric at morphine's 6-hydroxyl group, is μ-selective and surprisingly more potent than morphine (14,15). A series of 6,14-dideoxynaltrexones containing different electrophiles at position 6 has been prepared. These irreversible opiate antagonists possess profiles similar to the 14ß-hydroxy compounds (16). The monofunctional derivative (**7**) of ß-chlornaltrexamine has a qualitatively similar antagonist profile (17). Replacement of the chloroethyl side chain in **7** with aspartyl (**8**) provides a peripherally active full agonist equivalent to morphine and postulated to act at κ-receptors (18). A high level of interest continues in the hydrazones and azines of various opiates. The 6-phenylhydrazone derivatives of oxymorphone bind in a non-equilibrium fashion to opiate receptors and have analgesic potencies equivalent to their parent (19). The benzoylhydrazone derivative of naloxone has been described as possessing reversible κ and a slowly reversible μ-receptor affinity (20). The pharmacology of naloxonazine has been described (21). A series of 14ß-cinnamoylaminomorphinones (e.g., **9**) show potent, long acting, opiate antagonist activity after parenteral administration and may prove useful for treatment of narcotic dependence (22). The crystal structure of the clinical candidate, pentamorphone (RX 77989), has been described (23). Two studies on the conformational structure activity relationships for opiate agonists and antagonists have appeared (24,25).



1 R=CH_3
2 R=H



3 R^1=CH_3, R^2=α-OH
4 R^1=H,R^2=α-OH
5 R^1=H,R^2= =O



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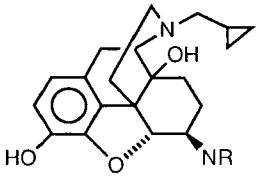
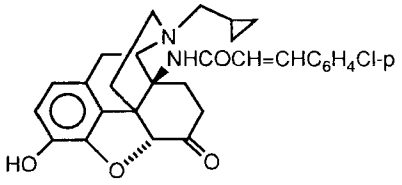
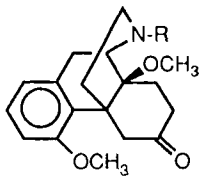


7 R=$(CH_3)CH_2CH_2Cl$
8 R=(H)Asp

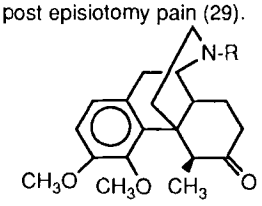


9

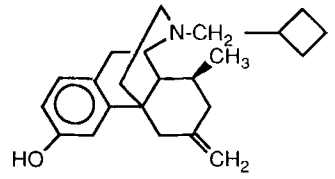
An earlier series of 14-methoxy-N-alkylmorphinan-6-ones with combinations of hydrogen, hydroxy, and methoxy substituents at C3 and C4 (e.g., **10**) had previously shown increased antinociceptive potency over morphine and increased opiate receptor affinity (26). Replacement of methyl by cyclopropylmethyl results in cyprodime (**11**), which lacks agonist potency, but has selective μ-opioid antagonistic activity in vitro and weak in vivo activity (27). N-allyl- and N-cyclopropylmethyl-5-methylmorphinan-6-ones (**12**) and (**13**) lack any antagonistic activity and are potent antinociceptive agents in the acetic acid writhing and guinea pig ileum assays, with compound **13** showing a preference for κ over μ-opioid receptors (28). Xorphanol (**14**) is undergoing clinical trials for post episiotomy pain (29).



10 R = CH_3
11 R = CH_2-c-C_3H_5

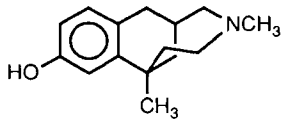


12 R = $CH_2CH=CH_2$
13 R = CH_2-c-C_3H_5



14

Eptazocine (**15**), a homobenzomorphan, has been launched in Japan for the treatment of postoperative pain. Chronic administration shows no cross-tolerance with pentazocine or morphine (30). Tonazocine (**16**), a μ-receptor agonist/antagonist and δ-agonist, is in phase II evaluation for the relief of postoperative pain (31). MR2033 (**17**) has been classified as a mixed μ/κ-agonist on the basis of dependence and cross-tolerance studies (32).



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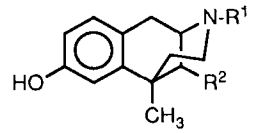
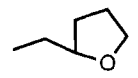
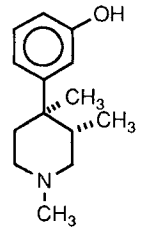


16 $R^1 = CH_3$, $R_2 = (CH_2)_2CO(CH_2)_4CH_3$

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

Research interests in piperidine analgesics have focused on both experimental and theoretical studies in the various alkyl, aryl and anilido structural classes. Energy conformational calculations and X-ray crystallography on a series of flexible 4-alkyl-4-(3-hydroxyphenyl)piperidines have been used to describe a "universal phenyl axial pharmacophore" which gives high affinity μ-receptor binding (33). NMR spectral studies have confirmed that 4-alkyl-4-(3-hydroxy- or 3-methoxyphenyl)piperidines preferring the axial 4-aryl chair conformation are opiate agonists, whereas an antagonist was found to favor an equatorial 4-aryl chair (34), also preferred in C4-hydrogen and C4-reversed ester analogs (33). Picenadol (**18**) a mixed opiate agonist/antagonist without psychotomimetic effects, is being studied clinically for postoperative pain (35). Among constrained 4-phenylpiperidines, the benzofuropyridine (**19**) has potent antinociceptive activity in vivo with a 2000-fold μ/κ-selectivity (36) and 3-hydroxyphenyl substituted diazabicyclanes (eg., **20**) are significantly more potent than morphine as analgesics in mice (37). Novel N-butyrophenone prodine analogs have displayed both opiate analgesic and neuroleptic activity (38).



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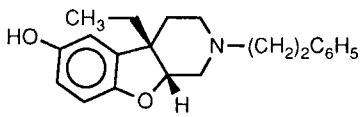

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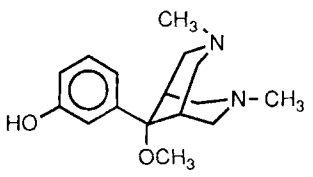
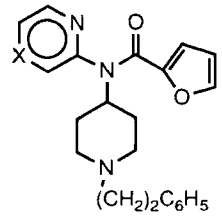


20

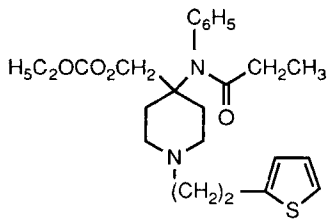
In 3-(3-hydroxyphenyl)-piperidines, theoretical studies on 2-methyl analogs, which show diminished μ-receptor affinity and analgesic activity, have demonstrated stabilization of the equatorial phenyl conformer (39). Several constrained 3-alkyl-3-(3-hydroxyphenyl)piperidines have proved to be pharmacologically interesting: a racemic N-phenethyl trans-4a-aryldecahydroisoquinoline (**21**) has 3-10 times the potency of morphine and high μ-affinity, while the 4aR,8aR-N-cyclopropylmethyl compound (**22**) has a mixed agonist/antagonist profile similar to pentazocine, with both μ and κ-receptor affinity (40). Pyranopyridine analogs of **21** and **22** retain significant analgesic activity and show varied receptor specificities (41,42).

21 R = $(CH_2)_2C_6H_5$
22 R = CH_2-c-C_3H_5



23 X = CH
24 X = CCH_3
25 X = N

^{13}C-NMR studies on substituted 4-anilidopiperidines, which have a common calculated minimum energy conformation (43), have shown solution conformations similar to fentanyl (44). Para-substitution on the anilino group of fentanyl gives derivatives with potencies greater than morphine, although less than fentanyl itself (45). Heterocyclic substitution for the phenyl ring has led to a potent opiate agonist (**23**) and a novel antagonist (**24**), which reverses both morphine-induced analgesia and respiratory depression (46). Notably, one 4-(heteroanilido)piperidine (**25**) possesses a good analgesic profile in several animal models, with little cardiovascular and respiratory depression compared to fentanyl. An anilidopiperidine 4-carbonate derivative (**26**) is potent and long acting (47). Several 4-phenyl- and 4-heteroaryl-4-anilidopiperidines possess high analgesic potency and favorable pharmacological profiles. For example, compound **27** has a short duration of action, with a more rapid recovery and less cardiovascular and respiratory depression than the clinically used fentanyl and alfentanil (48).



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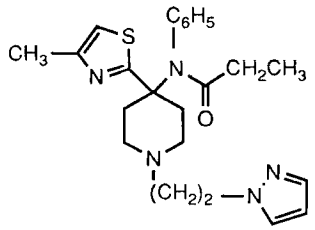
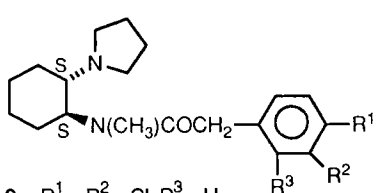
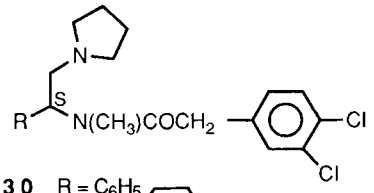


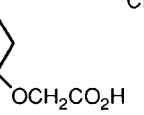
27

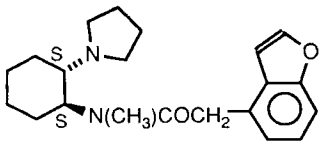
Kappa Agonists - Research on κ-opiate analgesics has been summarized (49). There is serotonergic involvement, both 5-HT_1 and 5-HT_2, in the nociceptive action of the κ-agonist U-50,488 and in the development of tolerance to it (50). The enantiomers of U-50,488 and its cis-diastereomer have been prepared. The highest receptor affinity and antinociceptive activity reside in the (-)-(1S,2S)-U-50,488 enantiomer **28** (51). Further investigation using **28** indicates the presence of multiple types of κ-receptors in brain, a result confirmed using the site specific acylating agent (**29**), which possesses the same absolute configuration as **28** (52). A variety of new κ-ligands primarily related to **28**, have been reported, and an interesting SAR has emerged concerning these analogs and their specificity for the various opiate receptor types. A series of κ-agonists, where the ß-amino-amide of **28** is not constrained by a ring, possess high κ-selectivity, with the most potent, ICI-199441 (**30**), being 275 times more potent as an analgesic than **28** (53). ICI-204448 (**31**) is a κ-agonist with low CNS penetration (54). When the phenyl ring in **28** is replaced by a benzofuran ring, an orally active κ-analgesic (**32**) with very high selectivity (κ/μ=780) is obtained (55,56). However, when the pyrrolidine ring is constrained in a spirocycle, the resulting compounds are potent μ-selective analgesics which have lost most of their κ-affinity (57). In a similar vein, the cis-diastereomer of **28**, **33**, has lost its affinity for the κ-receptor, but is a potent, high affinity σ-ligand (58).



28 $R^1 = R^2 = Cl$, $R^3 = H$
29 $R^1 = R^2 = H$, $R^3 = NCS$



30 $R = C_6H_5$
31 R = 



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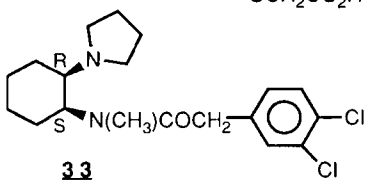
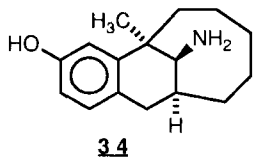


33

Miscellaneous Opiates - Dezocine (**34**), awaiting final approval in the U.S., has been recommended for Schedule V of the Controlled Substances Act as an injectable analgesic. Development of ciramadol (**35**) has been terminated due to side effects (59). A series of 1,2-diphenylethylpiperazine derivatives has displayed potent opiate agonist activity, with compound (**36**) being the most potent (60). The hexahydrodibenzofuran derivative (**37**) represents the most potent compound in its series (61).



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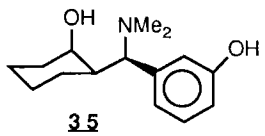

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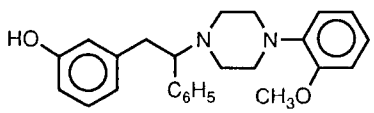

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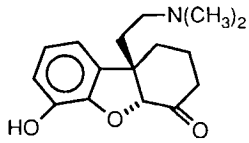
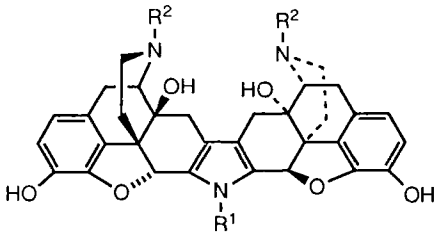
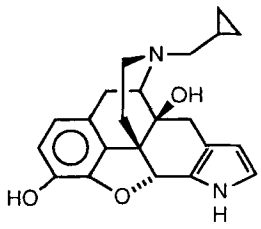


37

Opiate Receptor Probes - The selectivity of receptor ligands commonly used in opiate receptor subtype studies has been summarized (61). The "message-address" concept and its application to the design of subtype-selective ligands has been reviewed (63).

Binaltorphamine (**38**) and norbinaltorphamine (**39**) are κ-selective antagonists (64,65). Studies on analogs suggest that κ-selectivity is dependent on both the N-substituent and the presence of some element(s) of the second pharmacophore, since the N-methyl analog (**40**) and the truncated analog (**41**) are μ-selective agonist and antagonist respectively (66). However, the entire second pharmacophore is not required, as shown by the κ-selective antagonism and enhanced potency shown by the meso analog of **39** (67). This is in contrast to μ-selective ligand (**42**), in which a stereospecific interaction with the second pharmacophore has been demonstrated (68). Naltrindole (**43**) is a potent monovalent opiate antagonist with an appended "address" conferring δ-selectivity, and is the first non-peptide ligand to display such attributes (69,70). A hybrid oxymorphamine/enkephalin conjugate (**44**) has been shown to have enhanced potency relative to either of its constituent substructures (71). ß-Funaltrexamine has been shown to alter the affinity of a subset of δ-receptors, in support of the existence of a μ/δ-receptor complex (72). The (+)-trans isomer of 3-methylfentanyl isothiocyanate (**45**) has been shown to be a potent and selective inactivator of δ-receptors (73).





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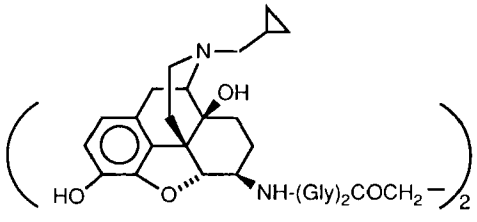
38 $R^1 = CH_3$, $R^2 = CH_2\text{-}c\text{-}C_3H_5$
39 $R^1 = H$, $R^2 = CH_2\text{-}c\text{-}C_3H_5$
40 $R^1 = H$, $R^2 = CH_3$



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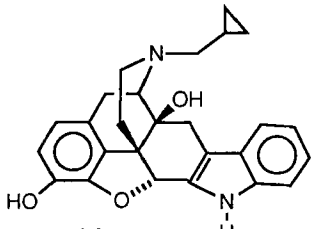

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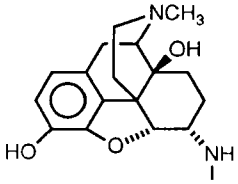
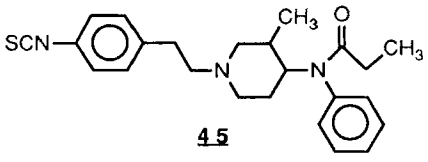


44 H-Tyr-D-Glu-Gly-Phe-Leu-OH



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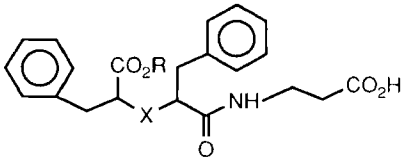
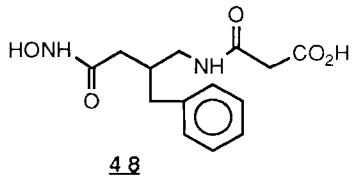
Enkephalinase Inhibitors - Reviews on the inhibition of proteases in general (74) and enkephalinase, in particular (75), have appeared. A study of the effects of selective and complete inhibitors of enkephalin-degrading enzymes on morphine withdrawal has demonstrated reduced severity of the morphine abstinence syndrome (76). SCH-34826 (46), an orally active enkephalinase inhibitor analgesic selected for clinical evaluation, is a prodrug of SCH-32615 (47), the active species (77). It inhibits enkephalinase, but neither aminopeptidase nor diaminopeptidase III, and lacks respiratory or gastrointestinal side effects, and neither tolerance nor dependence develops (78). SAR optimization of kelatorphan has resulted in 48, which inhibits all three enkephalin degrading proteases and is a more potent analgesic than kelatorphan by i.c.v. injection. Without the phenyl ring, analogs of 48 selectively inhibit enkephalinase (79). Newly reported enkephalinase inhibitory analgesics are the 2,4-dibenzylglutaric amide (49), specific for enkephalinase (80), and D-Phe-AHPA (50) (81). An actinomycete, *kitasatosporia setae*, has produced two new, selective enkephalinase inhibitors, propioxatins A (51) and B (52), which specifically inhibit the cleavage of the Gly-Gly bond in Met-enkephalin (74,82).



46 X = NH, R = CH_2
47 X = NH, R = H
49 X = CH_2, R = H



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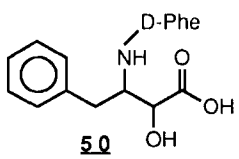

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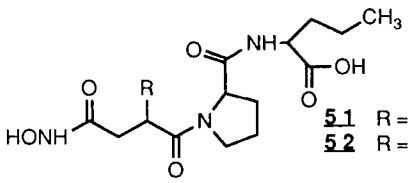


51 R = $(CH_2)_2CH_3$
52 R = $CH_2CH(CH_3)_2$

Opioid Peptides - A recent monograph on opioid peptides (83) includes articles on the role of various neuropeptides in nociception and analgesia, conformational analysis of opioid peptides, and thermoregulatory and immunological effects of opioid peptides. Another review covers the synthesis of neuropeptide precursors, metabolic activation, degradation, and interactions with postsynaptic receptors (84). A review of endogenous opioids and receptors, and their role in nociception has appeared (85). Studies of the uses of opioids in humans have been reviewed (86). An in-depth review of the design of receptor-specific opioid peptides includes extensive tabulations of peptide ligands, their potencies, and their receptor specificities (87).

Various opioid peptides, including novel C-terminal amides, isolated from a number of sources, have served as starting points for the design of novel receptor-specific ligands. A number of D-amino acid-containing opioids isolated from natural sources are interesting in that nature anticipated this stabilizing modification long before medicinal chemists employed it. The deltorphins, isolated from frog skin, having a D-Ala residue at the second position and a C-terminal amide, are the most potent and selective δ-receptor ligands from natural sources to date (88). Structure-activity studies on dermorphin and dermenkephalin (also D-amino acid-containing peptide amides from frog skin) have defined elements responsible for high μ- and δ-selectivity, respectively (89,90).

Analogs of the δ-selective cyclic peptide (D-Pen^2, D-Pen^5) enkephalin (DPDPE) have been prepared, including derivatives containing a cyclic pentamethylene penicillamine analog (91) and a p-chlorophenylalanine analog with enhanced affinity and selectivity for δ-receptors (92). A series of conformationally restricted analogs of DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) and DTLET (Tyr-D-Thr-Gly-Phe-Leu-Thr) bearing bulky hydroxylic substitution on the amino acids at the 2- and/or 6-positions have shown improved receptor selectivity relative to the parent systems (93).

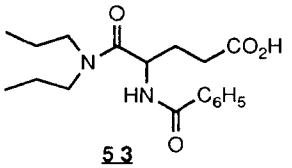
A number of polar enkephalin analogs have shown analgesic activity in vitro and in vivo which appears to be mediated by peripheral opioid pathways, in light of demonstrated antagonism by the peripherally-acting quaternary antagonist N-methylnalorphine (94,95). A series of N,N-dialkylated Leu-enkephalin derivatives have shown activity as δ-receptor antagonists (96); the corresponding affinity ligands with a bis(2-chloroethyl)amino substituent show lower affinity and only weak irreversible effects (97).

Among μ-selective peptides, modified analogs of dermorphin[1-4] have dermorphin-like activity in vitro and produce analgesia upon subcutaneous administration (98). A series of cyclic peptides based on somatostatin has shown very high selectivity for μ-receptors over both δ and somatostatin receptors (99,100).

The pharmacology of dynorphin has been reviewed. Evidence suggests it acts as a modulator of analgesia in the brain, with no intrinsic analgesic effects of its own (101). "DAKLI", a C-terminal extended derivative of dynorphin, has been labeled with various reporter groups and used to label κ-receptors (102).

Other Neuropeptides - A general review of tachykinins has appeared (103). SAR of substance P (SP) agonists and antagonists in a variety of systems has been reviewed (104), as have the various biological approaches used to assess analgesic effects of SP antagonists (105). The rat SP receptor has been cloned, and the deduced amino acid sequence shown to be similar to the bovine substance K receptor (106). Nociceptive and antinociceptive effects of selective agonists for neurokinin receptor subtypes have been characterized (107). The pharmacological effects and potential therapeutic applications of bradykinin (BK) antagonists have been summarized (108). BK antagonists have been shown to block BK-induced pain in vivo, and BK receptors are localized primarily on sensory neurons (109).

Proglumide (**53**), a cholecystokinin (CCK) antagonist, has been reported to potentiate morphine analgesia, but a recent clinical study of **53** found no significant reduction in postoperative patient-controlled morphine consumption (110). Another CCK antagonist, MK-329 (**54**), has been reported to enhance morphine analgesia (111). Intrathecal somatostatin is reported to produce analgesia in cases of both acute and chronic pain (112).



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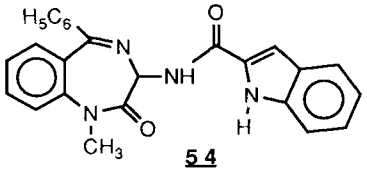

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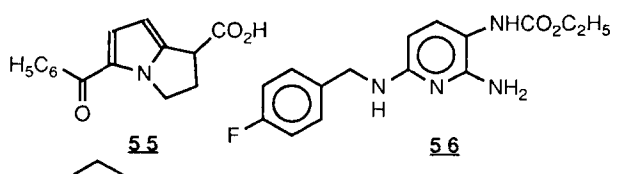
Biogenic amines in analgesia - Naloxone antagonism of the analgesic effects of clonidine suggest a role for opioid receptors in α_2-adrenergic analgesia (113). The ability of subanalgesic doses of intrathecal clonidine to potentiate intrathecal morphine analgesia and the somewhat reduced analgesic potency of intrathecal clonidine in morphine-tolerant mice further support interactions between these systems (114), possibly at the level of common G-protein-mediated signal transduction and activation of the same set of potassium channels (115).

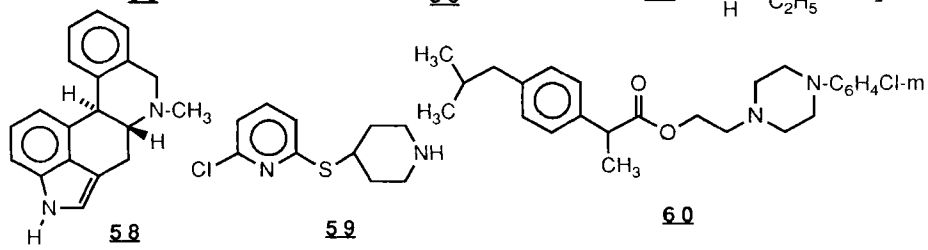
The complicated interactions of inhibitory GABAergic systems with other neurotransmitters and neuromodulators has been reviewed (116), and evidence concerning interactions with opioid systems has been summarized (117). Zopiclone, a benzodiazepine receptor agonist, potentiates morphine analgesia by a spinal mechanism which does not involve brain benzodiazepine receptors (118). Midazolam has been reported to produce hyperalgesia upon systemic administration, but to be moderately analgesic intrathecally (119), suggesting opposing roles for brain and spinal GABA receptors in nociception. Blockade of brain GABA receptors with bicuculline abolishes spinal dorsal horn nociceptive responses, suggesting that brain GABAergic activation results in inhibition of descending antinociceptive pathways (120).

Interest continues in the involvement of serotonergic pathways in opiate and non-opiate analgesia (121,122). In particular, 5-HT_3 receptor antagonists have shown efficacy in animal models of visceral pain (123,124), but have not been evaluated clinically.

Miscellaneous Analgesics - Ketorolac (**55**) has been approved by the FDA for short-term management of pain. Flupirtine (**56**) is under clinical evaluation for numerous types of pain (125). Drug discrimination studies suggest that it acts primarily by a central α_2-adrenergic mechanism (126). Pemedolac (**57**) is a potent analgesic with a profile different from that of opiates and nonsteroidal anti-inflammatories (127,128). SAR of pemedolac analogs for analgesic activity and inhibition of cyclooxygenase has been explored (129). CY 208-243 (**58**) is a novel compound which is thought to act by a combination of D_1-dopaminergic and atypical opioid pathways (130). Anpirtoline (**59**) is a potent non-narcotic analgesic with possible antidepressant effects (131). Lobuprofen (**60**) is an ester derivative of ibuprofen which is reported to produce analgesia by both central and peripheral mechanisms (132).



55 **56** **57**



58 **59** **60**

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Chapter 3. Cognition Enhancers

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Introduction - Cognitive dysfunction is a prominent symptom accompanying aging, stroke, head injury and neurodegenerative diseases including Alzheimer's disease. Medical advances and demographic changes have extended mean life expectancy and led to the aging of society. Because of this, the incidence of cognitive dysfunction in the population is likely to increase. Without adequate therapies for these disorders, society faces an increasingly difficult burden in the care and maintenance of afflicted individuals. The research community has responded to the problem and the development of compounds as potential palliative therapies for cognitive disorders has expanded rapidly (1). More recently, strategies which go beyond pallative therapy to attack the potential causes of these disorders have emerged. This chapter reviews progress in these areas occurring since the last review from this series which appeared in 1988 (2).

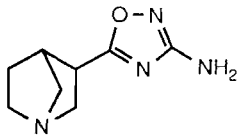
CHOLINERGIC AGENTS

A deficit in central cholinergic function may contribute to the cognitive dysfunction in both aging and Alzheimer's disease (3,4). Cholinergic agonists, acetylcholine releasing agents or acetylcholinesterase inhibitors (AcChEI) may provide symptomatic therapies for cognitive disorders.

Muscarinic Agonists - Development of muscarinic receptor agonists which overcome the limited oral activity, short duration of action, or lack of separation between central and peripheral effects of classical agonists (e.g. arecoline, oxotremorine) is currently in progress and was reviewed (5). Additional reviews on the design and evaluation of muscarinic agonists have appeared (6,7).

Recent advances in molecular biology have led to the discovery and expression, in transfected cells, of five subtypes (m1-m5) of muscarinic receptors (8,9). Based on their receptor localization in cortex and hippocampus, areas intimately involved in cognitive function, m1 and/or m3 subtype selective agonists may be useful for the treatment of cognitive disorders (10).

The structure-activity relationships (SAR) and biological characterization of oxadiazoles, 1 and 2, related to arecoline has been reported (11). These compounds are among the most potent and efficacious muscarinic agonists disclosed to date (K_i = 0.04 nM [^{3}H]-oxotremorine-M binding; 170% of the response to 1 nM carbachol in phosphatidyl inositol turnover). Prodrug forms, 3, of these compounds are claimed (12). Structurally-related thiadiazoles (13) and tetrazoles (14), as well as compounds with the substituent in the 4-position of the piperidine ring (15), have been described.



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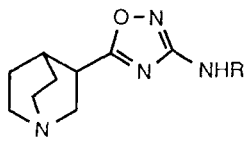
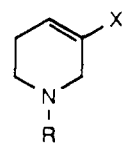


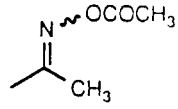
2 R = H
3 R = COC_7H_{15}

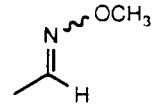
Oxime-containing derivatives of arecoline continue to generate interest. The pharmacological profiles of CI-969 (4) and CI-979 (5) have been described (16,17). Structurally-related compounds, 6 and 7, as well as the corresponding tetrahydropyridine carbamate, 8, have been claimed as muscarinic agonists (18-20).

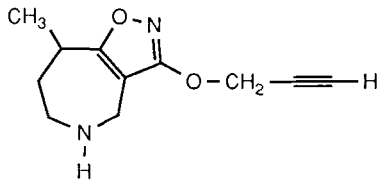
SAR studies have been reported with tetrahydropyridine-3-carboxylic acid propargylic esters, 9, with a range of activity from full agonists to antagonists (21,22). The seven-membered ring

analog, **10**, of the previously-described tetrahydropyridine isoxazole compounds (6) reportedly possesses high affinity for the central M_1-receptor with only limited toxicity (23) (M_1, M_2, etc. refers to the pharmacological characterization of muscarinic receptors, while m_1-m_5 refers to the cloned muscarinic receptor subtypes). The AF-102B analog, **11** (AF-122B), the tetrahydrofuran derivative, **12**, and the RS-86 analog, **13** (YM954) have been described as possessing muscarinic agonist activity (24-26).



4 R = CH_3 X = 

5 R = CH_3 X = 
6 R = H



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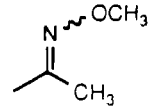
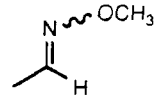
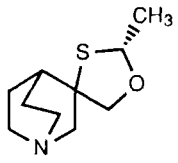
7 R = H X = 

8 R = pClPhCO$_2$ X = 

9 R = CH_3 X = $CO_2CH_2C{\equiv}CR'$



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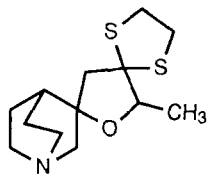

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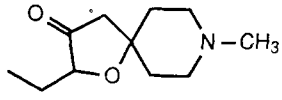
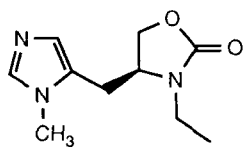


13

Examination of a series of pilocarpine analogs resulted in the identification of **14**, which is equipotent to pilocarpine but more stable to hydrolysis (27). Among compounds related to oxotremorine, an SAR study of McN-A-343 derivatives (**15**, a reported selective M_1 agonist)(1) revealed that the corresponding sulfur-containing analogs, **16**, were partial agonists while the 4-Br analog of McN-A-343, **17**, was three-fold more potent while retaining selectivity (28). Conformationally-restricted analogs of BM-5 with weak partial agonist or antagonist activities have been described (29).



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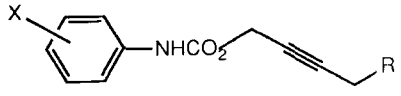
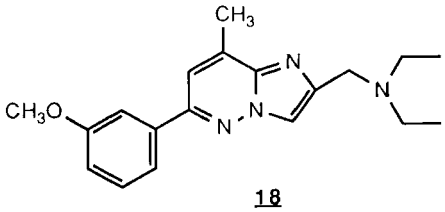


15 X = 3-Cl R = $\overset{+}{N}(CH_3)_3$

16 R = $\overset{+}{S}(CH_3)_2$

17 X = 4-Br R = $\overset{+}{N}(CH_3)_3$

Several structurally-unique agonists have been reported. SR 96095A (**18**) is claimed to be 162-fold selective for the M_1 receptor (30). SR 95639A (**19**) and SR 95777A (**20**), semi-rigid analogs of the antidepressant minaprine, have been described as selective M_1 agonists devoid of classical muscarinic side effects (31,32).



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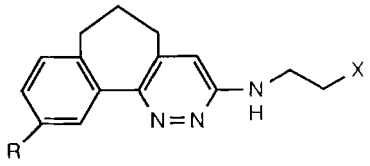
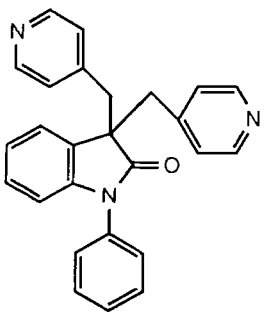


19 R = H X = 

20 R = NO_2 X = 

A pharmacophore which distinguishes high and low affinity state agonist binding has been described. This model led to the suggestion that agonists and antagonists bind at two independent sites on the receptor (11).

Several agents which enhance the release of acetylcholine in the brain have been discussed. DUP-996 (**21**) has undergone Phase 1 clinical trials which demonstrated an increase in the alpha and alpha-adjacent beta activity in the EEG suggesting vigilance-improving properties (33,34). The SAR of a series of ureas such as **22**, and a modified ACTH analog, **23** (HOE 427), which enhance acetylcholine release have been described (35,36).



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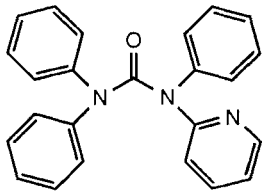
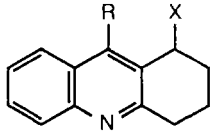


22

Met(O_2)-Glu-His-Phe-D-Lys-PheNH$(CH_2)_8$-NH_2

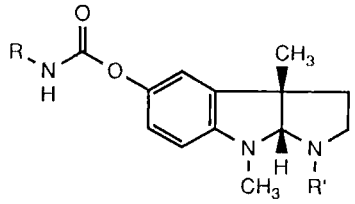
23

Acetylcholinesterase Inhibitors - Focus on the development of novel acetylcholinesterase inhibitors intensified with the report of clinical improvements with tacrine (THA, **24**) in Alzheimer's patients (37). While several clinical reports on tacrine have appeared, a large multicenter trial currently in progress will provide more definitive results on tacrine's efficacy (38,39). Other AcChEIs of the aminoacridine class are also under investigation. These include velnacrine (**25**, HP 029) and suranacrine (**26**, HP 128)(40-42). The SAR leading to these two compounds has been described (42). Additional derivatives of tacrine have been reported (43-47).



24 R = NH_2, X = H
25 R = NH_2, X = OH
26 R = $NHCH_2Ph$, X = OH

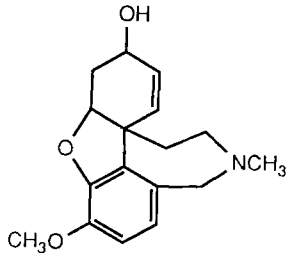
Physostigmine, **27**, and its analogs continue to generate interest. A controlled release form of physostigmine is being developed (48) and SAR studies of the carbamoyl group have resulted in the identification of heptylstigmine, **28**, as a longer acting derivative with a more favorable therapeutic index than physostigmine (49-51). The 3-chlorophenyl carbamate has been reported to be as potent as physostigmine but with improved selectivity for acetylcholinesterase over butyrylcholinesterase (52). SAR studies of substituents at N-1 and the carbamoyl group have revealed that the structure of physostigmine can be altered significantly while retaining AcChEI activity (53).



27 R = R' = CH_3
28 R = n-C_7H_{15}, R' = CH_3

Galanthamine, **29**, has been reported to reverse cognitive deficits induced by ibotenic acid-induced lesions and a limited SAR study of galanthamine has been conducted (54,55).

Several structurally-novel AcChEIs have been reported including N-benzylpiperidines (56-59). The N-benzylpiperidine benzamide **30**, resulting from a QSAR study, possessed an in-vitro IC_{50} of 0.6 nM (59,60). Huperzine A, **31**, improved performance on a discrimination test in aged rats with experimental cognitive impairment and the structure of a novel AcChEI, anatoxin-a(s) (**32**), has been described (61-63). Onchidal, **33**, a naturally-occurring irreversible AcChEI from molluscs has been reported (64). Finally, the antimalarial, amodiaquine, **34**, has been claimed as an AcChEI (65).



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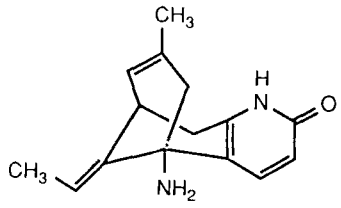

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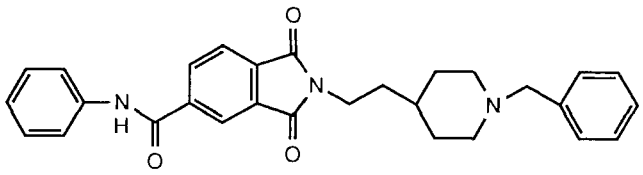

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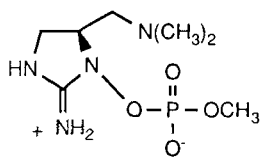

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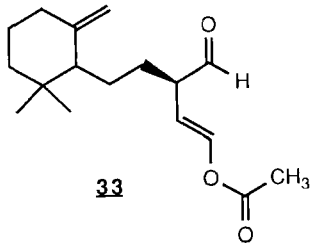

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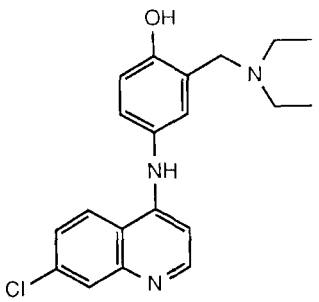

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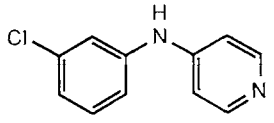
Recently, acetylcholinesterase (AcChE) has been described as a potential zymogen (precursor) for neuropeptide processing enzymes, which might help explain the existence of AcChE in tissues which do not contain cholinergic cell bodies or nerve terminals (66).

NON-CHOLINERGIC AGENTS

Biogenic amines - Modulation of brain catecholamines has been shown to affect cognitive performance. In cognitively intact rodents, monkeys and humans, enhancement of brain catecholamine activity leads to improvement (67,68) while reduction of brain catecholamine activity leads to impairment in cognitive performance (69-71). Somewhat less consistent changes in brain noradrenergic systems have been reported in normal aging and dementia. Decreases in brain norepinephrine content and activity of catecholamine synthetic enzymes are associated with cognitive decline in these conditions (72). In addition, there is a loss of norepinephrine-containing cells in the locus coeruleus with age and in dementia (73).

In humans and animals, the alpha-2 noradrenergic agonist clonidine impairs cognitive performance (70,74,75). However, in conditions where presynaptic elements have degenerated or been destroyed or an existing cholinergic dysfunction exists, clonidine and guanfacine, another alpha-2 antagonist, improved cognitive performance (76-79). In other studies alpha-2 antagonists such as PD 122655 (**35**) or L-654,284 (**36**) improve cognitive performance of mice (80).



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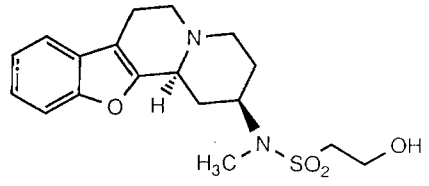

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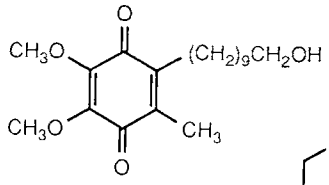
Reductions in serotonin (5-HT) and serotonergic binding sites have been reported in Alzheimer's disease and normal aging. Alaproclate, a serotonin uptake inhibitor, may improve the affect of Alzheimer's patients. Others report little cognitive improvement with this agent in dementia (81,82), however, the 5-HT reuptake blocker, zimeldine, had no effect on memory or reaction time in Alzheimer's disease patients (83). Other agents influencing serotonergic systems have had limited success in improving cognitive function in animals, but have not proved beneficial in treating human cognitive dysfunction (1).

Nootropics - Recent developments in 2-pyrrolidinone-containing nootropics have been reviewed (84). While clinical efficacy has not been proven, a hint of benefit has been seen. Preclinically, many workers continue to demonstrate the protective effects of nootropics against various insults to the central nervous system. The beneficial effects of idebenone (**37**), indeloxzine (**38**) and exifone (**39**) on memory in rodents have been discussed (85-88). An indeloxazine analog, Y-8894 (**40**), has been reported (89). Acetylcarnitine improved quality of life in 20 elderly subjects with no side effects and its effects on aged rats have been described (90,91).



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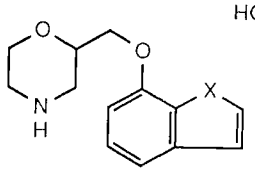
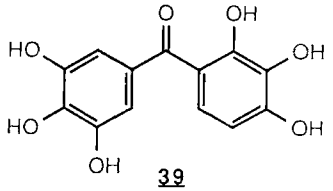


38 X = CH
40 X = S



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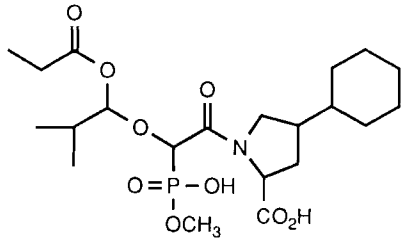
Angiotensin Converting Enzyme (ACE) Inhibitors - A number of reports have appeared suggesting that the angiotensin converting enzyme inhibitors, captopril and enalapril, improve the quality of life of patients undergoing treatment for hypertension and elevate mood in normal and depressed subjects (92-96). These actions may indirectly lead to improved cognitive performance. However, captopril and SQ 29,852 (**41**), another ACE inhibitor, directly improved cognitive performance in young and middle aged rodents and reversed scopolamine-induced cognitive impairments in young animals, properties shared by the ACE inhibitor **42** (Hoe 288)(97-99). Interestingly, **43** (Hoe 065) is structurally related to known ACE inhibitors and can enhance cognitive performance in rodents but is devoid of significant effects on plasma ACE activity or blood pressure (100,101). Thus, it is not known if the cognition enhancing effects of ACE inhibitors are mediated by inhibition of the conversion of angiotensin I to angiotensin II in the brain or by a more general modulation of protease function.

Miscellaneous Peptides - Prolyl endopeptidase inhibitors continue to generate interest (2). An SAR study has been reported on prolinal containing peptides (102). Additional prolinal derivatives as well as compounds such as SUAM 14496 (**44**) have been described (103,104). Several TRH (thyrotropin-releasing factor) analogs have been reported (105-107). YM-14673 is ten times more potent than TRH in reversing amnesia induced by anoxia or scopolamine (108). Other neuroactive peptides including vasopressin, alpha-MSH analogs and the nonapeptide V-9-M have been described as having positive effects on learning and memory (109-111). L-Pyroglutamyl-L-alanine amide has been reported to activate all phases of memory function (112).



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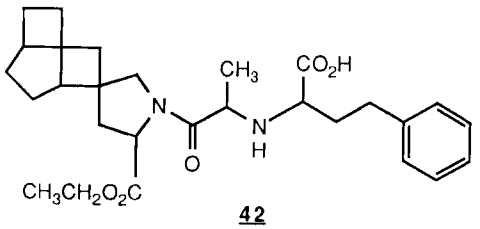

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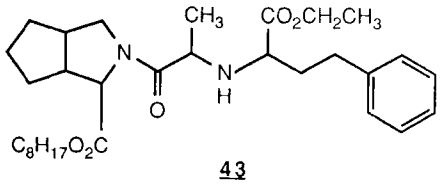

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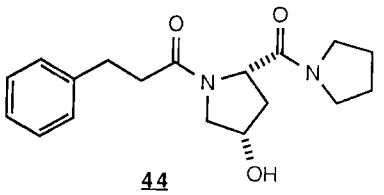

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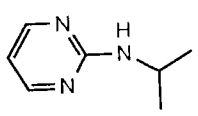
Future Directions - The preceding sections have focused on compounds designed to treat the memory deficits associated with aging and human neurological disorders. It is now possible to propose treatment strategies designed to halt or retard the progressive neurodegenerative changes mediating cognitive impairments in disorders like Alzheimer's disease. Endogenous trophic factors have been described which define the form and function of neurons in the CNS (113). The best characterized is nerve growth factor (NGF), but others with central activity have been described, including brain derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF)(See Chapter 26). Infusion of NGF, BDNF or bFGF into the lateral cerebral ventricles prevents experimentally-induced or age-related degeneration of basal forebrain cholinergic cell bodies in adult rats and monkeys (114-118). In the rat, NGF infusions can restore age-related or lesion-induced loss of cognitive function (119,120). It is not clear whether this latter effect reflects neurotrophic actions or the ability of NGF to pharmacologically increase cholinergic neuronal transmission.

These endogenous trophic factors are large proteins which are susceptible to rapid enzymatic degradation and inactivation and thus may not be suitable as drugs. Other agents which apparently modulate the activity of endogenous trophic factors may hold more promise. These include gangliosides (121,122), isaxonine (**45**) and a series of related pyrimidines, and **46** (123-125).

Catechols have also been described which enhance the release of NGF from glial cells (126,127).



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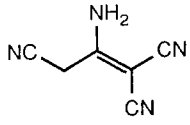

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Proteases and protease inhibitors appear to modulate neuronal growth and possibly CNS plasticity. Human protease nexin-I, a serine protease inhibitor with neurotrophic activity, is reduced in Alzheimer's disease (128). Alternative splicing of the gene encoding the amyloid precursor protein (APP) of senile plaques in Alzheimer's disease yields molecular forms with (APP 751 and 770) and without (APP 695) a Kunitz-type serine protease inhibitor domain (129). The deduced amino acid sequence of the protease inhibitor coded by APP 751 and 770 transcripts is similar to protease nexin-II, a potent antichymotrypsin (130). The APP-695 transcript is selectively decreased in cortex and hippocampus of Alzheimer's diseased brains relative to the APP 751 transcript (131). In addition, the serine protease inhibitor, alpha$_1$-antichymotrypsin, is associated with amyloid deposition in Alzheimer's disease (132), and portions of the A4 amyloid protein have been shown to be both neurotrophic and neurotoxic depending upon the fragment of the protein studied (133,134). Thus, a dynamic balance between the activities of proteases and protease inhibitors may be required for maintenance of normal neuronal connectivity. Disrupting this balance may lead to formation of aberrant neuritic processes like those associated with some senile plaques, or alternatively may lead to abnormal processing of multiple proteins in Alzheimer's disease (135). Reinstating this balance may yield useful therapies for the pathologies mediating the cognitive dysfunction that accompanies neurodegenerative diseases and aging.

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Chapter 4. Acute Ischemic and Traumatic Injury to the CNS

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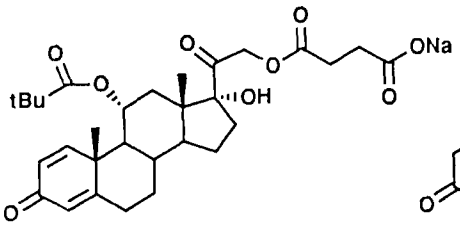
Introduction - Ischemic and traumatic injuries to the central nervous system are unmet medical needs. They include post-traumatic injury (head and spinal cord), focal ischemia, focal ischemia with reperfusion (stroke), global ischemia (CPR), hemorrhagic injury (subarachnoid hemorrhage) and radiation injuries. Although ischemia itself can cause irreversible injury, reperfusion of the ischemic tissue is particularly damaging, perhaps because of iron release, mitochondrial leakage with superoxide anion formation, and increases in xanthine oxidase or neutrophil infiltration. Traumatic injury can lead to free radical damage because of local ischemia or iron release from damaged cells or petechial hemorrhage.

Although the initial events vary, the secondary, progressive processes that lead eventually to cell death and neurologic injury overlap for these diverse maladies. The mechanisms and agents through which these injuries develop include lipid peroxidation, calcium influx, excitatory amino acids and arachidonic acid metabolites. A variety of therapeutic interventions are being actively investigated because of the desperate clinical need for new agents. For example, inhibitors of lipid peroxidation, iron chelators, superoxide dismutase, calcium channel antagonists, magnesium, excitatory amino acid antagonists, prostanoid modulators, adenosine agonists, opioids, diuretics, mitochondrial stimulants, monogangliosides, anti-cholinergics and hypothermics have all been evaluated in animal models of ischemia and trauma. These studies are described below. Excitatory amino acid antagonists (1,2,3) and lytic therapy (4) have been reviewed recently and are thus omitted from this chapter.

Lipid Peroxidation Overview - Lipid peroxidation at the cellular membrane level is a chain reaction that alters or destroys the polyunsaturated chains of membrane phospholipids. Lipid peroxidation occurs when a reactive oxygen species (superoxide anion, hydrogen peroxide with iron, hydroxyl radical, peroxyl radical) or a carbon radical attacks the fatty acid chains of membrane phospholipids. The unsaturated fatty acids of the phospholipids of the cell and organelle membranes are particularly susceptible to radical attack because of their allylic hydrogens. Hydrogen abstraction initiates a chain reaction; the allyl radical product of the initiation reaction can abstract a hydrogen from adjacent chains, react with oxygen to form a lipid peroxyl radical, couple with other radicals, or fragment. Iron is intimately involved in the lipid peroxidation process. Initial radical attack of unsaturated fatty acids may be catalyzed by iron near or within the cell membrane. Iron can decompose lipid hydroperoxides to peroxyl and alkoxyl radicals that in turn may initiate new lipid radical chain reactions. Iron can also interact with molecular oxygen or reduced oxygen radical species and deliver them to the membrane. Radical mediated neurologic injury occurs when the body's natural defenses (SOD, catalase, vitamin E, glutathione) are overwhelmed. Therefore, compounds which remove iron from the process (iron chelators) or eliminate the chain initiating or propagating events (superoxide dismutase, radical quenchers) are potential therapeutic agents (For a general discussion see ref 5). The structural integrity and the function of cell membranes are irreversibly changed during membrane lipid peroxidation. Extracellular calcium can enter the cell and activate calcium dependent phospholipases and protein kinases. Phospholipases, once activated, will cleave fatty acids from phospholipids and cause additional changes in the chemical composition and physiologic state of the cell membrane. Free fatty acids are converted to prostaglandins and thromboxanes. The sources and role of oxygen radicals in CNS damage were recently reviewed (6).

Lipid Peroxidation Inhibitors: Steroids - Large doses of either methylprednisolone or hydrocortisone improve neurologic outcome after CNS injury (7,8,9,10,11). The effects were probably not glucocorticoid related because of the high doses required and the need for maintenance dosing. Rather, a weak inhibition of lipid peroxidation was implicated (12). The steroid-lipid peroxidation hypothesis is supported by a series of non-glucocorticoids (1,2) which lack the 11-beta hydroxy group. This hydroxy group is one of the structural requirements of glucocorticoids. These compounds lack hormonal activities, weakly inhibit lipid peroxidation, and are active in models of CNS injury (13). Recently, a series of 21-aminosteroids which are termed lazaroids have been reported. Tirilazad (U74006F) and U74500A (3,4) are potent inhibitors of lipid peroxidation and are extremely effective in models of head and spinal cord injury, subarachnoid hemorrhage, and temporary and permanent central occlusion (14,15,16).



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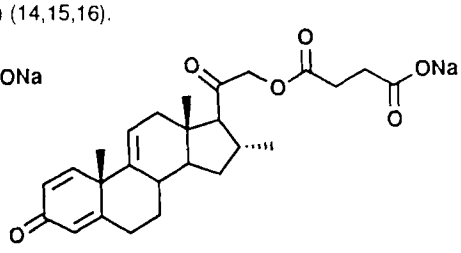

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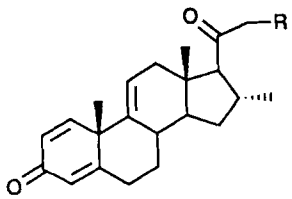

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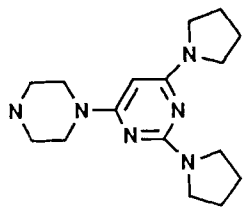

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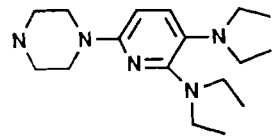
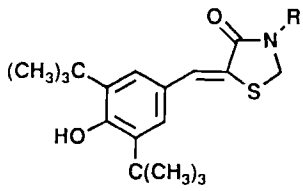


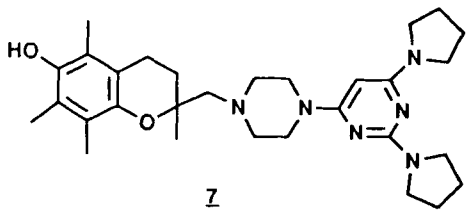
Lipid Peroxidation Inhibitors: Nonsteroids - Members of a series of 4-thiazolidinones (LY178002 and LY256548, 5,6) ameliorate neuronal necrosis in the hippocampal CA-1 region of rats and gerbils in models of transient global cerebral ischemia (17). LY256548 has also been reported to protect against ischemia-induced damage in the rat middle cerebral artery occlusion-reperfusion model (18).



5 R^2 = H

6 R^2 = CH_3

Vitamin E is the most important endogenous membrane antioxidant. The benzopyran portion of vitamin E has been included in several new lipid peroxidation inhibitors. 2-Methylamino analogs of trolox which inhibit lipid peroxidation with IC50s in the micromolar range have been prepared. Compounds U78517 and U78518 (7,8) are active in models of murine head injury at iv doses below 1.0 mg/kg (19).



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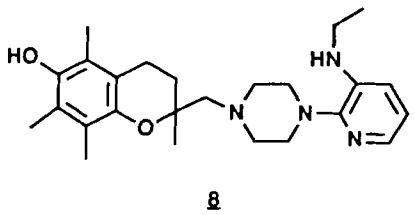

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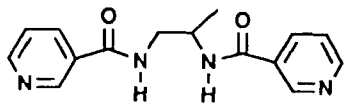
Other new antioxidants have been described. AVS (9) has shown utility in the suppression of chronic vasospasm in dogs following subarachnoid hemorrhage (20). Ionol displayed protective effects in a cerebral ischemia/reperfusion model (21). AJ-3941 (10), which combines anticonvulsant and antihypoxic properties with an antioxidant ability, protects against delayed neuronal death following transient ischemia (22). Sodium ozagrel (11) and nizofenone fumarate (12) are reported to be free radical scavengers; they are marketed in Japan for subarachnoid hemorrhage. The role of nizofenone as a cerebral protectant was recently reviewed (23). Nizofenone is effective in a cat model of subarachnoid hemorrhage (24). The effect of nizofenone on delayed ischemic neurological deficits following subarachnoid hemorrhage due to aneurysmal rupture was investigated by a cooperative double-blind clinical trial (25). Idebenone (13) is an inhibitor of lipid peroxidation (26) which is marketed in Japan for improving cerebral metabolism and improving performance after stroke. In a rat bilateral carotid occlusion model, pretreatment with idebenone prolonged survival time and delayed the onset of ischemic seizures (27). It also partially reversed ischemia-induced amnesia in one-trial passive avoidance in a rat model (28).



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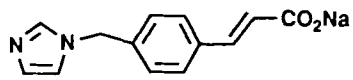

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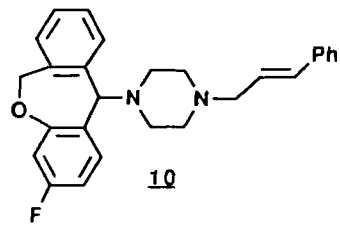

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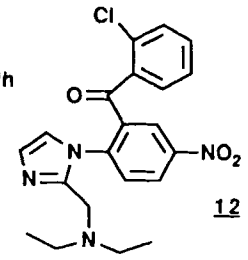

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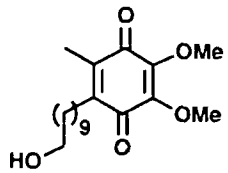

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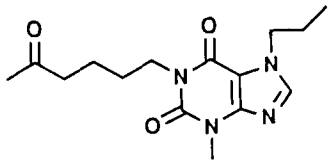
Mannitol, which is a scavenger of hydroxy radicals, displayed cerebral protective effects in dogs and rabbits after ischemic insults. In human studies, it lowered intracranial pressure while increasing cerebral blood flow (29,30).

Superoxide Dismutase/SOD and Catalase - Endogenous superoxide dismutase (SOD) and glutathione peroxidase were measured in male Wistar rats after right middle cerebral artery occlusion with temporary bilateral carotid artery occlusion. Cystolic CuZn-SOD decreased in the infarcted focus after cerebral ischemia, but glutathione peroxidase did not. This suggests a protectant role of SOD as a result of ischemia (31). Superoxide dismutase is being evaluated in many ischemic injuries. In a rabbit model of spinal cord ischemic injury, SOD given during reperfusion of the cord decreased paralysis and motor nerve damage (32). Recently, conjugates of SOD which extend its half-life have been developed. SOD and catalase that have been coupled to polyethyleneglycol are called PEG-SOD and PEG-CAT, while a pyran (divinylether maleic acid) copolymer with SOD is called Pyran-SOD. PEG-SOD and Pyran-SOD (33,34) both prolong measurable plasma half life of SOD. SOD and catalase may complement each other in ischemia.

In a rat model of focal cerebral ischemia, co-administration of PEG-SOD and PEG-CAT prior to the ischemic insult significantly reduced the infarct volume as compared to vehicle (35).

Xanthine oxidase inhibitors blunt superoxide anion production. Propentofylline (HWA-285, **14**) is undergoing Phase II clinical trials for cerebrovascular disorders. This xanthine derivative reduced infarct size by 27% in a gerbil model of focal cerebral ischemia and significantly protected the hippocampal CA1 neurons, even when drug was administered 1 hour after injury (36).



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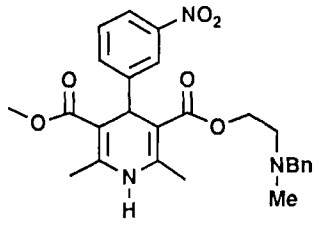
Iron Chelators - Iron chelators can impede iron-catalyzed lipid peroxidation (37). Deferoxamine, an iron chelator, has been attached to large biocompatible polymers such as hydroxyethyl starch. These derivatives have increased plasma retention and decreased toxicity (38). They are active in a murine model of head trauma and in a rat model of cerebral ischemia (39). However, the use of deferoxamine alone or in combination with lidoflazine (a calcium antagonist) did not show any cerebral or neuronal protection in two models of global brain ischemia in dogs (40).

Divalent Cation Modulation - Calcium's role in neuronal hyperexcitability and ischemic injury was recently reviewed in the context of seizures (41), and brain ischemia (42). The neuroprotective activity of the long acting calcium-entry blocker flunarizine was described (43). The radical scavenging action of the dihydropyridine calcium antagonists (44) suggests the possibility of multiple mechanisms for this class of calcium channel antagonist.

Nimodipine is a calcium antagonist which has been approved by the FDA for subarachnoid hemorrhage. It is being evaluated for stroke in the US and has been launched in Germany, Denmark, and S. Korea for the treatment and the prophylaxis of stroke (45). The cerebrovascular applications of nimodipine were recently reviewed (46). Nicardipine (**15**), another calcium entry blocker, is an antagonist of voltage sensitive L-type calcium channels. It reportedly reduced ischemic injury in the CA1 hippocampus of the gerbil (47). Nicardipine showed promise in an open-label stroke trial and is now being tested for reduced disability in a blinded, 3-month study in patients after an aneurysmal subarachnoid hemorrhage (48). A recent trial with the calcium antagonist PY108-068 (**16**) for the treatment of acute ischemic cerebral infarction showed no significant improvement (49).



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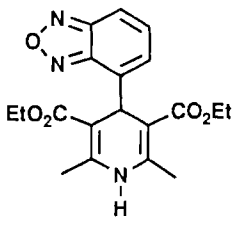

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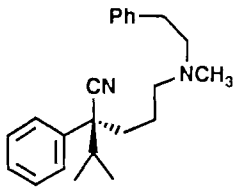

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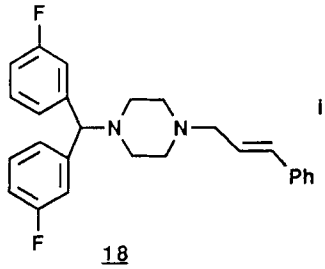
Several calcium antagonists that are in early stages of development have shown useful activity in animal models of stroke and trauma. Emopamil (**17**) is a calcium channel blocker/5HT2 antagonist which increased cerebral cortical blood flow. In a middle cerebral artery occlusion (MCAO) model in rats, emopamil when given after the insult significantly reduced the infarct size (50). It was also cerebroprotective in models of acute hypoxia and ischemia (51). Another calcium entry blocker, flunarizine (**18**), reduced neuronal necrosis in the hippocampus after forebrain ischemia (52) or global ischemia (53) caused by cardiac arrest. PN200-110 (**19**) prevented the formation of brain edema in an ischemic-reperfusion middle cerebral artery occlusion model (54). E-2001 (**20**) is a novel antioxidant/calcium antagonist. It inhibited lipid peroxidation and scavenged oxygen and nitrogen radicals. In animal models E-2001 protected hippocampal neurons following transient ischemia, prolonged survival following KCN injection or bilateral carotid

artery ligation in mice, and improved the symptoms of stroke produced by unilateral carotid artery ligation in gerbils (55).



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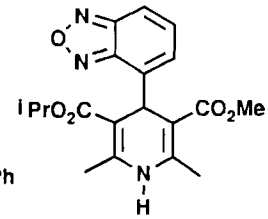

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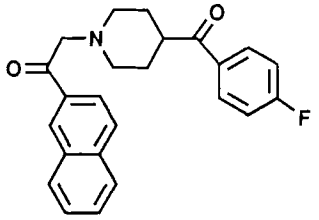

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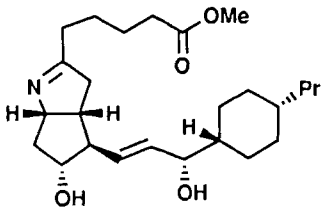
Arachidonic Acid Cascade Related - Arachidonic acid metabolites may contribute to brain edema, post ischemic hypoperfusion, and neuronal cell injury in cerebral ischemia (56). OP-2507 (21) is a PGI analog which increases survival time in animal models of cerebral anoxia produced by various insults. At very low subcutaneous doses, it also prevents edema and biochemical changes after bilateral carotid occlusion by an unknown mechanism (57). Thromboxanes have been proposed as being important in ischemia-induced damage (58). The thromboxane synthetase inhibitor OKY-046 (22) increases cerebral blood flow after bilateral carotid artery ligation in spontaneously hypertensive rats (59). Treatment with the selective thromboxane synthetase inhibitor 1-benzylimidazole increased cerebral blood flow and reduced thromboxane B2 levels after thirty minutes of transient forebrain ischemia in rats (60).



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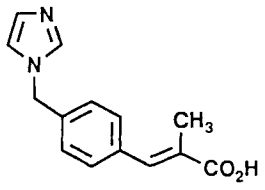

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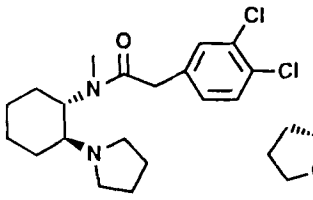
Opioids and Opioid Related Compounds - Traumatic brain injury increases endogenous opioids. In the past, naloxone has given mixed results in models of brain and spinal cord trauma (61). In an open label, 38-patient stroke trial, naloxone showed no improvement in neurological outcome scales or CT lesion volumes (49). Naloxone had some benefit in a rat spinal cord injury model (62); however, it had no detectable effect on stroke course or severity in an NINDS-sponsored trial in which patients received up to 5 g of drug. The average time to treatment was 14 hours (63). Naloxone is reportedly an inhibitor of lipid peroxidation (64). This complicates interpretation of the mechanism of action. Recently, kappa agonists and antagonists have been studied in ischemia and trauma. Kappa agonists U50488E (23) and U62066E (24) protected hippocampal CA1 neurons during temporary bilateral carotid occlusion in gerbils (65). The kappa agonist U50488H was effective in a feline middle cerebral artery occlusion model. It also reportedly worsened neurological outcome in a rat model of traumatic brain injury (66). Dynorphin also displayed neuroprotective ability in models of cerebral ischemia (67). In seeming contrast to the kappa agonist effect, the kappa antagonist WIN 44,441-3 (25) improved survival and cerebral blood flow in cats after a traumatic spinal brain injury (68). WIN 44,441-3 reduced motor dysfunction after ischemic spinal cord injury (69) and improved neurological function after a middle cerebral artery occlusion (70). Nor-binaltorphimine in a similar fashion significantly improved neurological recovery after spinal trauma in rats (71). Thyrotropin releasing hormone (TRH) has shown useful activity in animal models of CNS ischemia and in CNS and spinal trauma (72,73). The effects of TRH on blood flow and the microcirculation was recently reviewed (74).

Dextromethorphan is a non-opioid cough suppressant which is enantiomerically related to an opioid. High affinity sites were identified for dextromethorphan in 1981. In addition to binding at its own site, it may also be a sigma antagonist (75). Dextromethorphan was neuroprotective in glutamate-induced neurotoxicity in murine neocortical cell cultures (76). It reduced neuronal damage in a rabbit in vivo model of acute focal cerebral ischemia (77,78). Finally, it reduced post-ischemia hypoperfusion in a rat global ischemia four vessel occlusion model (34). The mechanism of action is unclear.



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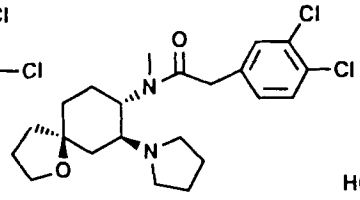

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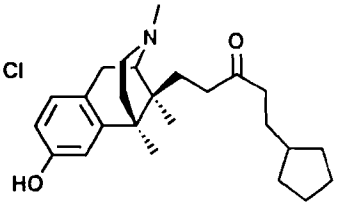

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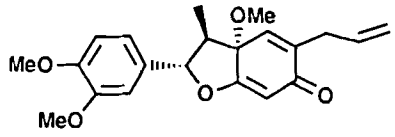
PAF Antagonists - Central production of PAF, PAF central binding sites and the effects of PAF on cerebral ischemia have been reviewed (79). Kadsurenone (26) reduced multifocal cerebral ischemia induced by air embolism in dogs and enhanced neuronal recovery (80). BN-52021 (27), a terpene isolated from Ginkgo biloba extract (GBE 761), improves regional cerebral blood flow and prevents the generation of free fatty acids following 10 minutes of transient global ischemia and 60 to 90 minutes of reperfusion in the gerbil when administered therapeutically (81). Recently, the PAF antagonists WEB 2086 (28) and Ginkgolide B were studied after permanent middle cerebral artery occlusion in the rat. Significant reduction in cortical infarct size was seen with both pre- and post-ischemic drug delivery (82).



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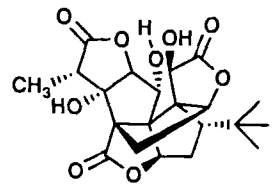

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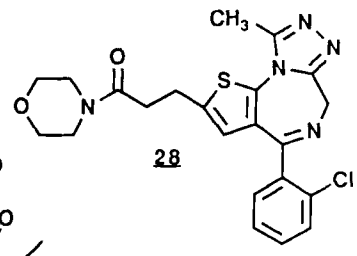

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Neurotransmitter Related Therapy - In vivo microdialysis studies with probes in the corpus striatum of rats or gerbils that had been subjected to either global cerebral ischemia or focal cerebral ischemia showed that massive amounts of dopamine were released into the extracellular fluid compartment (83,84,85,86,87). Further evidence that endogenous dopamine has an important role in causing selective neuronal necrosis in the striatum was obtained when substantia nigra lesions that destroy the nigrostriatal dopaminergic neurons protected against ischemic damage in the striatum (88,89). Dopamine, serotonin, and glutamate nerve terminals suffer less damage from ischemia when catecholamines are depleted using the methyl ester of alpha-methyl-p-tyrosine (90).

The injury-related release of dopamine can contribute to ischemic cell death by spawning toxic oxygen radicals when undergoing oxidation during reperfusion (91). It has also been suggested that dopamine may act as an excitotoxin or may potentiate glutamate's excitotoxic effects (92).

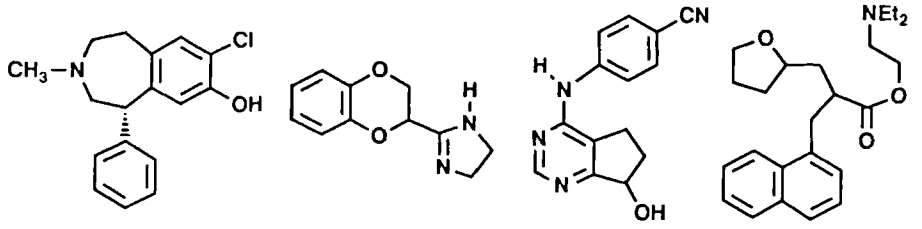
Recently, the dopamine D1 antagonist SCH 23390 (29), in combination with a NMDA receptor blocker (MK 801) had a protective effect in the hippocampal CA1 region following 10 minutes of

global ischemia in rats. However, neither SCH 23390 nor MK 801 had any effect when given alone (93).

Idazoxan (30) is an alpha-2 antagonist which enhances activity of locus coeruleus neurons, increases release of noradrenaline, and reduces damage assessed at one week after injury in the CA1 region of the hippocampus in gerbils which had been subjected to 10 minutes of transient cerebral ischemia combined with hypotension. Drug is given by constant infusion for 6 hours (94).

RS-8359 (31) is a reversible and selective inhibitor of MAO-A which ameliorates ischemia-induced brain damage in a 4-vessel occlusion model (rat) or a bilateral common carotid artery occlusion temporary occlusion model (gerbil). Hypoperfusion of the ischemic area was reduced and the CA-1 region of the hippocampus was somewhat protected. Blood flow and monoamine restoration are the reported mechanisms (95).

Mianserin and cyproheptadiene improve functional outcome after moderate, but not severe, experimental spinal cord injury in the rat. Drug was given at 15 minutes after injury by iv bolus. Postmortem 5HT and 5-HIAA levels were maintained below the lesion site. The authors argue that spinal 5HT elevation is part of the secondary injury mechanism (96). The serotonin S2 antagonist, naftidrofuryl (LS 121, 32), was markedly protective on CA1 pyramidal cells in a gerbil ischemic brain damage model (97).



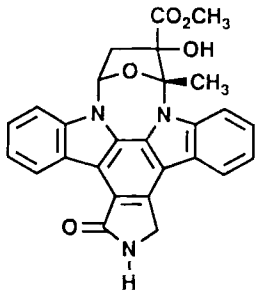
29 30 31 32

Adenosine - Adenosine is an endogenous purine which has profound neuromodulatory influences on central nervous system function (98, 99). Adenosine and its agonists are powerful inhibitors of excitatory neurotransmitter release (100). They also inhibit superoxide anion and hydrogen peroxide release from stimulated neutrophils, which is suggestive of a role in the protection of vascular endothelium from damage by neutrophils (101). Recently, the effect of cyclohexyl adenosine (CHA) was studied in gerbils subjected to 30 minutes of bilateral carotid occlusion. Post ischemia treatment with CHA significantly protected the pyramidal cells in the CA-1 region of the hippocampus (102) and improved survival (103). 2-Chloroadenosine, a stable analog of adenosine, protected the CA-1 region in the rat hippocampus following 10 minutes of transient forebrain ischemia (104). The adenosine A1 agonist, (R)-phenylisopropyladenosine (RPIA), showed a significant reduction in cortical infarct volume after permanent middle cerebral artery occlusion in the rat (105).

Gangliosides - Gangliosides are a class of sialic acids that contain glycosphingolipids. They are natural components of cellular membranes and are particularly abundant in neural tissue (106,107). GM_1 ganglioside or AGF_2 (an internal ester of GM_1 ganglioside) treatment has been shown to decrease mortality 52% and protect against loss of Na^+/K^+-ATPase in total plasma membrane fractions 48 hours after the induction of ischemia in gerbils by permanent unilateral ligation of the common carotid artery (108). In a middle cerebral artery occlusion/reperfusion model in cats, post-ischemic administration of ganglioside GM_1 resulted in significant recovery of neurologic deficits and less histologic damage than untreated cats (109). Recently, AGF_2 was studied in a transient global cerebral ischemia model in monkeys. The rate of neurologic recovery was accelerated by treatment with AGF_2 after ischemia (110). GM_1 and AGF_2 have been

shown to reduce Ca^{++} overload and K^+ efflux from cells and improve cerebral blood flow following transient global ischemia in the rat (111). GM_1 was studied clinically for acute stroke. Treatment was started ≤12 hours after onset of a hemispheric cerebral infarct. Outcome measures were mortality and neurological deficits. Ischemic stroke patients showed a significantly greater degree of neurologic improvement over the 15 days of drug administration than placebo-treated patients. At day 120 these improvements were no longer statistically significant. GM_1 ganglioside is currently being evaluated in studies where it is given for a longer period of time (112).

Gangliosides may play a role as endogenous modulators of protein kinase C translocation from the cytosol to the plasma membrane (113). This process involves the activation of calcium influx with the increase of phosphorylation of specific membrane proteins. This translocation involves glutamate or other excitatory neurotransmitter receptor operated cationic channels, which, with persistent stimulation, can result in activation of protein kinase C (114). Gangliosides have also been shown to be inhibitors of protein kinase C activity in vitro (115,116). Recently, the protein kinase C inhibitor K-252a (33) was studied in a global cerebral ischemia model in gerbils. Pretreatment with K-252a significantly protected neurons in the CA1 sector of the hippocampus (117).



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Chapter 5. Advances In Central Serotonergics

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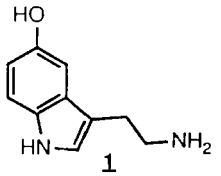
Introduction - Research on serotonin (5-hydroxytryptamine; 5-HT (1)) has been intensified during the last decade, mainly due to the development of new, selective compounds, both agonists and antagonists, which bind to different subtypes of 5-HT receptors. Most of this development has been focused on the central nervous system (CNS). The receptor types discussed are 5-HT1, 5-HT2 and 5-HT3, where the 5-HT1 receptors have been further subclassified into 5-HT1A, B, C and D (1-5). This subclassification is not unambiguous, and it was recently suggested that the 5-HT1C receptor subtype should more appropriately be placed in the 5-HT2 receptor family (4). This chapter will focus on compounds acting at the 5-HT receptors and will not consider agents, e.g. classical antidepressants acting via inhibition of 5-HT reuptake.

5-HT1 RECEPTORS

General background - 5-HT1A agonists with a high degree of intrinsic efficacy induce the so called 5-HT behavioral syndrome in the rat. The syndrome consists of a flat body posture, abducted hind- and forelegs, reciprocal forepaw treading, head weaving and tremor (6-8). However, interesting species differences exist with regard to this syndrome. For example, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT; 2) does not induce the 5-HT syndrome in mice after s.c. administration (8) but only after i.v. administration (9). In addition, no syndrome was seen after a high i.p. dose of 8-OH-DPAT to wild rats (10). Among several biological effects, 5-HT1A receptor agonists stimulate male rat sexual behavior and produce hypotension and hypothermia in various animal models (3). Interestingly, 8-OH-DPAT does reduce blood pressure in the normotensive rat. However, its effect is pronounced in the SHR, in the anesthetized cat and in the dog (11). Intracarotid infusions of 8-OH-DPAT did not affect arterial blood pressure in pigs (12). Although controversial, some reports suggest that the brain 5-HT1A receptors may be involved in the regulation of alcohol intake (13). A new, interesting and potentially useful clinical application of 5-HT receptor agonists is their involvement in the stimulation of natural killer cell activity. Recent in vitro studies showed that both 5-HT and 8-OH-DPAT possess such effects (14). However, most of the recent clinical interest in 5-HT1A receptor agonists is related to their putative involvement in depression, anxiety and obsessive compulsive behavior (15,16).



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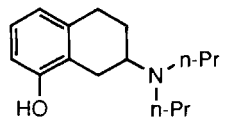
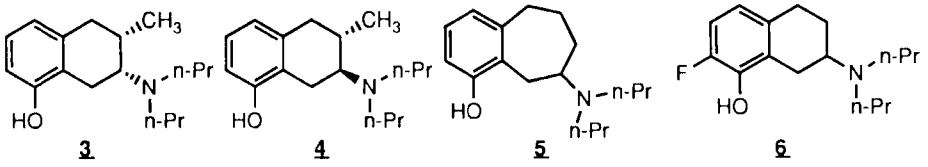


2

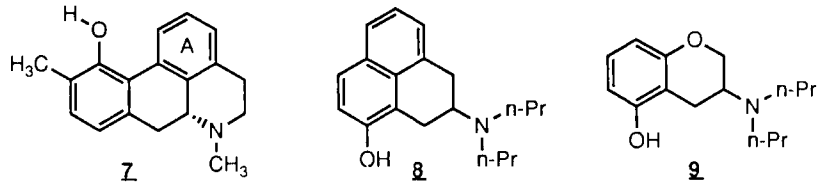
New 2-aminotetralins and analogs of 8-OH-DPAT - The reference 5-HT1A agonist is 8-OH-DPAT, and several analogs of this compound have appeared in recent years (17-19). For instance, both 3 and 4 have been resolved (20). (-)-cis-(2R,3S)-3 and (+)-trans-(2S,3S)-4 were both about 100 times less potent than 8-OH-DPAT in the 5-HTP accumulation assay. Both of these active enantiomers also induced the 5-HT behavioral syndrome while their optical antipodes were inactive. Interestingly, the stereochemistry at C2 in compound (-)-cis-(2R,3S)-3 is opposite to that of the more active enantiomer (R-form) of 8-OH-DPAT (17). The same research group also reported the central effects of the enantiomers of 5, where the activity resided in the R enantiomer (21). It is homochiral to (+)-R-8-OH-DPAT and is active in the biochemical assay in reducing brain 5-HTP-accumulation and in producing a clear 5-HT behavioral syndrome in reserpinized rats.



3 4 5 6

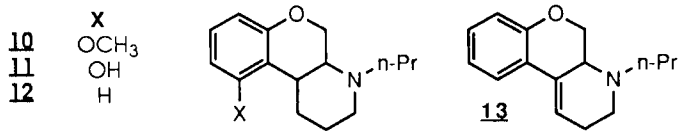
The ortho-fluorinated 8-OH-DPAT analog **6** has the same potency and selectivity at 5-HT1A receptors as 8-OH-DPAT itself (22). The internal H-bond between the 7-F and the 8-OH substituents is probably present also at the drug receptor interaction, providing indirect evidence for the suggestion that H-bond acceptance (oxygen lone pairs interacting instead of O-H) is sufficient for inducing 5-HT1A receptor interaction (22).

The aporphine **7** is not a dopamine (DA) receptor agonist; instead, it has the pharmacological profile of a selective 5-HT1A agonist (23). From NMR and molecular mechanics calculations it was suggested that, due to steric hindrance, the 11-OH group would not be coplanar with the A-ring of the aporphine nucleus, providing a tentative explanation for the lack of dopaminergic effects of **7** (23). U-67413B (**8**) is a new 5-HT1A agonist (24). It binds to 5-HT1A receptors with an IC50 of 22 nM and induces the 5-HT behavioral syndrome. In biochemical and electrophysiological assays **8** is also a partial DA receptor agonist. A series of hydroxylated or methoxylated 3-aminochromane analogs of 8-OH-DPAT (e.g. **9**) have been described, and striking similarities in the SAR of the two structural classes were noted (25,26).



7 8 9

Potent and selective ligands for D2, α2, 5HT1A and 5HT2 receptors may be obtained, by appropriate pharmacophoric modification, from compounds related to the 3-aminochromanes, namely **10-12** (27). Compound **10** (CGS18102A) is relatively selective for central 5-HT receptors, showing high affinity for central 5-HT1A and 5-HT2 receptors (28). Compound **10** also decreased 5-HTP accumulation in rat brain, however, the compound did not induce the 5-HT behavioral syndrome in doses up to 30 mg/kg (p.o.) in the rat. It blocked 5-HTP-induced head twitches in mice, a typical behavior elicited by 5-HT2 receptor agonists (28). Interestingly, the non-substituted analog **13** has a higher 5-HT1A affinity (IC50 = 74 nM) than **12** (IC50 = 219 nM) (27). Quite analogously, chemical manipulation of the ergolines can lead to compounds with increased potency and selectivity for 5-HT1A receptor sites as compared to 5-HT2 receptor sites (29). Thus, the 9-10 double bond was shown to be essential for high affinity at the 5-HT1A receptor, which can be seen when comparing compound **14** (IC50 = 17 nM in [^{3}H]-5-HT binding) with its saturated analog **15** (IC50 = 390 nM in [^{3}H]-5-HT binding).

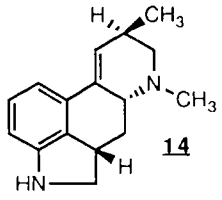


	X
10	OCH_3
11	OH
12	H

13

New octahydrobenzo[g]isoquinolines with 5-HT1A receptor affinity have recently been described (30). Compound **16** (IC50 = 18 nM) was the most potent analog but, in addition to its 5-HT1A affinity, **16** is non-selective and has high affinity for α1 and α2 as well as D2 receptors. Compounds **17-19** showed high 5-HT1A receptor binding affinity, indicating that 8-methoxy or 8-hydroxy substituents are not necessary for efficient binding to 5-HT1A receptors (31). A series of 8-methoxylated analogs of 8-OH-DPAT was described (e.g. the N,N-di-

8-methoxylated analogs of 8-OH-DPAT was described (e.g. the N,N-di-cyclopropylmethyl analog 20) (32). Compound 20 was resolved and the R enantiomer is a selective 5-HT1A agonist with high intrinsic efficacy, although its potency is 20-30 times lower than that of 8-OH-DPAT (32). S-20 is equipotent and equieffective to R-20 in the binding, biochemical (brain 5-HTP and 5-HIAA suppression) and rectal temperature assays. However, S-20 produces only weak signs of the 5-HT behavioral syndrome, even at comparatively high doses in both normal and reserpinized rats. Thus, R-20 is an agonist with high intrinsic efficacy, while S-20 seems to be a partial agonist at postsynaptic 5-HT1A receptors (32).



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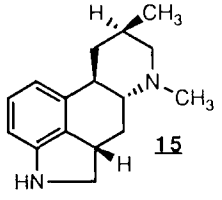

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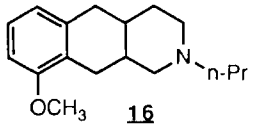

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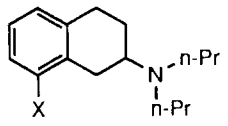
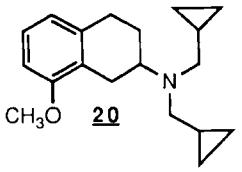


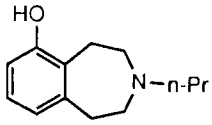
17
18
19

X
$CONH_2$
$O\text{-}(CH_2)_2NHCOOEt$
$(CH_2)_2NHCOOBz$



20

The new 5-HT1A agonist 21 (about 20 times less potent than 8-OH-DPAT in reducing brain 5-HTP accumulation) (33), can be viewed, due to its symmetry, as an analog of both the potent DA receptor agonist 5-hydroxy-2-(di-n-propylamino)tetralin (5-OH-DPAT; 22) and the 5-HT1A agonist 8-OH-DPAT. Compound 23 and its N,N-di-n-propyl analog (24) were shown to be orally active dopamine receptor agonists in rats and mice (34). Interestingly, 23 is non-selective and equipotent at DA and 5-HT1A receptors (35). Resolution and testing in vivo and in vitro indicated that 23 is active per se and that the active enantiomer is (+)-R-23 (35).



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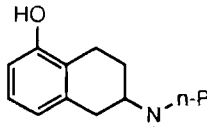

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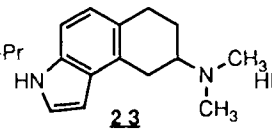

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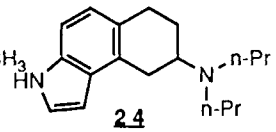
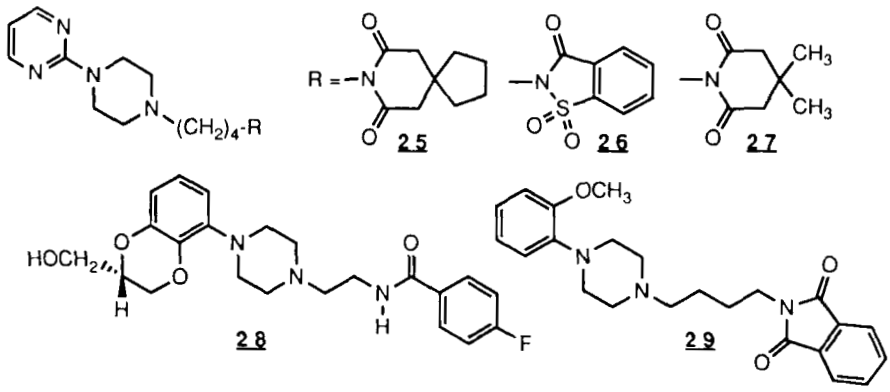
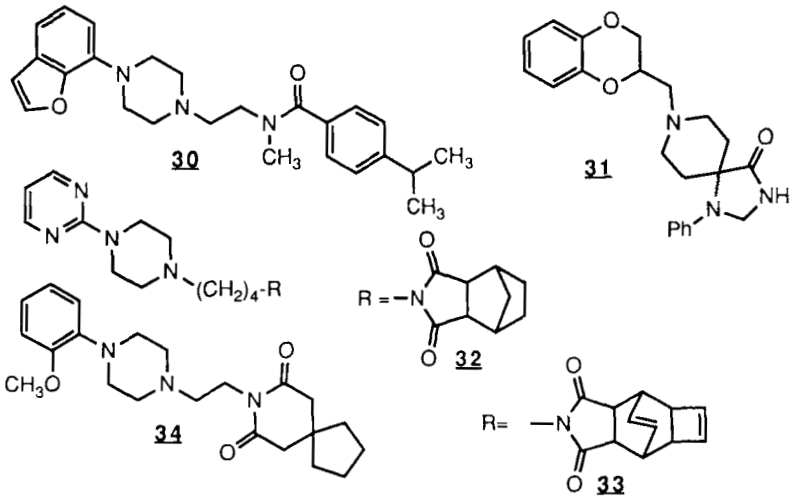


24

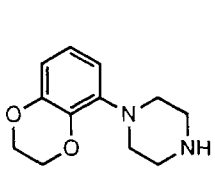
Arylpiperazines - Several research groups are working on new compounds closely related to the arylpiperazines buspirone (Buspar®; 25), ipsapirone (26) and gepirone (27) (16). The stimulation for these efforts is the growing evidence for anxiolytic and also antidepressant properties of these compounds in man (36-39). The new arylpiperazines are generally more selective (as compared to e.g. 25) for the 5-HT1A receptors. The efficacies of the new analogs differ. Some appear to be agonists with a high intrinsic efficacy (e.g. flesinoxan (28)), while others have a low intrinsic efficacy and have been classified as potential antagonists (e.g. NAN-190, 29) (40). Flesinoxan (DU 29373; 28) is a potent and long-acting 5-HT1A ligand with antihypertensive effects in the SHR (41). No active metabolites are produced from flesinoxan in the species studied (42). Notably, flesinoxan displayed only weak and short-lasting hypotensive effects in healthy volunteers (43) and, likewise, ipsapirone seems to be devoid of cardiovascular effects in normotensive patients too (44). Befiperide (30) has been reported to be a partial 5-HT1A receptor agonist (45). However, the compound has antidopaminergic (and thus potential antipsychotic) properties in some animal models. Thus, befiperide blocks apomorphine-induced stereotypies and inhibits the conditioned avoidance response in rats (45). The arylpiperazine spiroxatrine (31) was developed from



spiperone. On the basis of the in vitro binding data and effects on the activity in a canine basilar artery preparation it was suggested that spiroxatrine is an antagonist at 5-HT1A-like receptors (46). However, a recent study clearly shows that spiroxatrine is a 5-HT1A receptor agonist and a DA receptor antagonist (like buspirone) in the CNS (47). This study was based on behavioral (conflict behavior) and biochemical (monoamine metabolite levels in the CSF and in vitro binding) experiments in the pigeon. Several other arylpiperazines with different intrinsic efficacies have been presented recently, e.g. SM-3997 (**32**) (48), WY-47846 (**33**) (49), and BMY7378 (**34**) (50).



5-HT1B receptors - In the rat, 5-HT1B receptors are mostly concentrated in the basal ganglia and the substantia nigra (51). In cow, pig and human tissue these brain areas contain almost exclusively 5-HT1D receptor binding sites (52,53). A 5-HT1B ligand, eltoprazine (**35**), is in early clinical development for its potential use in disorders where aggression is a symptom, e.g. in schizophrenia (54). Eltoprazine is structurally similar to quipazine (**36**), mCPP (**37**) and TFMPP (**38**), and it possesses affinity for the different subtypes of the 5-HT1 receptor (54). The behavioral effects of activation of 5-HT1B sites are less well defined, and more selective agonists and antagonists are needed (7). The selectivity of the different ligands for the 5-HT1 receptor subtypes has been challenged (55). In particular, the previous classification of mCPP and TFMPP as being relatively selective for the 5-HT1B binding sites in rat frontal cortex was discussed (56). TFMPP has a very specific anti-aggressive profile in several animal models and may belong to a new class of compounds, the serenics (57,58).



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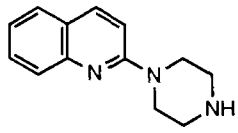

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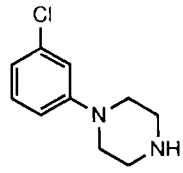

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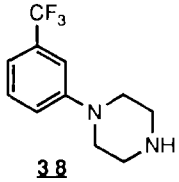

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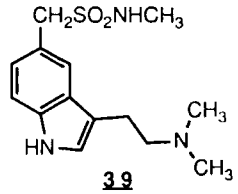


39

5-HT1C receptors - In most species studied, the 5-HT1C binding sites are found in the choroid plexus and cortex (59). No compound seems to possess selectivity for this receptor subtype, though mCPP has previously been classified as such a candidate. However, the affinity of mCPP for 11 neurotransmitter receptor binding sites was determined in human brain membranes (60). The results showed that mCPP has no selectivity, but is essentially equipotent at all 5-HT receptor subtypes (IC50 = 360-1300 nM). More interestingly, mCPP displays a very high affinity (IC50 = 15 nM) for central [^{3}H-]-quipazine labeled 5-HT3 sites (61). Based on similarities between the 5-HT1C and 5-HT2 receptor types with regard to their molecular biological, pharmacological and biochemical properties, the 5-HT1C receptor has recently been suggested to belong to the 5-HT2 receptor family (4).

5-HT1D receptors - The majority of [^{3}H]-5-HT-labeled recognition sites in the human cortex, caudate, and globus pallidus represent 5-HT1D binding sites (52). Sumatriptan (**39**) was shown to have high affinity for 5-HT1D (Ki = 17 nM) and 5-HT1B (Ki = 27 nM) binding sites in animals (62). It was slightly less potent at 5-HT1A binding sites (Ki = 100 nM) and was essentially inactive at ten other binding sites, suggesting that the interactions with 5-HT1B and D sites may be the reason for its apparent efficacy in the acute treatment of migraine (63).

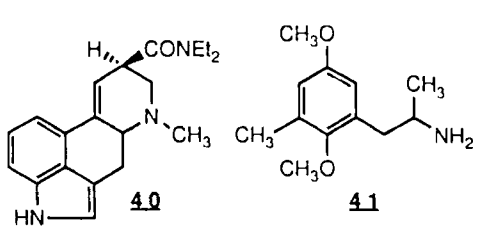
5-HT2 RECEPTORS

5-HT2 receptor agonists - The induction of head twitches in mice by 5-HT2 receptor agonists and its blockade by selective 5-HT2 receptor antagonists represents an easily quantifiable model of 5-HT2 receptor activation in vivo (7,64). Analogs of the classical human hallucinogens d-LSD (**40**) and DOM (**41**) are potent 5-HT2 receptor agonists (64-68).

5-HT2 receptor antagonists - Selective antagonists for the 5-HT2 receptor are available, e.g. ritanserin (**42**). However, this compound produces only weak behavioral and biochemical effects in various species. Because of this, it has been suggested that the 5-HT2 receptor might be a binding site without an important physiological function, or a redundant receptor (69). In man, the best documented clinical effect of ritanserin appears to be an increase in slow-wave sleep, improving the overall sleep quality (70,71). A mixed D2/5-HT2 blocking effect has been suggested to contribute to the beneficial clinical profile of atypical antipsychotics (72).

Amperozide (**43**) is a 5-HT2 receptor antagonist with strong anti-aggressive properties in animals (73). Electrophysiological studies of amperozide have been performed in rats and it increased the firing rate of noradrenergic neurons of the locus coeruleus (74). It has also been shown that amperozide is able to regularize the firing pattern of midbrain DA neurons (75). Preliminary, open clinical studies have shown that amperozide is effective against both positive and negative symptoms in schizophrenia with only few side effects (76,77). 5-HT2 receptor antagonists block the discrimination stimulus properties of the known hallucinogens (65,67,68). Although no human data have been reported, these studies strongly indicate that centrally acting 5-HT2

antagonists like ritanserin would be able to block human hallucinations elicited by 5-HT2 agonists like the street drugs d-LSD, DOM and analogs (78,79).



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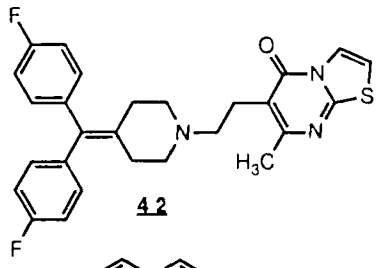

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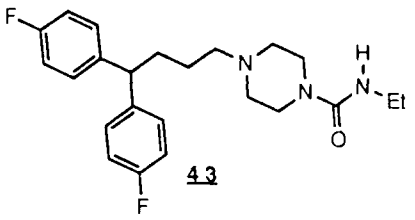

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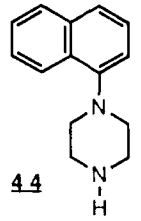

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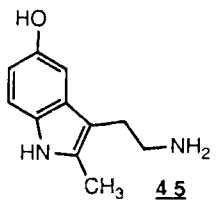
A series of arylpiperazines was studied in [^{3}H]-5-HT (5-HT1) and [^{3}H]-ketanserin (5-HT2) binding. The most potent compound in this study was 1-[α-naphthyl]piperazine (1-NP, **44**) displaying Ki values (nM) of 5 and 18 for 5-HT1 and 5-HT2 sites, respectively. 1-NP produces stimulus generalization effects similar to those of TFMPP and can antagonize the stimulus effects of the 5-HT2 receptor agonist DOM, indicating that 1-NP has 5-HT2 receptor antagonist properties (80). Interestingly, 1-NP has a lower affinity (IC50 = 110 nM) for 5-HT3 sites in [^{3}H]-quipazine binding (81).

5-HT3 RECEPTORS

Due to the development of new ligands, mainly antagonists, for the 5-HT3 receptor type, this research area has expanded enormously lately (82).

Peripheral 5-HT3 receptors - The peripheral 5-HT3 receptor has been suggested to be identical with the M-receptor (1,83).The prototypical 5-HT3 agonist is 2-methyl-5-hydroxytryptamine (2-Me-5-HT; **45**), which binds with an IC50 of 430 nM in [^{3}H]-zacopride (**46**) binding (84). It has been shown that 5-HT3 antagonists reduce cisplatin-induced emesis in both animals and man (85,86). Interestingly, BRL43694 (**47**) does block cisplatin-induced emesis, but not apomorphine induced emesis in dogs, indicating that its anti-emetic effect is not due to a blockade of the motor component of the emetic reflex. Neither the mechanism of cisplatin-induced emesis nor the site of action of the 5-HT3 antagonists are clearly understood (87).



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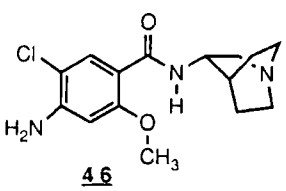

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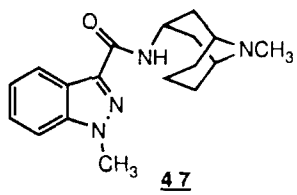
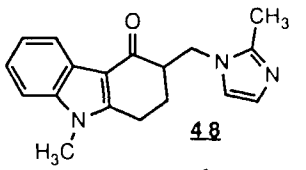


47

Central 5-HT3 receptors - The first direct evidence that 5-HT3 receptor sites exist in the post-mortem human brain has been described (88). The receptors were labeled with [^{3}H]-zacopride and displaced with several agents known to be potent 5-HT3 antagonists, e.g. BRL43694, ondansetron (GR38032F; (**48**)), and ICS205-930 (**49**). The effects of three of the these 5-HT3 receptor antagonists (BRL43694, ondansetron and zacopride) were examined for anxiolytic effects in low and high light social interaction tests and in the elevated plus-maze test with negative results (89). This would indicate caution when considering 5-HT3 receptor antagonists as potential anxiolytics; however, preclinical data on the 5-HT3 antagonists reports activity in several animal models of anxiety and in one model of psychosis (82,90). In these studies zacopride, ondansetron, granisetron (**50**), MDL72222 (**51**) and ICS205-930 show very potent anxiolytic activity. They appear to display an inverted U-shaped dose-activity relationship. Interestingly, a similar dose-effect relationship was found for the anxiolytic effects of some 5-HT1A receptor agonists in the elevated plus-maze model (91).



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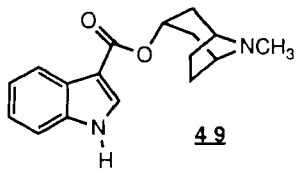

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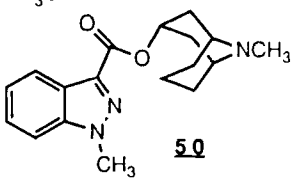

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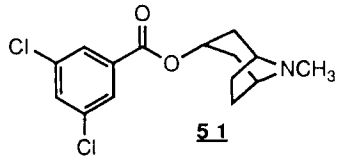
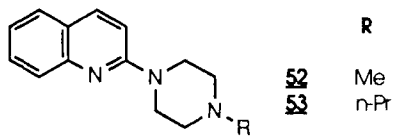
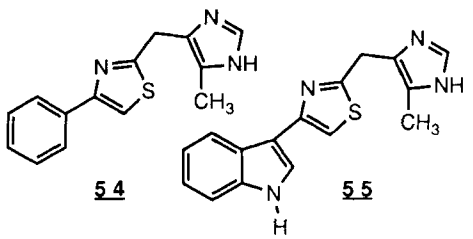


51

As mentioned above, it has been shown that [^{3}H]-quipazine can be used to label binding sites in rat brain membranes that display 5-HT3 receptor characteristics. The pharmacological profile of the binding site studied was in excellent agreement with that of 5-HT3 receptors characterized in other models (61,81,92). A series of 21 compounds related to the arylpiperazines were investigated in a structure-affinity study ([^{3}H]-quipazine labeled central 5-HT3 sites) (81). It was shown that these simple analogs can bind with high affinity, provided they carry a meta-chloro substituent on the aromatic ring. N4 alkylation of quipazine with methyl yields a derivative (**52**) with somewhat reduced 5-HT3 affinity, as compared to quipazine itself, but with greatly improved selectivity. In particular, the 5-HT2 and 5-HT1B binding affinities are reduced by a factor of 3 and more than 50, respectively. The N-n-propyl analog of quipazine (**53**) also has lower 5-HT3 affinity (by a factor of 10, as compared to quipazine).



	R
52	Me
53	n-Pr



54 **55**

A computer generated model for the 5-HT3 receptor was recently described (93). Nineteen compounds from 7 different chemical classes (IC50<10 nM) were selected and manipulated to place the outmost basic nitrogen atom in a position where it is in the same plane as the aromatic ring system of the molecule studied. No energy minimizations were performed, which the authors concede makes it virtually impossible to judge if the conformations selected for fit may exist at all. A novel class of 5-HT3 ligands (aromatic thiazole derivatives) has been reported (94). This SAR study resulted in compound **54**, which possesses potent 5-HT3 receptor antagonist properties. In this series, an aromatic portion consisting of a phenyl group as in

series, an aromatic portion consisting of a phenyl group as in **54**, instead of the usual 3-indolyl group (c.f. **55**), yielded a more potent compound (by a factor of about 7).

SUMMARY

Research in the 5-HT field, in particular in the CNS, is under active investigation. The clinical trials with 5-HT1A receptor agonists with different degrees of intrinsic efficacies and with 5-HT3 antagonists will give useful feedback to the medicinal chemists and pharmacologists for further improvements, and for the development of selective and more potent ligands. In particular, clinical results in depression and anxiety are crucial, since the predictive value of animal behavioral models seems to be very low in that area.

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SECTION II. CARDIOVASCULAR AND PULMONARY AGENTS

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Chapter 6. Antihypertensive Agents

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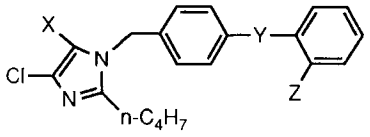
Introduction - Pharmacologic therapy of hypertension decreases overall cardiovascular morbidity and mortality, but fails to show a clear reduction in the risk of coronary heart disease (CHD) (1-4). Although many explanations have been offered for the lack of demonstrated benefit for CHD, one hypothesis in continued focus involves the unfavorable metabolic and biochemical changes caused by diuretics or ß-adrenoceptor antagonists devoid of intrinsic sympathomimetic activity (5). These drugs were used in the majority of the hypertensive clinical trials completed to date and adversely affect many of the known risk factors for CHD, including hyperlipidemia, hyperglycemia and elevated plasma catecholamine levels. These potentially deleterious changes might have offset expected benefits of blood pressure reduction.

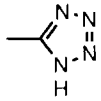
Since CHD is still the primary cause of death in the United States, long-acting calcium entry blockers, angiotensin converting enzyme (ACE) inhibitors, α_1-adrenoceptor antagonists and centrally acting α_2-adrenoceptor agonists, which have less negative impact on risk factors, continue to gain support as alternate choices for first-line therapy (4,6). Proponents of the traditional drugs argue that diuretics and beta-blockers have been shown to reduce cardiovascular morbidity and mortality in long-term clinical trials and that the lower doses of diuretics now used may not have persistent adverse effects on serum lipids (7). Recognizing the potential drawbacks of the traditional drugs, yet acknowledging the lack of long-term clinical trials demonstrating a protective effect of the newer agents, the 1988 report of the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure retained its previous endorsement of diuretics and ß-adrenergic blockers, but now includes ACE inhibitors and calcium entry blockers as recommendations for initial pharmacologic therapy of hypertension (1,8). Thus, an ideal regimen for treating mild to moderate essential hypertension has yet to be established.

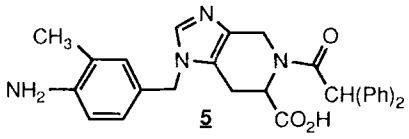
An important milestone of the past year was the introduction of orally active nonpeptidic angiotensin II (A-II) receptor antagonists. Besides offering the promise of a new approach to antihypertensive therapy, these compounds allow study of the renin-angiotensin system via blockade of the effector hormone rather than inhibition of its production. Progress in the area of renin inhibitors was marked by reports showing the first clinical evidence of efficacy of this class of compounds, although oral bioavailability was low for most of these agents. Research in the area of calcium channel antagonists was highlighted with the disclosure of a number of compounds having a long duration of action.

Angiotensin II Receptor Antagonists– New benzylimidazole A-II antagonists include EXP 6803 (**1**), a specific competitive antagonist of A-II in vascular smooth muscle (9-12). It blocks A-II-induced pressor responses without the agonist activity typically shown by peptide antagonists, and causes a hypotensive response in renal-artery ligated rats (RALR) after i.v. but not oral administration. Despite its nonpeptidic nature, **1** is poorly absorbed and rapidly metabolized and secreted in bile. EXP 7711 (**2**), EXP 9020 (**3**), and DuP 753 (**4**) are orally active in RALR and sodium-depleted dogs (12, 13). Removal of the amide linkage between the phenyl rings appears to be the critical factor which leads to oral activity for **2**, **3**, and **4** relative to **1**.

Two A-II receptor subtypes have been characterized by their differing sensitivity to dithiothreitol and their distinct structure-activity requirements for binding of nonpeptide antagonists (13, 14). In rat adrenal cortex, 80% of the binding of ^{125}I-A-II is inhibited by **4** and 20% by EXP 655 (**5**), an agent which does not lower blood pressure. The **4**-insensitive binding is also inhibited by CGP 42112A (**6**), and this ligand has been used to detect similar binding sites in human uterus, where only one receptor subtype is apparently present, as well as in other tissues where both receptors are present (15). These receptor subtypes are not distinguishable by profiling with angiotensin peptides. The receptor which shows high affinity for **4** is probably responsible for the stimulation of aldosterone biosynthesis by A-II in the adrenal cortex, while the function of the other receptor is unknown at this time.



	X	Y	Z
1	$-CH_2COOCH_3$	$-NHCO-$	$-COOH$
2	$-CH_2OH$	—	$-COOH$
3	$-CHO$	—	$-COOH$
4	$-CH_2OH$	—	



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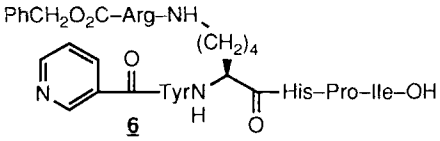
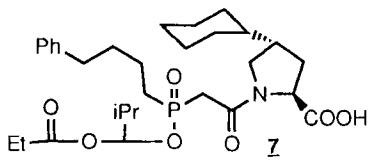


6

ACE Inhibition – A review of the biological role of ACE in the circulating and tissue renin-angiotensin systems, the immune system, and in neurotransmission concludes that only bradykinin and substance P are plausible alternative substrates (16). While the physiological relevance of bradykinin potentiation remains debatable, ACE may play a role in neurotransmission due to its ability to inactivate substance P. Pretreatment of RALR with the A-II antagonist **1** or a monoclonal antibody to A-II eliminates any further hypotensive response to captopril, suggesting that blockade of the circulating renin-angiotensin system, but not that found in tissue, is more important in short-term control of blood pressure in this model (17). The involvement of kinins, prostanoids, and other factors in the action of ACE inhibitors has been reviewed (18). Beneficial effects on coronary hemodynamics in myocardial ischemia may offer new therapeutic gains for both sulfhydryl- and non-sulfhydryl inhibitors.



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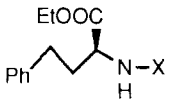
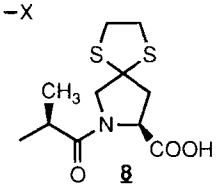


X=



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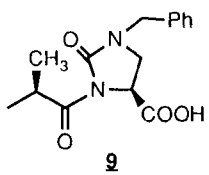

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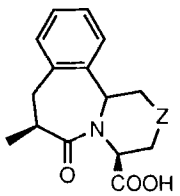
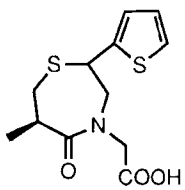


10 Z=CH_2

11 Z=S



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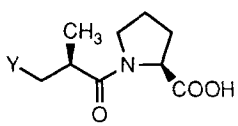
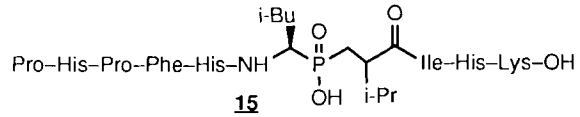


13 Y= ON-S-

14 Y= HS-

The prolonged duration of activity found preclinically for fosinopril (**7**) was reflected in initial clinical results (19, 20). Spirapril (**8**) is an analog of enalapril in which lipophilic substitution in the proline 4-position leads to enhanced activity in vitro and in vivo (21). Compound **9** incorporates an oxoimidazolidine ring in place of the proline ring of enalapril which leads to slightly lower potency in vivo (22). MDL 27,088 (**10**) and MDL 27,788 (**11**) exhibit extremely high affinity for ACE, probably due to optimum fit of the conformationally-constrained dipeptide surrogate (23). The potent inhibitor CS-622 (**12**) decreases calcium ion–dependent aortic tone in spontaneously hypertensive rats (SHR), a mechanism that may contribute to long-term efficacy of ACE inhibitors (24). The S-nitroso derivative (**13**) of captopril (**14**) retains ACE-inhibitory activity in vitro and in vivo and exhibits the direct vasodilator activity associated with organic nitrates (25-27).

Renin Inhibition – The crystal structure of recombinant human renin has been refined to 2.5 Å resolution (28). Comparison of renin to pepsinogen and three fungal aspartic proteases shows that renin has the same relatively hydrophobic core as the other enzymes with substantial similarity in the region of the active site. However, surface residues which are critical for renin's extreme substrate specificity vary greatly from corresponding regions of the other proteases. Most renin inhibitors contain an alcohol function in the configuration found in the prototype inhibitor pepstatin which binds to the catalytic aspartate carboxyls of renin (29). Compound **15**, which incorporates a hydroxyphosphinyl function in place of the statine alcohol, shows inhibitory potency comparable to corresponding statine-containing peptides, but analogs with shorter peptide sequences showed substantially reduced activity (30). The main barrier to clinical utility of renin inhibitors has been poor oral bioavailability. Therefore, most recent reports of new renin inhibitors describe molecular modifications designed to improve oral activity. BW-175 (**16**) shows 2.8-9.7% oral bioavailability in rats (31). Incorporation of the morpholino urea in **17** enhanced its aqueous solubility and improved intestinal permeability, but biliary excretion still limited oral bioavailability (32). The morpholino carbamate in SC-46944 (**18**) also increased oral activity relative to the Boc-NH compound (33).



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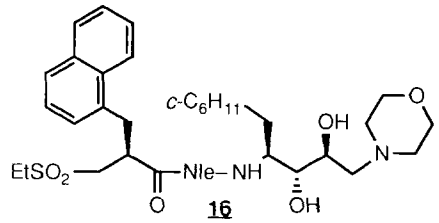

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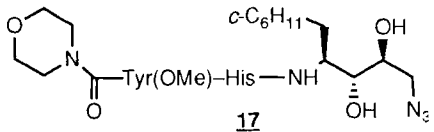

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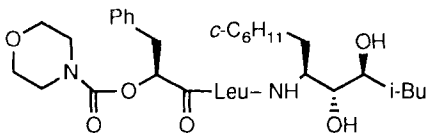
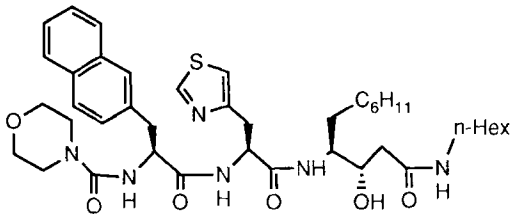


18

Initial clinical trials of four new renin inhibitors have been reported. ES 8891 (**19**) at 60 mg orally reduced plasma renin activity (PRA) 75% without further indication of efficacy in normal volunteers (34). Ro-425892 (structure not disclosed) caused a 47% reduction in plasma A-II concentration after 600 mg orally (35). Administration of CGP 38560A (**20**) (up to 0.25 mg/kg i.v.) to normal volunteers led to inhibition of PRA which lasted longer than reductions (ca. 90%) in plasma A-II concentration (36). Oral administration of 200 mg **20** to volunteers caused inhibition of PRA; plasma A-II concentration was not measured but lack of an increase in plasma active renin indicated little oral efficacy. Plasma drug levels were 25-fold lower after oral administration of **20**, indicating <1% oral bioavailability; the elimination half life for **20** was about 1 hr (36-38). Enalkiren (A-64662, **21**) displayed a similar ability to inhibit PRA and reduce plasma A-II concentration at i. v. doses up to 0.1 mg/kg in volunteers; elimination half life was about 2 hr (39). Oral doses of 10 to

40 mg inhibited PRA for up to 12 hr but plasma A-II concentration and oral bioavailability were not determined (40). In salt-depleted hypertensive patients, an ascending-dose i. v. bolus study showed **21** to have slightly greater effect than enalaprilat (41). In another i.v. bolus study, hydrochlorothiazide increased the maximum antihypertensive effect of 1 mg/kg **21** from 15 to 27 mm Hg (42). Once-daily i.v. administration of 1.2 mg/kg **21** for 7 days caused progressively greater effect with diastolic blood pressure reduced 24 mm Hg 24 hr after the last dose (43). The duration of effect of **21** appears to be longer than indicated by its plasma half life. However, i.v. administration of **21** to heart failure patients caused favorable vasodilator and hemodynamic effects which were of substantially shorter duration than the prolonged inhibition of PRA (44).



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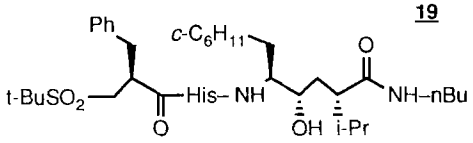

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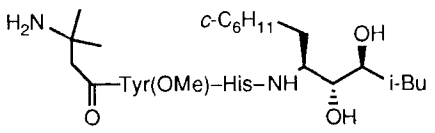
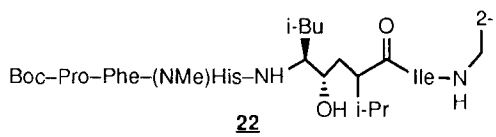


21

The substrate analog inhibitor U-71038 (**22**) was found to be resistant to biotransformation but rapidly cleared from plasma via biliary clearance after i. v. administration (45). Surprisingly, the primate renin-specific inhibitor L-157,119 (**23**) caused a greater hypotensive response than did ACE inhibitors in rats (46). This result may indicate a hypotensive mechanism for **23** which does not involve the renin-angiotensin system.



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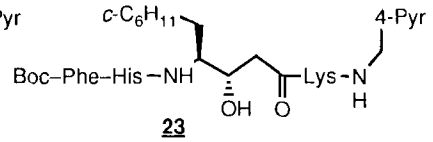
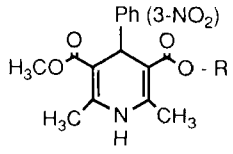
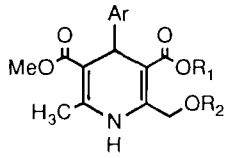


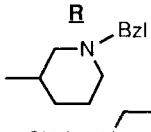
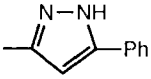
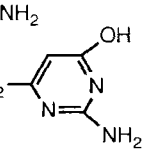
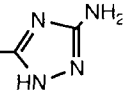
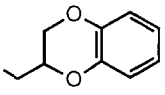
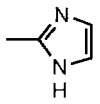
23

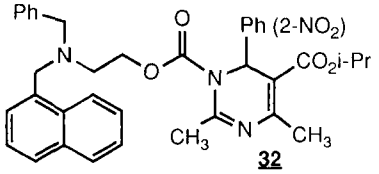
Calcium Channel Antagonists – New dihydropyridines have been found to have more potent and specific vascular effects than prototypes in this class. Compounds related to nicardipine, i.e. benidipine (**24**), manidipine (**25**), CV-159 (**26**), and P-0285 (**27**), have been reported to have a slow onset of action and long duration of effect in animals without the cardiodepressant effects characteristic of other dihydropyridines (47-55). Analogs of amlodipine (**28**), including NB-818 (**29**), UK 56,593 (**30**), and UK 52,871 (**31**), are more potent and have a slower onset of effect than **28**, and have a longer duration of effect in vivo than agents such as nifedipine and nicardipine (56-61). Compound **29** has also been reported to increase cerebral cortical blood flow (62) and to improve memory in certain models. (63).

Two dihydropyridines were designed to have additional mechanisms of action. Compound **33** blocks calcium channels nearly as well as nicardipine and blocks α-adrenoceptors with 100-fold lower potency than prazosin (65). This combination of activities might lead to antihypertensive activity without activation of the sympathetic nervous system. Since an agent with antithrombotic activity would be of potential benefit to hypertensive patients, Wy 27569 (**34**), which is 20 times less potent than nifedipine as a calcium channel blocker, was designed to inhibit thromboxane synthetase also (66). Another novel class of compounds structurally similar to the dihydropyridines, the dihydropyrimidines, such as **32**, have been reported to have a longer duration of effect in SHR than either nifedipine or nicardipine (64).

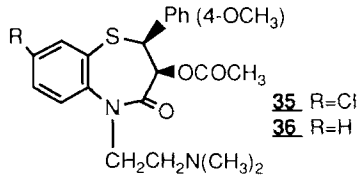




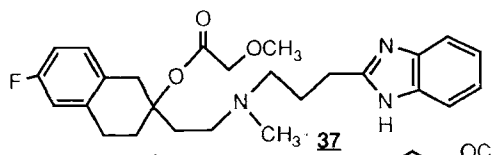
	R				R1	Ar	R2
24				28	Et	Ph (2-Cl)	$O(CH_2)_2NH_2$
25	$-(CH_2)_2-N\langle\rangle N-CHPh_2$			29	i-Pr	$Ph(2,3-Cl_2)$	$OCONH_2$
26	$-(CH_2)_6-$ 			30	Et	$Ph(2,3-Cl_2)$	OCH_2- 
27	$-(CH_2)_2-N\langle\rangle N-Ph(3,4-Cl_2)$			31	Et	$Ph(2,3-Cl_2)$	$O(CH_2)_2-NH-$ 
33	$-(CH_2)_2NH-$ 			34	Et	$Ph(3-NO_2)$	



TA-3090 (35, 8-chlorodiltiazem) has 4-fold higher affinity for the myocardial receptor and longer duration of effect in anesthetized dogs than diltiazem (36), and a prolonged hypotensive effect in SHR (67-70). Additionally, 35 increased vertebral blood flow and protected against strokes in stroke prone SHR (71, 72). RO- 5967 (37) and KT-362 (38) bind to the verapamil binding site, improve myocardial function and exhibit more cardioprotective *effects than verapamil in models of ischemia and arrhythmias* (73-78). *The indolizine sulfone* SR 33557 (39), inhibits binding of dihydropyridines, benzothiazepines and phenylalkylamines to their receptors (79).



35 R=Cl
36 R=H



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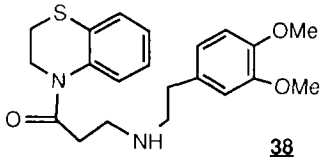

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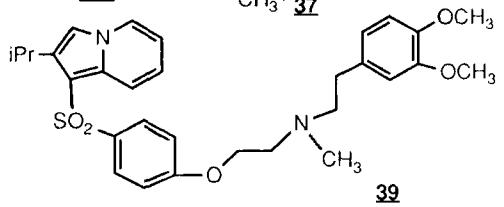
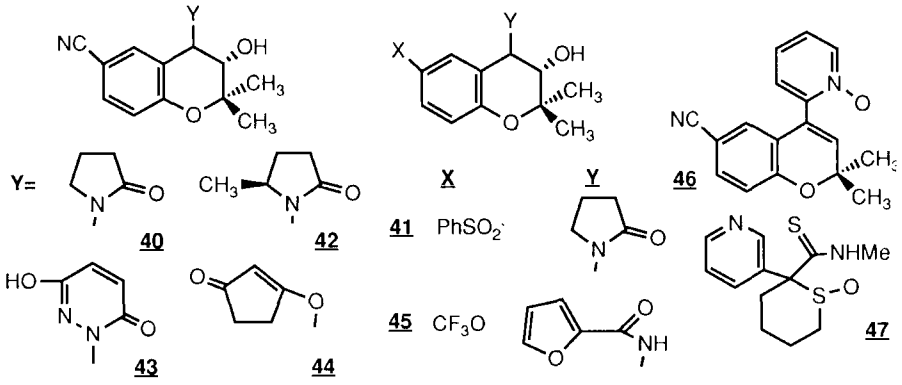
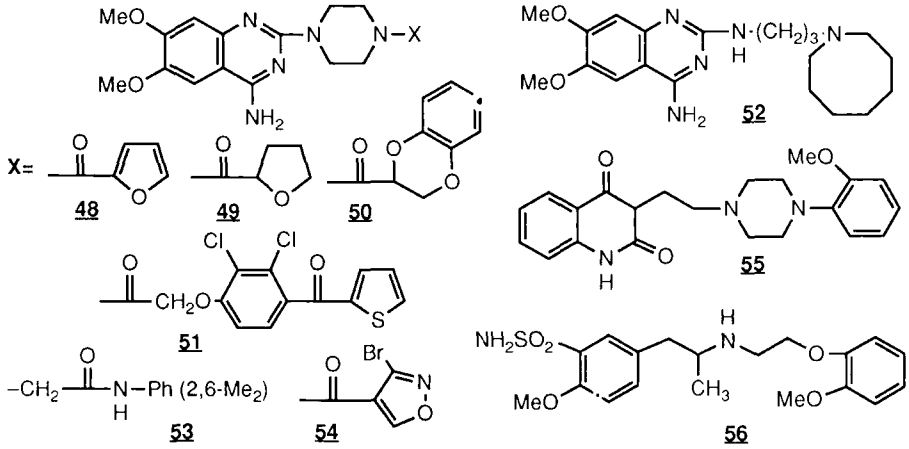


39

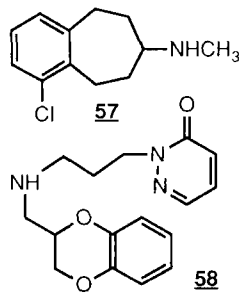
Potassium Channel Openers – Recent reviews describe the class of compounds which depolarize vascular smooth muscle and cardiac myocytes by opening potassium channels (80-84). Most new agents of this class have been analogs of cromakalim (40). The C-6 nitrile of 40 can be replaced with phenylsulfonyl (41) without loss of potency; the methyl analog 42 is more potent than 40 and possibly tissue-selective as well (85, 86). Various substitutions at C-4 of the benzopyran ring of 40 are tolerated as exemplified by compounds 43, 44, and 45 (87-91). Compound 46 shows that the C-3 alcohol can be replaced by a C-3-C-4 double bond (92). RP 49356 (47), like other potassium channel openers, may interact with the ATP-sensitive channel in cardiac tissues (94).



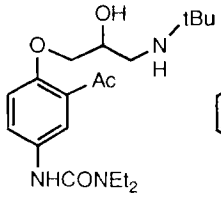
α_1-Adrenoceptor Antagonists - The antihypertensive effects of the prazosin (**48**) analogs, terazosin (**49**) and doxazosin (**50**), were associated with favorable changes in serum lipid profile (95-96). The substitution of the C-2 side chain of **48** with components of tienilic acid (diuretic and uricosuric), guanethidine (hypotensive), and lidoflazine (vasodilator, calcium-antagonist) yielded **51**, **52** and **53**, respectively (97). Compound **51** had the greatest and longest lasting depressor effect following oral administration to SHR. The isoxazole **54** maintained good affinity and selectivity toward α_1-adrenergic receptors and exhibited antihypertensive activity in SHR similar to **48** but had lower bioavailability (98). SGB 1531 (**55**) and YM 12617 (**56**) are undergoing clinical evaluation, with **56** being studied for treatment of dysuria (99,100).



α_2-Adrenoceptor Antagonists – Theoretically, these agents could cause adrenergic stimulation (resulting from reduced negative autofeedback of norepinephrine (NE)) as well as postjunctional α-adrenergic blockade. Hence, the net circulatory and endocrine effects of different α_2-adrenergic antagonists would be determined by the magnitude of these opposing effects within various tissues. Oral doses of SK&F 86466 (**57**) in normal subjects caused an increase in stimulated (postural and cold challenge) and supine plasma NE concentrations; these changes were associated with increased heart rate and PRA but little increase in blood pressure (101). In contrast, the new α_1- and α_2-adrenoceptor antagonist, GYKI 12743 (**58**) which does not block cardiac presynaptic α_2-adrenoceptors, has antihypertensive effects without tachycardia in animals (102).



ß-Adrenoceptor Antagonists – Third-generation ß-adrenoceptor antagonists, which combine $ß_1$-selective antagonism with peripheral vasodilatory activity, may cause less cardiodepression, have less negative impact on exercise tolerance and lipid profile, and have reduced incidence of bronchospasm compared with older ß-blockers. Celiprolol (**59**), a $ß_1$-antagonist/$ß_2$-agonist exhibited a favorable side-effect profile in the clinic (103). In other clinical studies, the effects of the nonselective ß-antagonist/$ß_2$ agonist, dilevalol (**60**), on arterial compliance, renal function, and usefulness in severe hypertension were reported (104-106). At high concentration, the ß-adrenoceptor antagonist/vasodilator, carvedilol (**61**) inhibited calcium influx through potential-operated channels in isolated canine coronary arteries (107). Cicloprolol (**62**), which has intrinsic sympathomimetic activity, showed less evidence of reduced cardiac performance in ischemic patients as compared with atenolol (108).



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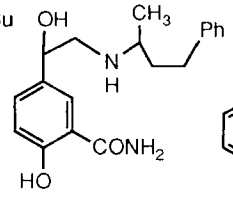

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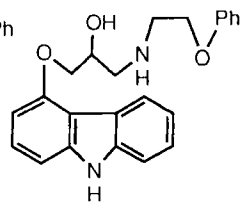

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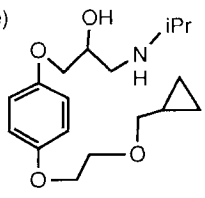

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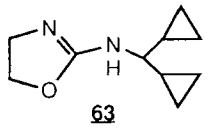
α_2-Adrenoceptor Agonists – The clinical pharmacology of centrally acting antihypertensives was reviewed with attention placed on sustained efficacy, regression of left ventricular hypertrophy and lack of adverse effects on sodium retention, glucose metabolism and lipid profile (109). These advantageous attributes, coupled with physicians' concern over treatment-induced metabolic effects, have rekindled interest in these agents. Rilmenidine (**63**) effectively lowered blood pressure in several clinical evaluations (110). The α_2–agonist, monoxidine (**64**), caused no deterioration in renal function in 24 hypertensive patients (111).



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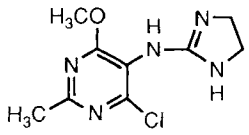

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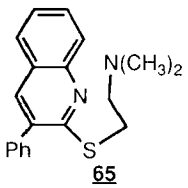
Miscellaneous Agents – Multiple receptor subtypes partially account for the mixed depressor and pressor actions of serotonin (5-HT) and the vasodilator and cardiac conduction effects of adenosine. Therefore, selective inhibition or stimulation of specific 5-HT and adenosine receptor subtypes may lead to novel antihypertensives. ICI 169,369 (**65**), a serotonin antagonist with selectivity for the 5-HT_2 and 5-HT_{1C} receptor subtypes, had no consistent effects on blood pressure in hypertensive patients (112). Preclinical studies showed the 5-HT_{1A}-receptor agonist flesinoxan (**66**) to be hypotensive and sympatholytic (113). The selective A_2-adenosine receptor agonist, CGS 21680C (**67**), increased coronary flow in the isolated perfused working rat heart model and lowered blood pressure after oral dosing in SHR (114). Cicletanine (**68**), a furopyridine derivative with undefined mechanism of action, attenuated the hypertension and cardiac hypertrophy in DOCA-salt rats, reduced the incidence of cerebral damage and increased the survival rate in stroke-prone SHR, and had a gradual antihypertensive effect in patients (115-117).



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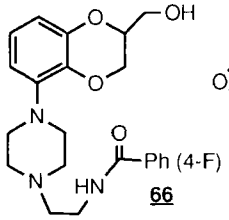

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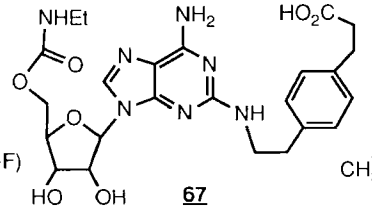

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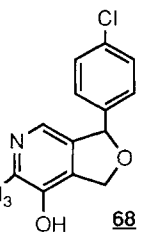
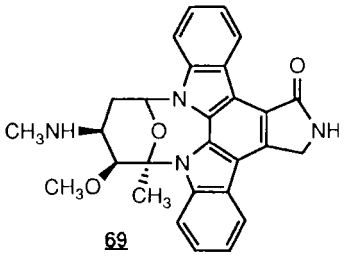


68

Antihypertensive agents with novel mechanisms include inhibitors of neutral endopeptidase 24.11, which degrades atrial natriuretic peptide (Chapter 10) and staurosporine (**69**) which inhibits various protein kinases (118, 119). Staurosporine-related compounds had long-lasting oral antihypertensive activity in SHR and relaxed isolated aortic preparations.



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Future Directions – Cardiovascular disease accounts for more expenditures on health care in the United States than any other diagnostic category, and hypertension is the most prevalent cardiovascular condition. While unit costs for hypertension treatment are low, aggregate costs are enormous due to the large and growing population of patients who are treated. Thus, economic considerations will have increasing impact on all future aspects of hypertension research (120). Greater efficiency in the application of antihypertensive therapy to the goals of reduction of cardiovascular morbidity and mortality will require research in a number of areas, including determination of pathophysiological mechanisms which define subsets of the hypertensive population, and development of highly specific drugs aimed at these patient groups (121). Specific gene markers may be identified that would be more useful than currently identified risk factors for indication of a predisposition to hypertension (122). Development of transgenic animal models may aid in this approach (123). Initial characterization of rats made hypertensive by transfer of the mouse renin gene represents an important early milestone in this exciting field (124).

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Chapter 7. Pulmonary and Antiallergy Agents

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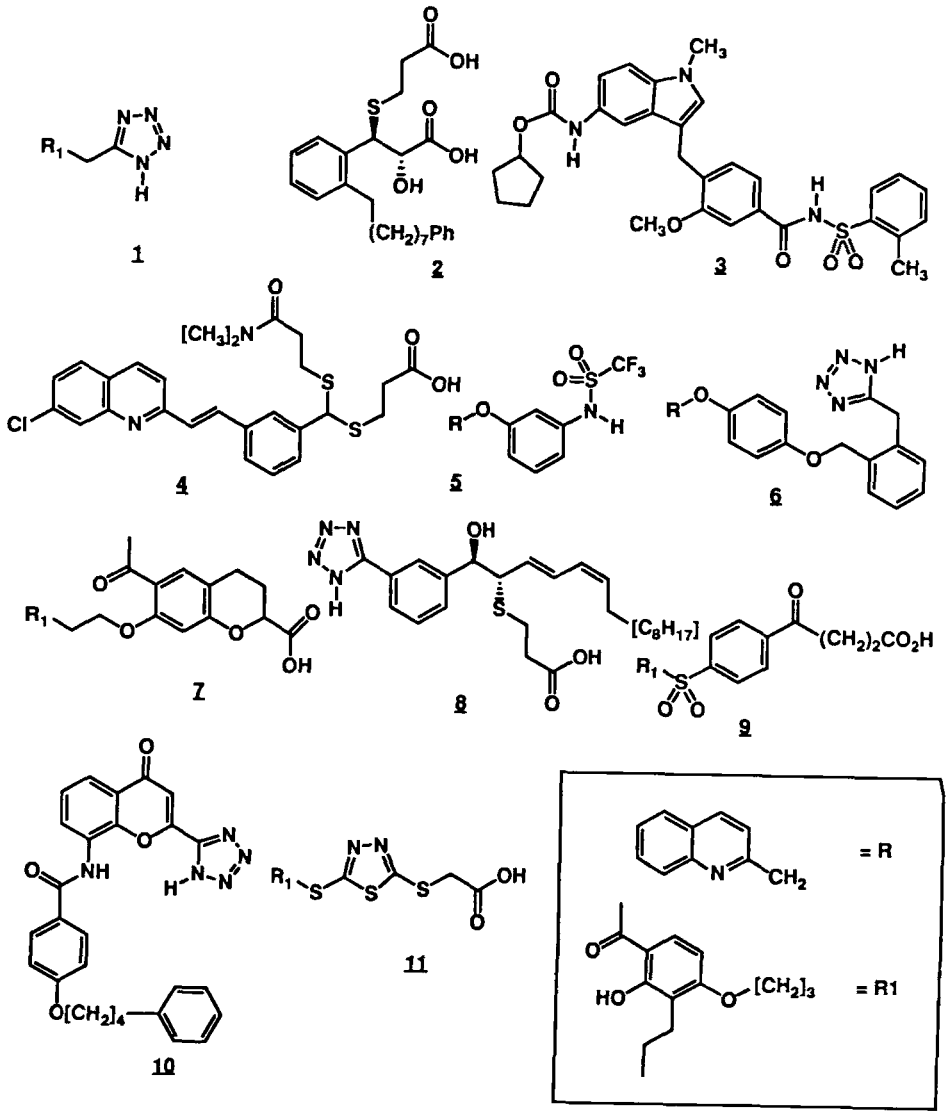
Introduction - Drug discovery efforts in the pulmonary area were predominantly aimed at the asthma/allergy market, with some activity evident against the adult respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), pulmonary hypertension (PH) and cystic fibrosis (CF). Asthma remains an attractive area for the introduction of new therapy as there is a growing sense that current treatment is inadequate and that the incidence is growing (1,2). A useful recent review of current therapy emphasized the need for new prophylactic, anti-inflammatory medications (3) and a provocative population analysis questioned the validity of the "extrinsic" and "intrinsic" division of asthma, suggesting that all asthmatic symptoms are associated with serum IgE levels (4). Atopy, as defined by specific IgE-mediated responsiveness, was shown to be linked to a marker on human chromosome 11 and to be inherited in an autosomal dominant manner (5). New directions for asthma research could be indicated by recent novel observations. Human asthmatic lung tissue obtained at autopsy or lobectomy was shown to be devoid of the bronchodilatory neuropeptide VIP (6), monoclonal antibodies to IL-5 were shown to block antigen-induced lung eosinophilia in mice (7), the rat basophil high affinity IgE receptor complex was expressed in COS-7 cells and a structure proposed (8), while a model of the attachment site on IgE itself was presented (9). Endothelin (10) and IL-8 (11) joined the ranks of putative pathological bronchoconstrictors, and ANP was shown to be a bronchodilator in asthmatics (12).

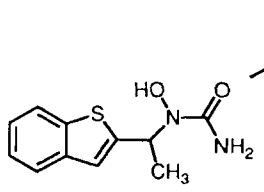
REGULATORS OF LIPID MEDIATORS

LTD_4 Antagonists - While definitive clinical evidence of a major role for the peptide leukotrienes in asthma/allergy was not presented this year, many antagonists are in the clinic and expectations remain high. A perceptive overview of the area appeared recently (13). The orally active LTD_4/LTE_4 antagonist LY171883 (1) showed small beneficial effects on pulmonary function (increase in FEV_1, decrease in use of bronchodilators) in mild asthmatics given 600 mg. b.i.d. for six weeks (14). SKF 104353-Z2 (2) has been shown to block LTD_4- and antigen-induced bronchoconstriction in asthmatics upon aerosol administration (15,16). Early clinical data for ICI 204219 (3) and MK-571 (4), previously known as L-660711, are now available; after oral administration of 3 or i.v. infusion of 4, both drugs were effective blockers of aerosol LTD_4 challenges (17, 18). The chemistry leading to the novel unsymmetrical dithioacetal unit present in 4 was described (19). Chemistry (20) and pharmacology (21,22) of Wy-48,252 (5) has now appeared, with an emphasis on oral potency: 5 has an ID_{50} of 0.6 mg/kg against antigen-induced bronchoconstriction when given intragastrically to guinea pigs. RG12525 (6) is another new LTD_4 antagonist showing good oral activity in animal models (23). The discovery and initial clinical results of RO 23-3544 (7) was described (24,25). LY170680 (8) is in Phase II evaluation, and

the preclinical pharmacology of this aerosol LTD_4 antagonist has appeared (26). L-648051 (9) is another aerosol-delivered antagonist in the clinic and relatively large doses (12 mg) were shown to inhibit LTD_4 bronchoconstriction in volunteers (27). A preliminary clinical report on ONO-1078 (10) shows it to be effective orally (300,450 mg) in blocking early and late responses to antigen challenge (28). The orally-active compound YM-16638 (11) has been profiled in the allergic sheep model, and shares with many other peptide LT antagonists the ability to block early and late bronchoconstriction responses in this model (29).



5-LO Inhibitors - Clinical evaluation of 5-lipoxygenase (5-LO) inhibitors has generally lagged behind that of LTD_4 receptor antagonists, due to the difficulty of finding appropriately safe and selective compounds. This situation is changing, and the recent demonstration that human urinary LTE_4 levels rise upon antigen challenge should aid trials which probe the importance of 5-LO in human diseases (30). Chapter 7 in Volume 24 highlighted the ex vivo 5-LO inhibition shown by oral A64077 (**12**) in man; preliminary pharmacological profiling has now appeared (31), showing **12** to inhibit 5-LO in broken RBL-1 cells (IC_{50} 0.5μM) and to block ionophore-stimulated LTB_4 production by whole human blood (IC_{50} 0.5μM). L-663,536 (**13**), now known as MK-886, marks an intriguing and promising new development in the area; it is a potent, orally-active inhibitor of leukotriene biosynthesis in several inflammatory models and blocks allergically-mediated bronchoconstriction in two species, whilst being inactive against isolated 5-LO (32). The protein to which MK-886 binds, and which is an accessory to 5-LO activity, has been isolated and its sequence deduced (33). Structure-activity relationships have been revealed for the series containing the potent, orally-active 5-LO inhibitor L-656224 (**14**), which inhibits antigen-induced bronchoconstriction in hyperreactive rats and shows extensive inhibition of pulmonary responses to ascaris antigen in the squirrel monkey (34). Data showing potent in vivo evidence of antiflammatory/antiallergic activity have been published for several new 5-LO inhibitors, including RG 6866 (**15**), AHR-5333 (**16**), E6080 (**17**), Wy-49,232 (**18**) and A-65260 (**19**) (35-39).



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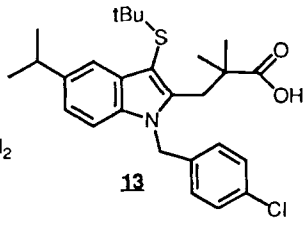

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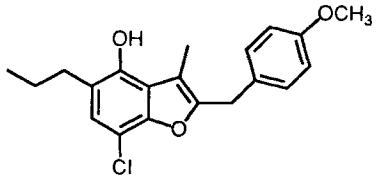

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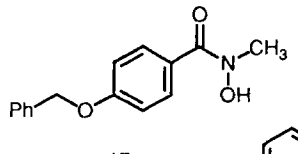

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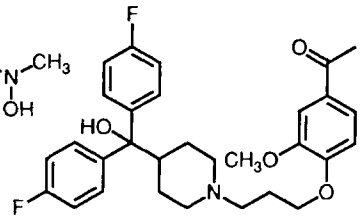

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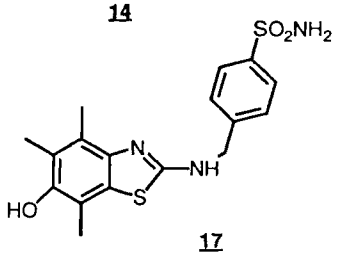

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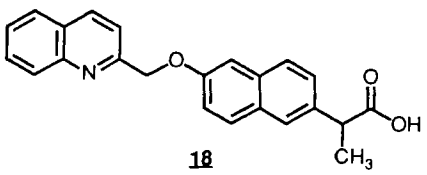

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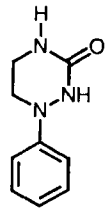

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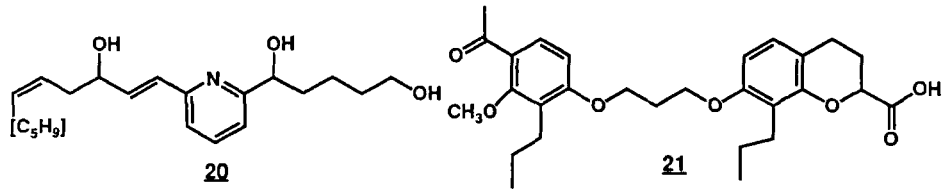
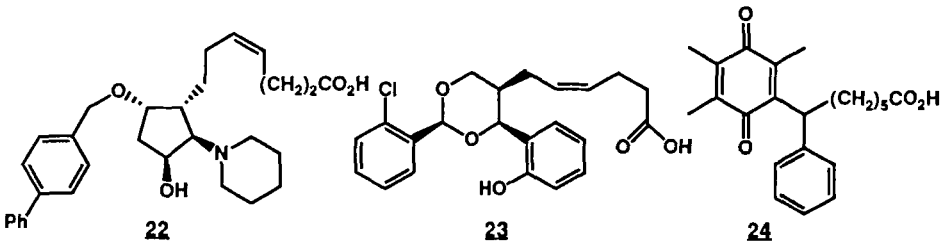
LTB_4 Antagonists - The role of LTB_4 in the inflammatory process of asthma, and the putative extra benefit that a 5-LO inhibitor might have over an LTD_4 antagonist, might be revealed by LTB_4 antagonists. U-73502 (20) given orally was shown to substantially inhibit the bronchoalveolar eosinophilia caused by antigen inhalation in sensitized guinea pigs (40), suggesting that the late-phase eosinophilia of human allergic asthma could be LTB_4 mediated. SC-41930 (21) is a new, orally-active LTB_4 antagonist whose synthesis and properties were described recently (41).

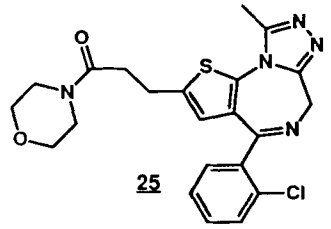
Thromboxane Antagonists - This general area is covered more fully in Chapter 11. The role of prostanoid constrictors in asthma remains uncertain, although thromboxane has been implicated in animal models of bronchial hyperreactivity. GR32191 (22) is a leading clinical TX antagonist, and was recently shown to be effective at 80 mg p.o. in inhibiting the bronchoconstriction caused by inhaled PGD_2 in asthmatics (42). However, its effect on the early constriction response to inhaled allergen was not particularly marked, and the conclusion was reached that histamine produces more of the early drop in FEV_1 than do prostanoid constrictors. ICI 192605 (23) has reached human studies and in ex vivo studies is a potent and orally-active inhibitor of U-46619 induced platelet aggregation (43). AA-2414 (24) has a pA_2 of 8.29 against U-46619 contractile responses of guinea pig parenchymal strips, and is active at 0.3 mg/kg p.o. in blocking IgG_1-induced bronchoconstriction in guinea pigs (44). It is now in clinical trial in asthma patients in Japan, reportedly showing beneficial effects at 40 mg. p.o.





PAF Antagonists - Platelet-activating factor (PAF) has attracted recent attention as a possible mediator of asthma because of its unique ability to produce pulmonary eosinophilia (guinea pigs) and bronchial hyperreactivity (man). This latter finding has recently been questioned, however (45). The PAF antagonist WEB 2086 (25) has been the best characterized agent in this area for some time; it has been shown to be well tolerated on semi-chronic oral dosing in man, and to show good ex vivo inhibition of PAF-induced platelet aggregation (46). A substantial review of the hetrazepine PAF antagonist work related to WEB 2086 has appeared (47). The identification was detailed of compound 26 as an orally-active antagonist of PAF-induced bronchoconstriction in guinea pigs (48). YM-461 (27) has a pA_2 of 7.29 against PAF-induced human

platelet aggregation and potent effects against PAF- and antigen-induced bronchoconstriction (49). SCH-37370 (28) is an intriguing new structural class of PAF antagonist which retains the H1 antagonism of its congeners, azatidine and loratidine, at PAF-inhibitory concentrations (50). The suggestion has been made that a number of prophylactic anti-asthma drugs act by functional antagonism of PAF-induced hyperreactivity (51). The PAF antagonist SDZ-64412 (29) was shown to block development of hyperreactivity in sensitized guinea pigs exposed to ovalbumin (52). The chemistry and pharmacology of the series containing RP-59227 (30) was concisely reviewed (53).



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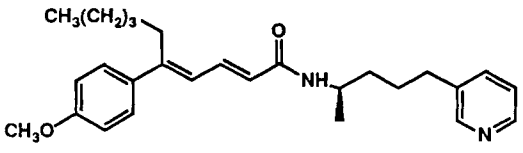

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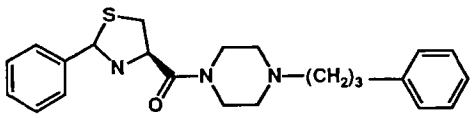

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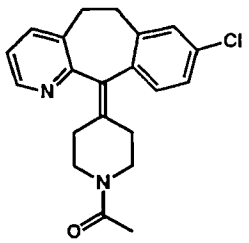

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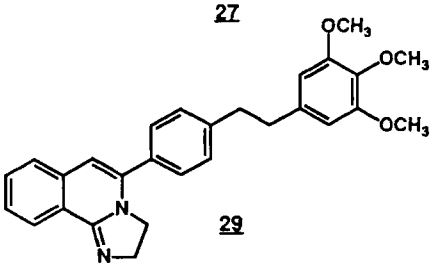

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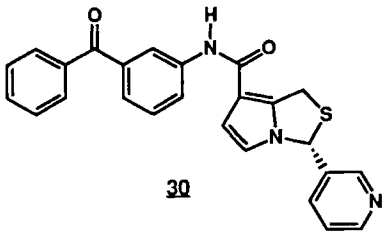

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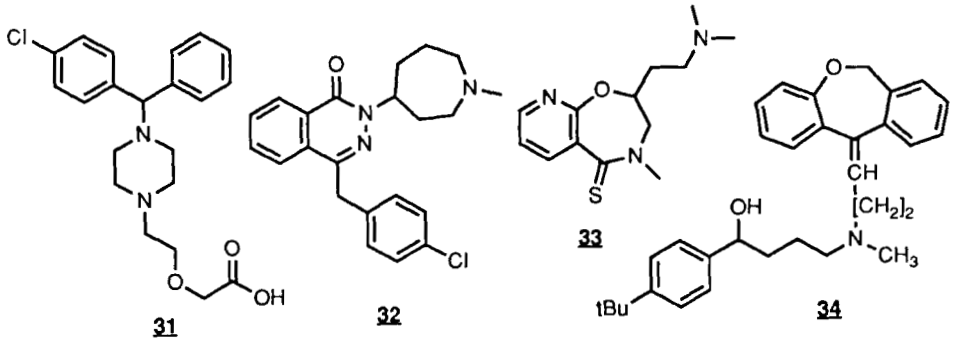
ANTIALLERGICS

Non-sedating Antihistamines - The traditional view that H1 antihistamines do not work in asthma is being questioned as the second wave of non-sedating compounds undergoes clinical trial (54,55). Pharmacological properties in addition to histamine antagonism are frequently invoked to support possible added clinical effects of the newer agents. Cetirizine (31) inhibited mediator release in a cutaneous late-phase response model (56), and azelastine (32) blocked granulocyte superoxide production in vitro at clinically relevant concentrations (57). Preclinical pharmacology on AHR-11325 (33) and PR1036-654 (34) has appeared (58,59); both compounds profile as potent, long-acting H1 antagonists.

Mediator Release Inhibitors - Further preclinical data have appeared for SCH-37224 (35), a potent antiallergic agent which prevents mediator release from anaphylactic guinea pig lung (60), and blocks both early and late responses to antigen challenge in allergic sheep after oral dosing (61). When dosed 50 mg orally to asthmatics, 35 was effective in blocking the bronchospasm of cold air isocapnic hyperventilation (62).



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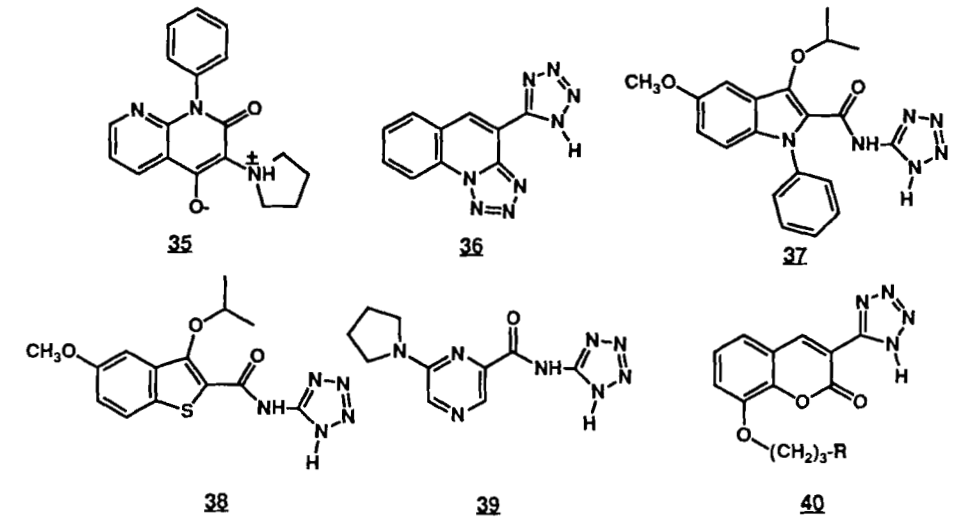
MDL-26024GO (36) is another mediator release inhibitor capable of blocking constriction in the allergic sheep model (63). CI-959 (37) appears to block release of multiple mediators from human leukocytes and mast cells, showing an IC_{50}~1μM for blockade of anti-IgE induced contraction of human bronchus (64). More complete chemical and pharmacological details on the related agent CI-949 (38) are available (65). HSR-6071 (39) is a highly potent new antiallergic, blocking IgE-mediated histamine release from rat peritoneal exudate cells with an IC_{50} of 0.46nM and showing potent activity in the rat PCA test (66). KP-136 (40, R=Et) is in clinical trial, and its major metabolite (40, R=CO_2H) retains activity in PCA and allergic asthma models (67).



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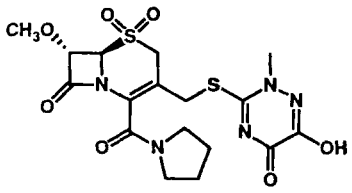
OTHER TOPICS

Nearly twenty-five years after the association of α_1-antitrypsin deficiency and genetic emphysema was noted, the American Thoracic Society endorsed the use of Prolastin, natural human α_1-antitrypsin, for i.v. augmentation therapy of genetic emphysema. Recombinant α_1-antitrypsin is unsuitable for i.v. administration, but the feasibility of aerosol delivery of sufficient material to the lower airways to restore protease-antiprotease balance has been demonstrated (68,69). The serine protease human neutrophil elastase (HNE) continues to be viewed as the prime

pathological agent in genetic emphysema, and its potential involvement in the lung damage of ARDS (70) and COPD (71) make it a target for intervention. One recent study evaluated drug effects on neutrophil chemotaxis or degranulation as a means of reducing lung HNE burden (72), whereas other work concentrated on active-site inhibitors of HNE. Intratracheal (i.t.) recombinant secretory leukocyte protease inhibitor (SLPI) inhibited HNE-induced damage to hamster lung (73). The modified β-lactam HNE inhibitor L-659286 (41) selectively inhibits HNE in a slowly reversible manner, has good lung residency after i.t. administration, and by this route blocks HNE damage to hamster lung (74). ICI 200,880 (42) shows long lung residency and potent ability to protect against HNE challenge after i.t. dosing (75). SC-39026 (43), a mixed non-competitive HNE inhibitor showed effects in a sheep model of ARDS suggestive of a protective action on elastase-mediated increases in microvascular permeability (76). X-ray crystal structures of HNE-inhibitor complexes and of inhibitor complexes with the related protease porcine pancreatic elastase have been discussed (77-80), and some new chemical approaches to serine protease inhibition were described (81,82). Almitrine (44) represents an alternative approach to therapy in COPD, and is now undergoing chronic studies in the U.S. (83). Further evidence of its ability to raise PaO_2 in hypoxemic patients was presented (84). In the area of PH, ligustrazine (45) prevents and reverses the pulmonary vascular changes caused by chronic hypoxia in rats (85) and an excellent overview of strategies for defending the lung from oxidative damage appeared (86). The CF gene was identified during the year (87) and further progress may be anticipated in understanding the epithelial ion transport deficit and abnormal mucus production in the disease. It has been known for some time that i.t. amiloride (46) can have a beneficial effect on mucus clearance (88) and there are indications the drug may be developed for use in CF (89).



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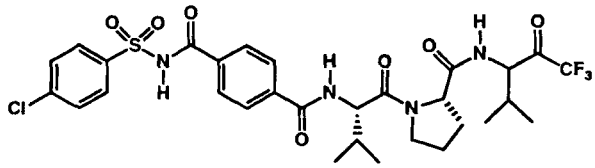

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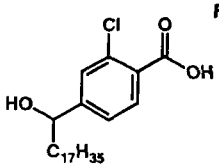

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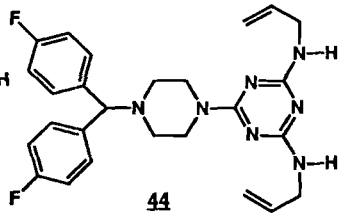

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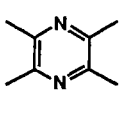

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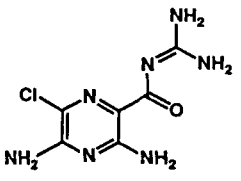

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Chapter 8. Treatment of Acute Myocardial Ischemia

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Introduction - Early reperfusion during acute myocardial infarction represents an effective means to limit tissue necrosis and preserve myocardial function (1,2). Restoring blood flow to ischemic tissue, however, may paradoxically have deleterious effects on reversibly injured cells within the region of the myocardium at risk of infarction (3,4). Consequently, with the development of thrombolytic agents and mechanical methods such as percutaneous transluminal coronary angioplasty (PTCA) for emergency revascularization, there is intense interest in identifying potential adjunctive pharmacotherapies which could limit the progression of tissue injury during reperfusion and enhance functional recovery of the postischemic myocardium.

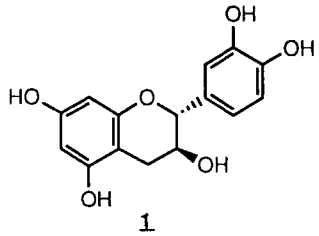
A number of important advances have been made toward understanding the mechanisms underlying ischemia-induced myocardial damage since the last review in this series on anti-ischemic agents eight years ago (5). The primary focus of investigation in this area over the past few years has been concerned with the importance of oxygen-derived free radicals, ion fluxes and the inflammatory response, in particular the role of the neutrophil, in mediating ischemia-induced cell injury and cardiac dysfunction. This chapter will summarize the important pharmacological approaches and agents reported in the recent literature for limiting acute myocardial ischemia and reperfusion injury.

Free Radicals - A brief period (< 20 min) of coronary artery occlusion followed by reperfusion results in a prolonged and eventually reversible depression of contractile function (myocardial stunning) (6). Although a number of causative mechanisms have been proposed, myocardial stunning is generally believed to be mediated in part by reactive oxygen species (7,8). The evidence for free radical involvement in postischemic contractile dysfunction and its clinical implications have been reviewed (9). A longer period of ischemia (20 to 60 minutes or longer) results in irreversible cell injury where the extent of tissue necrosis is related to the severity and duration of the ischemic episode. There is ample evidence to suggest that reoxygenation of the ischemic myocardium is accompanied by free radical generation from a variety of intracellular and/or extracellular sources, and that oxygen-derived free radicals may contribute to tissue injury. The role of free radicals and their possible contribution to reperfusion injury was reviewed in this series three years ago (10), and a number of reviews have since appeared in the literature (11-18). A review on coenzyme Q as a possible therapeutic agent in myocardial ischemia has also appeared (19).

Superoxide dismutase (SOD) was reported to reduce infarct size in an occlusion-reperfusion model of myocardial damage in the anesthetized dog (20), and was the first antioxidant or free radical scavenger to undergo clinical trials for treatment of acute myocardial infarction (21). Significant controversy exists, however, regarding the efficacy of SOD in experimental models of myocardial infarction (22-24). Since there may be a time window during which neutrophil-suppressive therapy must be administered for containment of ultimate infarct size quantitated several days after reperfusion (25), a potential complication in these models may be the relatively short pharmacologic half-life of both human recombinant and bovine erythrocyte SOD. Consequently, a short treatment period may not provide a sustained beneficial effect (26). Conjugation of SOD to PEG, however, was demonstrated to prolong the pharmacologic half-life of the enzyme from less than 10 minutes to greater than 30 hours, to provide sustained protection after 4 days of reperfusion, and to prevent rather than simply delay irreversible myocardial damage in canine models of myocardial infarction (27,28). Thus, effective therapy may be dependent on assuring that an appropriate concentration of the pharmacological agent be present in the circulation during the critical time period of reperfusion.

A number of agents have been described which exhibit beneficial effects in experimental models of myocardial stunning. These include the antioxidant cyanidanol-3 (1) and the iron

chelator 1,2-dimethyl-3-hydroxy-4-pyridone (**2**), both of which enhanced recovery of contractile function in postischemic buffer-perfused isolated rat hearts (29). Hydroxyethyl starch conjugated desferal also enhanced recovery of stunned myocardium in anesthetized dogs (30). In addition, captopril attenuated postischemic contractile dysfunction perhaps through a sulfhydryl-mediated free radical scavenging mechanism that was independent of ACE inhibition (31,32). A series of imidazol-2-thiones (e.g. **3**) structurally related to the positive ionotropes enoximone and piroximone reportedly improved contractility in stunned canine myocardium independent of ionotropic activity (33).



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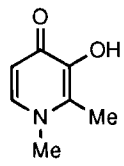

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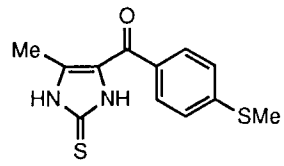

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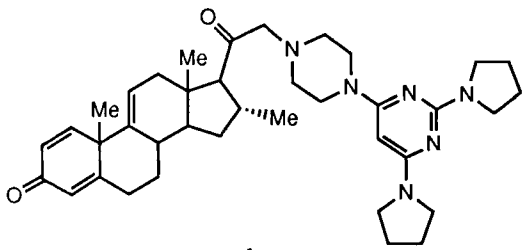
Since cell membrane phospholipids are particularly vulnerable to free radical mediated damage which results in increased membrane fluidity and permeability, agents which protect cell membrane structure and function during oxidative stress may limit ischemia-induced cellular injury (34). The lipid peroxidation inhibitor U74006F (**4**) enhanced functional recovery of stunned canine myocardium, without affecting systemic hemodynamics or myocardial blood flow (35).



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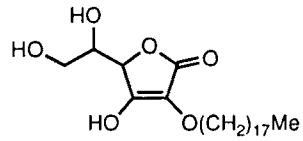

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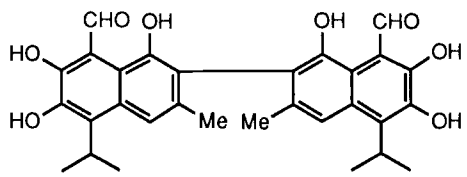

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Other lipid peroxidation inhibitors recently described include a series of 2-O-alkylascorbic acids (36). A free 3-hydroxyl group and a straight alkyl chain consisting of 11-20 methylene units on the 2-hydroxyl moiety were optimal for inhibiting lipid peroxidation in vitro. One of the most potent representatives from this series, 2-O-octadecylascorbic acid (CV-3611, **5**), decreased the incidence and duration of ventricular fibrillation following oral administration to a rat model of myocardial ischemia and reperfusion. This agent also reduced ultimate infarct size orally in dogs subjected to 60 minutes of coronary artery occlusion followed by 14 days of reperfusion (37). When administered intravenously just prior to reperfusion, CV-3611 also reduced infarct size and neutrophil infiltration in the area of myocardium at risk of infarction (38).

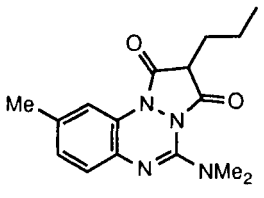
Gossypol (**6**), a polyphenolic component of cottonseed oil, was also recently reported to inhibit peroxidation of myocardial membrane phospholipid (39,40).

Increasing endogenous myocardial defense mechanisms against oxidative stress has been reported to reduce postischemic abnormalities in isolated rat hearts. For example, exposure to elevated temperatures is known to induce both cultured cells and whole animals to synthesize a

number of highly conserved proteins (heat-shock response) (41). Although the precise function of these heat-shock proteins is not completely understood, cells that synthesize and accumulate these substances are more resistant to oxidative stress. Heat-shocked isolated rat hearts subjected to 30 minutes of low-flow ischemia possessed elevated catalase activity which, upon reperfusion, exhibited enhanced recovery of function relative to control hearts (42). Endotoxin pretreatment also resulted in elevated catalase activity in rat myocardium which was accompanied by an increase in myocardial function after ischemia and reperfusion (43). These observations suggest that endogenous catalase levels may influence susceptibility to ischemia-induced myocardial injury.

Endogenous myocardial glutathione (GSH) may also play an important protective role in the heart. Enhanced myocardial GSH content by continuous intravenous GSH infusion was associated with a reduction in infarct size in pigs subjected to 45 minutes of coronary artery occlusion and 2 hours of reperfusion (44). Treatment with N-acetylcysteine, on the other hand, did not reduce infarct size in dogs subjected to 90 minutes of coronary artery occlusion and 24 hours of reperfusion (45), although an improvement in ventricular function was observed which may have been due to direct free radical scavenging by this agent (46). A 65% depletion of GSH with diethylmaleate impaired functional recovery of buffer-perfused isolated rat hearts subjected to ischemia and reperfusion (47). However, a 70% GSH depletion with L-buthionine-S,R-sulphoximine had no apparent adverse effect on cardiac energy metabolism during total global ischemia followed by reperfusion (48).

Neutrophils - Reviews on the role of infiltrating leukocytes and the inflammatory response (24,49,50), as well as the relationship between neutrophils and platelets (51) in postischemic myocardial injury have appeared. Activated neutrophils release a variety of mediators that may contribute to ischemia-induced myocardial damage (52,53). Leukocytes may also exacerbate ischemic injury through capillary plugging in the coronary microcirculation (no-reflow phenomenon). Although neutrophil depletion by extracorporeal filtration initiated at the time of reperfusion reduced canine infarct size (54), the role of the neutrophil in myocardial stunning is presently unclear (55-58). A recent clinical study examined several aspects of neutrophil function in patients with stable angina, unstable angina and acute myocardial infarction (59).



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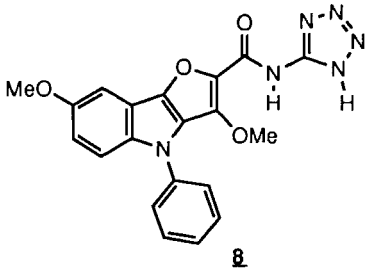

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Adenosine has been proposed to regulate the ischemia-induced inflammatory reaction in the microcirculation by linking ATP catabolism with inhibition of granulocyte activation and adherence (60). Intracoronary adenosine administration reduced infarct size (61) and attenuated microvasculature abnormalities in canine models of regional myocardial ischemia and reperfusion (62). In addition, augmenting the local concentration of adenosine with 5-amino-4-imidazole carboxamide (AICA) riboside pretreatment decreased granulocyte accumulation and increased collateral flow in the myocardium of dogs subjected to 60 minutes of coronary artery occlusion (60). Enhanced adenosine release by α_1-adrenoceptor stimulation also attenuated myocardial stunning in dogs (63). Several agents which inhibit functional responses of neutrophils have recently been reported to reduce infarct size in canine models of ischemia-induced myocardial damage. These include azapropazone (7), Fluosol-DA and CI-922 (64-66). The protective effect of CI-922 (8) was attributed to suppressed neutrophil release of various mediators including activated oxygen species, lysosomal enzymes and leukotrienes.

Lidocaine has been shown to inhibit neutrophil function in vitro (67,68) and more recently, to decrease mitochondrial dysfunction and leakage of lysosomal enzymes in a canine model of permanent coronary artery occlusion (69). However, lidocaine pretreatment (beginning 30 minutes before coronary occlusion) in dogs subjected to 2 hours of regional myocardial ischemia and 6 hours of reperfusion, failed to reduce neutrophil accumulation or infarct size (70). In

contrast, lidocaine pretreatment (beginning 90 minutes before coronary occlusion) in dogs subjected to a shorter period (90 minutes) of regional myocardial ischemia and 5 hours of reperfusion, limited infarct size without reducing neutrophil accumulation in the area at risk (71). Since lidocaine reduced coronary sinus levels of conjugated dienes, a potential marker of lipid peroxidation, the protective effect by this drug was attributed to cell membrane stabilization.

The monoclonal antibody 904 which binds to and interferes with the function of the alpha subunit of the leukocyte adhesion-promoting glycoprotein CD11b/CD18, reduced myocardial infarct size when administered to dogs subjected to 90 minutes of coronary artery occlusion followed by 6 hours of reperfusion (72). Circulating neutrophil count was not different from controls, but neutrophil accumulation within the myocardium was significantly attenuated when assessed histologically. In a subsequent study, myocardial infarct size was reduced in dogs treated with the $F(ab')_2$ fragment of 904 after 90 minutes of coronary occlusion and 72 hours of reperfusion (73). The $F(ab')_2$ fragment was ineffective for reducing the prolonged ventricular dysfunction associated with a short period of regional ischemia in a canine model of myocardial stunning (55).

The role of arachidonic acid metabolites in myocardial ischemia and reperfusion injury has been reviewed (74,75). The nonsteroidal antiinflammatory drugs indomethacin and aspirin were recently shown to have detrimental effects manifested by a further decrease in contractility and increase in coronary perfusion pressure in isolated buffer-perfused rabbit hearts subjected to low-flow ischemia and reperfusion (76). Stimulation of prostaglandin synthesis by defibrotide had a beneficial effect, suggesting a protective role for PGI_2 and PGE_2. In a canine model of myocardial stunning, iloprost improved postischemic contractile function (77). PGI_2 (78), iloprost (79,25), PGE_1 (80) and defibrotide (81) have also been shown to reduce infarct size in animal models of ischemia-induced myocardial damage, effects which may be related to reduced neutrophil activation. Extended inhibition of neutrophil activation and accumulation (between 2 and 48 hours of continuous iloprost infusion), however, was necessary for a reduction in ultimate infarct size quantitated 72 hours post-reperfusion (25).

Thromboxane - A number of thromboxane receptor antagonists and thromboxane synthetase inhibitors have been recently reported to exhibit beneficial effects in experimental models of myocardial ischemia (see Chapter 11). These include the thromboxane receptor antagonists BM-13,505 (82-85), SQ-30,741 (86) and AH-23,848 (87) as well as the thromboxane synthetase inhibitors OKY-046 (88), HOE-944 (89), CV-4151 (90) and RS-5186 (91). In particular, CGS-13080 (a thromboxane synthetase inhibitor) in combination with streptokinase enhanced reperfusion and prevented reocclusion in a canine model of coronary artery thrombolysis (92). Moreover, CGS-13080 in combination with t-PA synergistically attenuated creatine kinase release and reduced infarct size in cats subjected to 2 hours of coronary artery occlusion followed by 4 hours of reperfusion, whereas neither tPA nor CGS-13080 alone was effective for reducing tissue damage (93). Dazmegrel (a thromboxane synthetase inhibitor) but not BM-13,505 (a thromboxane receptor antagonist) enhanced postischemic recovery of canine myocardial function (94). Thus, PGI_2 elevation through metabolic precursor shunting (endoperoxide shunting) rather than thromboxane reduction *per se* may underly the beneficial effects of thromboxane synthetase inhibitors in this model of myocardial stunning (95). In addition, thromboxane synthetase inhibitors may reduce infarct size through a platelet-dependent, aspirin-sensitive mechanism (96). Thromboxane receptor antagonists and thromboxane synthetase inhibitors are reviewed in greater detail in Chapter 11 of this volume.

Leukotrienes - The peptidoleukotriene receptor antagonist SK&F-104,353 did not protect against ischemia-induced myocardial damage or reduce infarct size in rat models of coronary artery occlusion-reperfusion (97,98). However, in dogs subjected to 2 hours of coronary artery occlusion and 5 hours of reperfusion, the peptidoleukotriene receptor antagonist ONO-1078 and the lipoxygenase inhibitor AA-861 reduced myocardial infarct size (99).

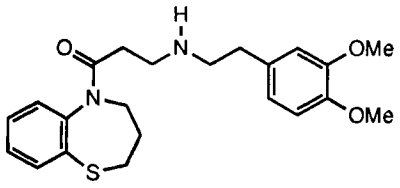
Calcium - The role of calcium (100,101) and calcium antagonists as potential adjunctive agents during reperfusion of ischemic myocardium have been reviewed (102). A discussion on the therapeutic potential of cytoprotective calcium antagonists with little or no primary hemodynamic effects has also appeared (103). Studies with buffer-perfused isolated rat hearts suggest the presence of contraction dependent and contraction independent routes of reperfusion-induced calcium entry (104), and that interventions introduced at the time of reperfusion might only delay rather than abolish reperfusion-induced calcium gain (105). Low concentration calcium

reperfusion has been investigated but with variable results (106).

Pretreatment with diltiazem reduced infarct size in a canine model of myocardial occlusion and reperfusion (107). More recently, intracoronary administration of diltiazem, but not verapamil or nifedipine, at the moment of reperfusion reduced infarct size in pigs subjected to 45 minutes of occlusion followed by 3 days of reperfusion (108). The salutary effects of diltiazem may be related to reduced myocardial lipid peroxidation (109). Verapamil administered either before coronary artery occlusion, at the moment of reperfusion or 30 minutes after reperfusion reduced myocardial stunning in dogs (110). Low-dose intracoronary nifedipine administered after reperfusion also enhanced postischemic contractile function in the absence of systemic hemodynamic effects (111).

When infused 30 minutes after occlusion, nisoldipine reduced myocardial infarct size independent of myocardial oxygen demand in cats subjected to 2 hours of coronary artery occlusion and 4 hours of reperfusion (112). If administered 90 minutes after occlusion, no protection was observed.

Pretreatment with the intracellular calcium antagonist KT-362 (9) reduced infarct size and myocardial stunning in canine models of ischemia-reperfusion (113,114). The relevant beneficial effects of this agent were attributed to a reduction in myocardial energy and oxygen demand.



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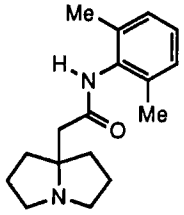
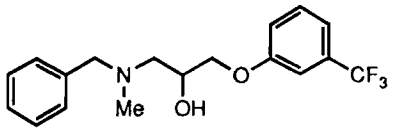


10

Platelets - Reviews on platelet-endothelium interactions in the pathophysiology of myocardial ischemia (115) and the relationship between platelets and thrombolysis (116) have appeared. Recent studies suggest that PAF is released during reperfusion of isolated ischemic rabbit hearts (117), and may contribute to ischemia and reperfusion-induced arrythmias in anesthetized dogs (118). In a preliminary report, the PAF antagonists BN-52021 and CV-3988 reduced infarct size in dogs subjected to 90 minutes of coronary artery occlusion followed by 3 hours of reperfusion (119).



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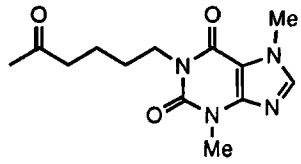
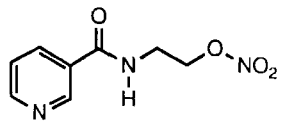


12

Other Agents - In a canine model of permanent coronary artery occlusion, the sodium channel blocker SUN-1165 (10) reduced ischemia-induced myocardial damage assessed by mitochondrial dysfunction and lysosomal enzyme leakage (120), while the PLA_2 inhibitor, 11, limited canine infarct size when assessed 6 hours post-occlusion (121). The hemorrheological agent pentoxifylline (12), however, was ineffective in reducing infarct size or enhancing collateral blood flow in dogs following 6 hours of coronary artery occlusion (122). Nicorandil (13) exhibited beneficial effects on metabolic and functional recovery of stunned canine myocardium (123) without increasing PGI_2 levels (124), and reduced infarct size in dogs subjected to 6 hours of coronary artery occlusion (125). Pinacidil (14) and cromakalim (15), two agents that activate ATP-dependent potassium channels, reduced myocardial infarct size when administered to dogs via the intracoronary route before the induction of 90 minutes of regional ischemia and 5 hours of reperfusion (126). The protective effects were not due to increasing collateral blood flow to the ischemic myocardium.



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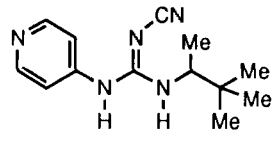

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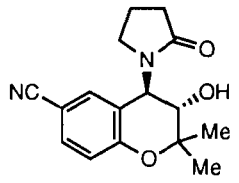

15

Conclusions - Many of the biochemical and functional abnormalities associated with myocardial ischemia and reperfusion are actively being studied experimentally. A number of agents appear to improve postischemic contractile function and/or reduce infarct size in experimental models. These encouraging results suggest that ischemia-induced myocardial injury may be further attenuated by appropriate pharmacological intervention in conjunction with early reperfusion. The role of oxygen-derived free radicals and the efficacy of SOD in myocardial ischemia with evolving infarction in humans may be clarified as clinical trials progress. Newer pharmacological interventions aimed at inhibiting neutrophil functional responses during the process of myocardial infarction may offer the most promise for the future pharmacotherapy of this disease.

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Chapter 9. Antiarrhythmic Agents

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Introduction - Class I and Class III anti-arrhythmic drugs were last reviewed in this series 4 years ago (1). Since that time other reviews on the use of these agents and their mechanism of action have appeared (2, 3, 4). However, the recent results from the Cardiac Arrhythmia Suppression Trial (CAST) have markedly affected the way some Class I compounds are now viewed. This review therefore will focus on the CAST study and its implications, and will then give an update on the Class I and Class III agents.

The CAST Study - After a myocardial infarction (MI), there is an increased risk of death from arrhythmia. Although many factors contribute to this risk, suppression of spontaneous arrhythmias after MI was thought to reduce the incidence of sudden cardiac death (SCD). The CAST study (5) was designed to test the hypothesis that suppressing asymptomatic premature ventricular beats with Class I anti-arrhythmic drugs would prolong survival. The three drugs studied (encainide, flecainide and moricizine) were chosen because they already had been shown to suppress premature ventricular beats in post MI patients. Other anti-arrhythmic drugs, such as quinidine, procainamide, disopyramide mexilitene, and tocainide were not used because previous studies suggested that they did not suppress arrhythmias as effectively, or that adverse effects precluded their long-term use in most patients.

Patients were entered into the trial between six days and two years after MI, and 2309 people were recruited for the initial drug-titration phase. Of these 1727 (75%) had demonstrable suppression of their arrhythmias through use of one of the three study drugs, and were then randomly assigned to receive either active drug or placebo.

After an average of 10 months treatment, the patients receiving either encainide or flecainide had a significantly higher mortality rate than patients assigned to placebo (7.7% compared to 3.0%). The exact causes of the deaths associated with encainide and flecainide treatment were not always known, but death from arrhythmia, as well as from non-arrhythmic cardiac disease were more common in the treated group. Because of these results, the treatment groups receiving encainide and flecainide were immediately discontinued. No significant difference was observed between moricizine and its placebo control. Accordingly, the Data and Safety Monitoring Board recommended that the study with moricizine continue.

It has long been known that some anti-arrhythmic drugs used to suppress arrhythmias can actually cause them (6), but the frequency and clinical implications of these pro-arrhythmic effects have only recently been appreciated (7, 8). Encainide and flecainide are drugs with a Class IC anti-arrhythmic mode of action (2, 4), i.e. they produce marked slowing of conduction and have little effect on action potential duration. Many deaths in the CAST study appear to have been due to pro-arrhythmic events, and the primary risk factors identified were, structural heart disease, sustained ventricular tachycardia, in-patient initiation, and dose escalation. The CAST researchers concluded that neither encainide nor flecainide should be used in patients with asymptomatic, or minimally symptomatic, ventricular arrhythmias following MI, even though these drugs may be effective in initially suppressing ventricular arrhythmias. It was speculated that because these two drugs had such a profound slowing effect on conduction velocity, under certain conditions this could facilitate re-entry and induce a lethal ventricular arrhythmia.

The consequences of CAST are under review and, since encainide and flecainide are potentially fatal in a particular group of patients, questions are being asked with regard to other classes of anti-arrhythmic drugs. However, CAST was the first large controlled study to examine

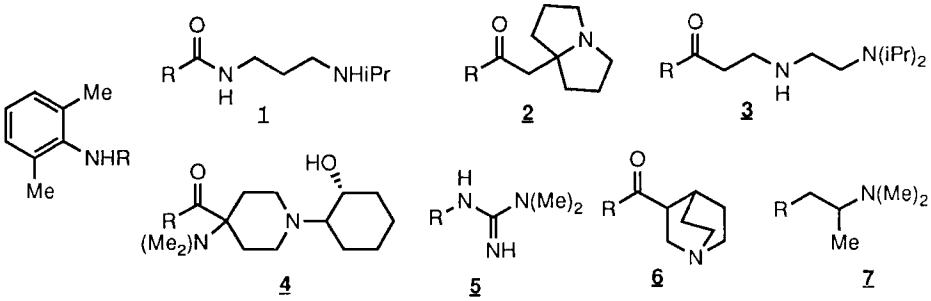
the effect of this type of drug upon survival, and there is no good information about effects of agents other than those of the Class IC sub-type. However, disopyramide (Class IA) did not reduce mortality in patients at relatively high risk when given for periods up to 14 days after MI (9), and mexiletine (Class IB) did not have a favorable effect on mortality after MI in a small trial (10). In both of these studies, although drug treatment was associated with a suppression of ventricular arrhythmia, there was a trend towards a worse prognosis (11).

The results from CAST have led the FDA's cardiorenal advisory committee to recommend that the CAST conclusions now be extended to cover all Class IC drugs. The committee suggested that the study results should also appear in some manner as a warning on all Class IA and IB anti-arrhythmic drugs - including quinidine, procainamide, disopyramide, lidocaine, tocainide and mexiletine. In addition, it was suggested that the labelling of all Class IA and IB drugs include the statement that "no benefit with respect to mortality has been shown for any anti-arrhythmic drug" (12).

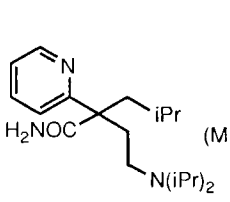
The CAST results emphasize the fact that clinical judgements made in the absence of large, well controlled, studies are often incorrect. Also, it demonstrated that the side-effects of some Class I anti-arrhythmic drugs are significant, and sometimes even fatal (13).

Class I Agents - The Class I antiarrhythmic agents pirmenol, cibenzolin and ethmozine (moricizine), the IC agents encainide, flecainide and propafenone, and the IB agents mexiletine and tocainide have been reviewed (2). All Class I drugs are Na^+ channel inhibitors and they decrease the fast inward Na^+ current; they reduce conduction velocity (V_{max}) in those cardiac structures in which resting potential is negative to -60 mV. The sub-classes differ with respect to their mode of action on Na^+-channels and in their effects on action potential duration (APD) (4).

A number of compounds possessing the 2,6-dimethylanilide substructure of lidocaine have been reported. Recainam (Wy-42,362, **1**) is said to possess a novel cardiac cellular electrophysiological profile since it has characteristics of all three Class I anti-arrhythmic subclasses (14). Results on SUN-1165 (**2**) suggest that the compound is likely to be of value in correcting not only ventricular but also supraventricular tachyarrhythmias (15). In addition, **2** was reported to have a cardioprotective effect on the ischaemic heart (16). Studies on AN-132 (**3**) suggest a high therapeutic potential, with the drug having slower offset kinetics than lidocaine (17, 18). Furthermore, the reported anti-cholinergic effects of **3** appear to be very specific for cardiac muscarinic receptors since the compound did not antagonize acetylcholine-induced contractions of intestinal smooth muscle (19). Transcainide (R-54,718, **4**) abolished ventricular arrhythmias in patients after intravenous or oral administration, but side effects, observed in 6 out of 41 patients, included Mobitz Type I AV-block, complete bundle branch block, hypotension and increased frequency of arrhythmias (20). It was recommended that **4** be used with caution in patients with conduction disorders and impaired cardiac function. Although TYB-3823 (**5**) shares certain electrophysiological characteristics normally associated with Class IA, IB and IC drugs (21), other mechanisms of action such as blockade of α and β-adrenoreceptors, and interference with the Ca^{++} intracellular stores, may account for the drug's actions (22). EO-122 (**6**) blocks both the fast inward Na^+ current and the slow inward Ca^{++} current at therapeutic concentrations (23). Electrophysiological studies on GYKI-23107 (**7**) in rabbit and canine ventricular muscle and Purkinje fibres have categorised the compound as a Class IB agent (24).



The disopyramide structure continues to serve as a prototype for new Class I agents. Pentisomide (CM-7857, **8**) had an acute haemodynamic profile similar to that of disopyramide, but the negative inotropic effect at equipotent doses was much reduced and was of shorter duration. However, the findings indicated that the drug has to be administered cautiously, and in low doses, to patients with reduced left ventricular function (25). FPL-13,210 (**9**) also profiled similar to disopyramide but was without the anti-cholinergic effects and hemodynamic liabilities of the latter (26). The cardiovascular profile of the Class IA/IB agent actisomide (SC-36602, **10**) has been compared to that of several marketed Class I drugs at equipotent anti-arrhythmic doses in anaesthetised dogs. It was reported that **10** produced no significant negative inotropism, and had no adverse haemodynamic side effects at its therapeutic dose (27), so indicating an advantage over the currently used agents. The cardiovascular pharmacology of indecainide (**11**) has been studied in intact animals, and the autonomic effects were reported as being slight, with no adverse effects on peripheral haemodynamics, QT interval, or the CNS (28).



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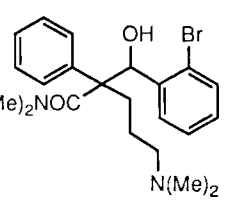

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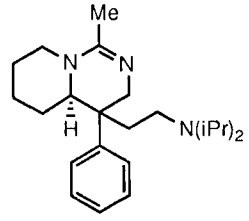

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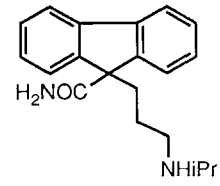

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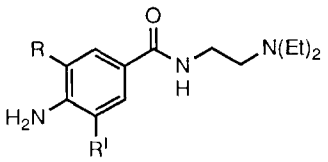
The widely used Class IA drug procainamide (**12**) has therapeutic limitations which include a short half-life, complex pharmacokinetics, production of autoimmune antibodies, and a lupus-like syndrome. The rate of generation of its major metabolite, N-acetylprocainamide (NAPA) is under genetic control and at least half of the patient population are rapid acetylators. In an attempt to overcome these drawbacks, analogues of procainamide possessing one (**13**) or two (**14**) methyl substituents ortho to the 4-amino substituent were synthesized (29). It was considered that this structural modification might eliminate the autoimmune toxicity, since the cytochrome P-450 derived metabolites of procainamide have been implicated in this process (30). It was found that the electrophysiological characteristics of procainamide were favorably altered by the addition of the methyl substituents, N-acetylation was prevented, and the plasma half-life was increased, as was potency against ouabain-induced arrhythmias in vivo. However, since there is no relevant animal model for procainamide-induced lupus, only clinical trials will be able to ascertain the degree of autoimmune mediated toxicity.

Ethacizin (**15**) is some 2-3 times more potent, and 4-5 times longer acting, than its structural analogue ethmozine, and its major metabolites have been isolated from human urine (31). In patients, ethacizin was well tolerated, and showed good efficacy in reducing ventricular premature beats (32). The pharmacological profile of AHR-10718 (**16**) was shown to be similar to that of disopyramide, procainamide and SUN-1165, but in the dog the correlation coefficients between the plasma concentrations of **16** and the anti-arrhythmic effect was not marked (33).



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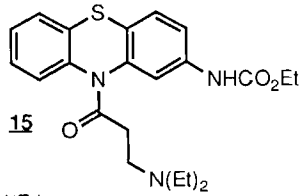
R = Me, R^1 = H **13**
R = R^1 = Me **14**



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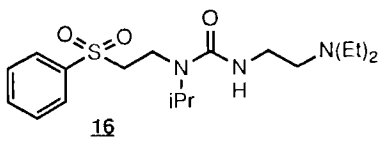

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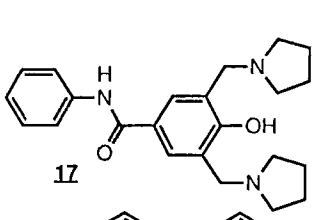
ACC-9358 (**17**), a compound related to changrolin, is an orally active Class IC agent with a profile similar to that of flecainide, but having less negative inotropic activity and devoid of CNS side effects (34). A series of ester derivatives of **17** have been described with very short plasma half-lifes, and biologically inactive metabolites, the aim of the work being to produce a quick onset/offset drug suitable for intravenous use in an acute situation (35). The Na^+ channel blocking properties of flecainide have been investigated in isolated guinea-pig papillary muscle, and it was concluded that the drug was primarily an open channel blocker, since its actions were independent of APD or the time period of inactivation (36). E-0747 (**18**) has been classified as a IC anti-arrhythmic agent since it depressed V_{max} by closely associating with open and/or inactivated Na^+ channels (37). The electrophysiology of AFD-21 (**19**), and its active metabolite AFD-19 (**20**), shows that while the effects of **19** on V_{max} are APD dependent, those of **20** are not, suggesting that **19** and **20** block open and inactivated Na^+ channels respectively (38). In clinical terms this implies that **19** may be effective against early and late ventricular extrasystoles, whilst **20** may be expected to be effective against atrial and ventricular tachycardias. In patients carocainide (**21**) increased sinoatrial conduction time, and QRS duration. Sinus rate, and sinus node recovery time, were not influenced, and the refractory period was unchanged. No major adverse effects were observed, and carocainide was effective in the short term treatment of patients with paroxysmal supraventricular tachycardias (39).



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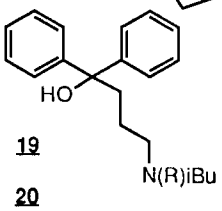

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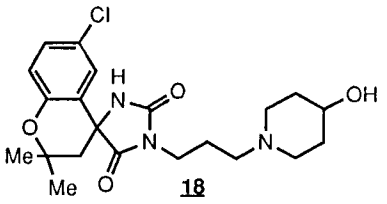

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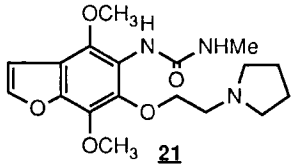
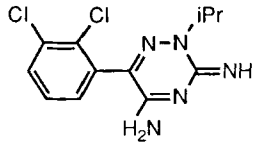


21

Following an observation that certain phenyldiamino-1,2,4-triazines had anticonvulsant activity, attributable to their ability to modulate neuronal Na^+-transport, it was established that these agents also possessed Class I anti-arrhythmic activity (40). Thus, BW A256C (**22**) was identified as being significantly more potent than quinidine, lidocaine and flecainide in reducing V_{max} in guinea-pig ventricle and dog Purkinje fibres. In dogs **22** caused a dose-dependent suppression of induced ventricular ectopic activity, with no evidence of peripheral or CNS toxicity. However, plasma levels of **22** only 3-4 times greater than the required anti-arrhythmic level produced pro-arrhythmic effects. Electrophysiology studies on nicainoprol (RU-42,924, **23**) proved that the compound was a typical Class I agent, but in addition had a minor inhibitory effect on the slow inward Ca^{++}-current (a Class IV effect) (41). In dogs, the pharmacological profile of nicainoprol was similar to that of aprindine and propafenone; CNS effects, including vomiting, were observed (42). Other agents with the β-antagonist pharmacophore continue to be investigated, and 711,389-S (**24**) elevated the adenylate energy charge and phosphorylation potential in the guinea-pig myocardium (43). Studies of the electrophysiological effects of E-3753 (**25**) in guinea-pig papillary muscle showed that the compound exhibited Class IC activity (44) In order to avoid the cardiodepressant effects of the Class I agents, it has been suggested that drug combinations from different Class I subgroups be used (45). Mexiletine (Class IB) and propafenone (Class IC) have been shown to be just such a synergistic combination, affecting only the onset kinetics of use-dependent V_{max} block, without detracting from the characteristics of propafenone (46).



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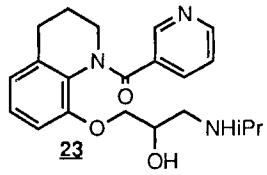

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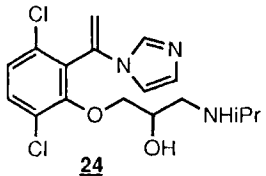

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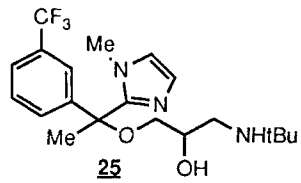


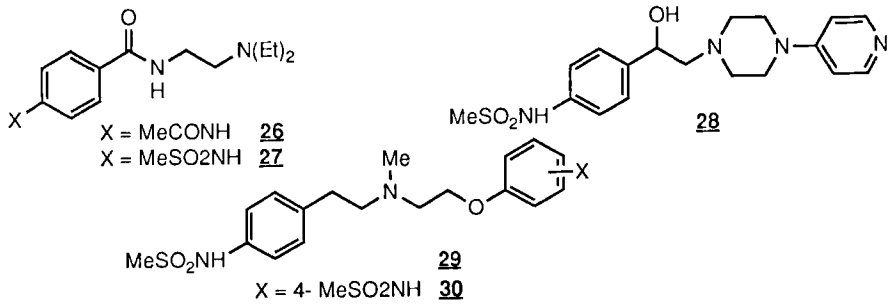
25

Class III Agents - Class III agents exert their primary anti-arrhythmic activity by prolonging cardiac action potential duration, and thereby the effective refractory period (ERP), with no effect on conduction. These electrophysiological changes are brought about by blockade of cardiac potassium channels; this mechanism is not associated with depression of the contractile function of the heart and may result in a slight enhacement of contractile force (47). The combination of pharmacological properties associated with a Class III agent increases the probability that a reentrant impulse, during its circuit, will encounter tissue that is still refractory, thus causing the arrhythmia to be extinguished and normal sinus rhythm to be restored. These agents increase refractoriness homogeneously across the whole myocardium, and should therefore be effective against arrhythmias whether ventricular, supra-ventricular or junctional in origin.

Since the last review (1), no new Class III agents have been launched to join the only two marketed agents bretylium (i.v. only) and amiodarone (i.v. and p.o.). Updates on the use of amiodarone continue to appear, including a report on its clinical efficacy versus malignant arrhythmias in patients (48). A recent study of ventricular fibrillation survivors who were placed on amiodarone therapy showed there was a reduced survival for the group of patients who discontinue amiodarone therapy because of side effects (49). Pulmonary toxicity is potentially the most severe adverse effect, affecting 5-10% of patients. Clinical studies using lower doses of amiodarone, to minimise side effects, are showing promising results in the control of ventricular arrhythmias (50). The β-blocker sotalol has also demonstrated anti-arrhythmic efficacy (51) but its utility is compromised by its β-blockade. When resolved it was found that the d-isomer retained Class III activity while having a lower affinity for β-receptors than the racemate (52). A 2 mg/kg infusion of d-sotalol, in patients undergoing programmed electrical stimulation (PES), caused an increase in QTc and refractoriness, but with a decrease in heart rate and also prevented the induction of ventricular tachycardia in 18/38 patients (53).

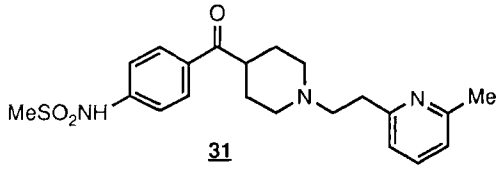
Because existing Class III agents are limited in their use by additional pharmacological activities, lack of good oral bioavailability, or a poor side effect profile, there have been intense efforts to discover new orally active, selective Class III anti-arrhythmic agents. Procainamide (**12**) is a relatively pure Class I agent, but its N-acetyl derivative **26** is primarily a Class III agent. It was found that replacing the labile N-acetyl group with the metabolically stable methanesulphonyl group (as found in sotalol) gave a potent and selective Class III agent sematilide (CK-1752, **27**). In vitro, sematilide (3.5 μM) caused a 20% increase in APD in canine Purkinje fibres without altering the the maximum rate of depolarisation. Similarly, at 17 μm a 20% increase in the functional refractory period in isolated canine ventricular muscle was measured; conduction times were not altered at concentrations up to 100 μm. These results show the compound to be a Class III agent, with no Class I activity over the concentration range measured (54). In a canine PES model, sematilide was also effective in preventing ventricular arryhthmias in 50% of the animals and was reported to be effective in a model of reentrant arrhythmias in the conscious dog. In dogs sematilide has 41-95% oral bioavailabilty with an elimination half life of 2.7 hr. (55). In patients with non-sustained ventricular arryhthmias, sematilide produced dose related increases in QTc, without altering PR, QRS or heart rate, thus providing electrophysiological evidence for Class III activity in man (56).

A sotalol analogue in which the isopropylamino function had been replaced by a 4-piperazinopyridine moeity has been reported (57). This compound (UK-66914, **28**) was markedly more potent than sotalol, producing dose dependent increases in APD and prolongation of ERP in canine venricular muscle and Purkinje fibre from a threshold concentration of 0.1 μm. At concentrations up 20 μm there was no effect on Vmax or the amplitude of the action potential, thus confirming the potency and selectivity (vs Class I) of **28** in vitro. In instrumented anaesthetised dogs **28** increased the ventricular fibrillation threshold by up to 6 fold; in conscious dogs the compound (0.25 - 1 mg/kg, p.o.) increased ventricular ERP by up to 17 ms and QTc by up to 43 ms. A more recent publication reports a series of achiral Class III anti-arryhthmic agents based on bis-arylalkylamines of the general structure **29** (58). The functional group X can be a variety of small polar functionalities; however both potency and selectivity are optimal where X is a second methanesulphonamido group attached specifically to the 4 position of the aryl ring. This compound, (UK-68798, **30**) concentration dependently increased the APD from canine ventricular muscle and Purkinje fibre in vitro over the concentration range 5 nm - 1 μm (59). The resting potential, amplitude and upstroke velocity remained unaffected. In isolated guinea-pig atria, the compound prolonged ERP at concentrations of 5 nm and above. These data indicate that in vitro **30** is a highly selective and potent (approximately 2,000 x sotalol) Class III agent. In anaesthetised dogs the compound increased both atrial and ventricular refractory periods dose-dependently; in addition the ventricular fibrillation threshold was elevated over the dose range of 3 μg/kg (74% increase in VFT) to 100 μg/kg (683% increase). No hemodynamic effects were observed at any of these doses (60). Compound **30** is orally active in the conscious dog (bioavailabilty = 72%, plasma half life = 4.6 hr) producing dose related increases in ventricular ERP and QTc, the only hemodynamic change being a slight increase in contractility (61).



E-4031 (**31**) prolongs APD and ERP in isolated guinea-pig ventricles at concentrations above 0.1 μm without altering Vmax (62,63). At doses above 3 μg/kg i.v. in anaesthetised dogs the compound increased QTc and ventricular ERP, and associated with this was an increase in cardiac contractility and a decrease in heart rate (64). In an anaesthetised dog model of sustained venticular tachycardia (SVT), **31** significantly decreased the incidence of SVT relative to control animals (62, 65). Again, a key structural feature of **31** is the methanesulphonamido- phenyl group, as it is in risitolide (Wy-48,986, **32**). The biological results show that risitolide and Wy-47,792 (**33**) are both Class III agents while the amino analogue Wy-47,804 (**34**) is clearly a Class I agent; these findings mirror the SAR reported for procainamide and sematilide (vide supra) (66). Risitolide produced a 50% increase in ventricular effective refractory period at 5 mg/kg i.v. in the anaesthetised dog. The same dose, in anaesthetised guinea pigs, is effective in preventing arrhythmias produced by coronary artery occlusion (67).

A benzopyran derivative RP-58866 (**35**) prolongs the cardiac APD and ERP in vitro without affecting upstoke velocity over the dose range 3-300 nm (68). In anaesthetised dogs, **35** dose dependently increased atrial, nodal and ventricular refractory periods. In the conscious dog the compound was ineffective against experimental arrhythmias produced by ischaemia, however it was effective (0.1-3mg/kg i.v.) in preventing atrial fibrillation induced electrically or by local application of acetylcholine. Compound **35** produced a mild, dose related bradycardia but was devoid of other pronounced haemodynamic effects below a dose of 1 mg/kg i.v. (68).



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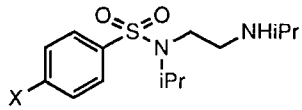
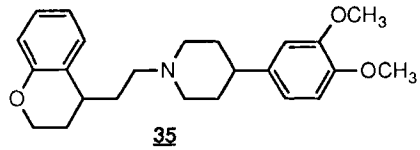


X = $MeSO_2NH$ **32**
X = NO_2 **33**
X = NH_2 **34**



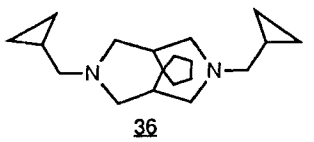
35

Agents with mixed activities - The bradycardic agent tedisamil (KC-8857, **36**) was reported to slightly prolong the APD in isolated guinea pig papillary muscle at 1 μm without affecting Vmax; an activity indicative of a Class III agent (69). However at 10 μm the APD shortened, as it did at all drug concentrations in isolated rabbit sino-atrial node. These results indicate the drug has a mixture of Class III/I activities, which is supported by the findings that tedisamil blocks both sodium and potassium currents (70, 71).

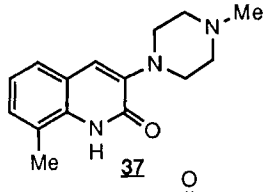
The 2-quinolone OPC-88117 (**37**) at concentrations above 3 μm causes dose-dependent increases in APD in isolated guinea pig papillary muscle; however it is not until the drug concentration exceeds 30 μm that Vmax is significantly decreased. These results, together with voltage clamp experiments, suggest that the compound has Class III activity and that at a higher (x10) concentrations it may also have Class IB activity (72). Similar conclusions were derived from electrophysiological studies in rabbit isolated hearts. The plasma concentration required to suppress ventricular arrhythmias in dogs was 1.7-3.3 μm (73).

In isolated guinea pig papillary muscles ,the piperazinoguanidine RS-87337 (**38**) (0.1-10 μm) prolonged APD and at higher concentrations (10-30μm) reduced Vmax. The mechanism of action of **38** was established as a mixture of Class III/IA on the basis of in vitro experiments in comparison with the standard agents flecainide, lignocaine, disopyramide and sotalol (74). In conscious dogs with a 2 stage coronary ligature, i.v.(3 mg/kg) and p.o.(30 mg/kg), **38** reduced ectopic ventricular complexes and i.v. reduced oubain induced arrhythmias (75).

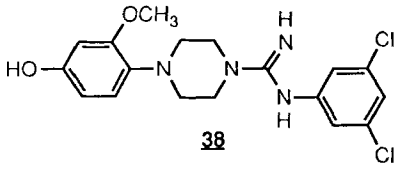
In the previous review in this series of anti-arrhythmic agents (1) the current literature suggested that the $5HT_3$ antagonist ICS205-930 (**39**) had Class I activity. A recent publication now reports that its effect on APD in guinea pig papillary muscle is similar to sotalol and that these effects are reversed by the potassium channel agonist cromakalim (76). The conclusion drawn from these new results is that ICS 205-930 inhibits potassium channels and therefore it is Class III activity that accounts for its anti-arrhythmic properties in dogs and rats.



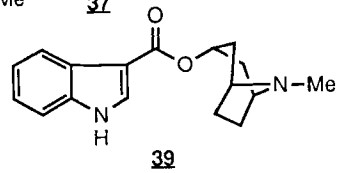
36



37



38



39

The aminoguanidine TYB-3823 (5) has been tentatively assigned as a Class I agent (21), however on the basis of its effect on cardiac action potentials in vitro and voltage clamp experiments (showing that at low concentrations the compound reduced the time dependent potassium current as well as blocking sodium channels) it is concluded that the compound has some Class III activity (77).

Finally, the psychotropic agent amperozide (2 mg/kg, i.p.) was compared with melperone, bretylium, lignocaine and thiorizide in a digoxin-induced arrhythmia model in guinea pigs. The conclusion that amperozide had Class III activity was based on its anti-arrhythmic effect being similar to that of melperone and bretylium (78).

Mechanism of action of Class III antiarrhythmic agents. The Vaughan-Williams classification of anti-arrhythmic agents is based on the electrophysiological effects of the various agents on action potentials. Thus a selective Class III agent will prolong APD without altering Vmax. However this electrophysiologic definition says nothing about the actual mechanism of action. Increasingly, the mechanism of action of Class III agents is being associated with the blockade of potassium ions associated with rectifying currents. Thus sotalol, (79), **30** (80), tedisamil (70), risotilide (81), and 35 (82), block a time dependent rectifier channel. This drug-sensitive channel has recently been characterised (83). Amiodarone blocks time dependent and time independent potassium channels as well as affecting sodium and calcium channels (84). Despite Class III activity becoming almost synonomous with potassium channel blockade, prolongation of APD without altering V_{max} can also result from prevention of inactivation of depolarising sodium channels as seen with S-DPI 205-430 (85). Nonetheless, these newer highly potent agents which are selective potassium channel blockers will help elucidate the mechanism of cardiac repolarisation and improve our understanding of the location, structure and gating mechanism of potassium channels. Recent publications give a further insight into the possible tertiary structure of the highly symmetrical tetrameric peptides that surround the potassium channel (86). Such studies are more advanced for the sodium channel where models of the tertiary structure (87) (deduced from the primary sequence) and gating mechanisms (88, 89) have been published.

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Chapter 10. Endogenous Vasoactive Peptides

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Introduction - In recent years, many endogenous vasoactive peptides have been isolated and characterized, several of which are involved in the regulation of cardiovascular and body fluid homeostasis (1,2). Some of these peptides, such as angiotensin II, vasopressin and atrial natriuretic peptide, function as hormones within the body, and modulation of their function has wide-ranging therapeutic implications (3-6). Endothelial cells also release substances that regulate vascular smooth muscle tone and platelet function (7-9). Many endothelium-dependent vasodilators act by formation of endothelium-derived relaxing factor (EDRF)(10,11). The recent discovery of endothelin-1, a potent vasoconstrictor peptide released from endothelial cells, has attracted great interest as a possible target for therapeutic intervention (12,13).

Although many other vasoactive peptides have been implicated in cardiovascular regulation, this chapter will focus on the newly discovered endothelin class of vasoactive peptides and on the atrial natriuretic peptide hormones; both types of peptides are of considerable interest at the present time.

ENDOTHELIN

Identification and Pharmacological Action - Endothelin is a 21-amino acid vasoconstrictor which does not belong to any previously described peptide class. It was originally discovered in the supernatant of cultured bovine aortic endothelial cells, and subsequently isolated from cultured porcine aortic endothelial cells (14,15). The primary structure of human endothelin has been deduced from a human placental cDNA library and found to be identical to that of porcine endothelin, now referred to as endothelin-1 (ET-1) (16-18). Numerous reports have described the effects of endothelin on the cardiovascular system. These actions are summarized in Table 1 and have been reviewed recently (19). In addition to its in vitro effects, infusion of endothelin to conscious, normotensive rats produces a sustained hypertensive response (20).

Table 1. Effects of endothelin on the cardiovascular system

Tissue/Organ	Effect	Reference
Vascular Smooth muscle	- long-lived constriction of isolated vascular muscle	12,21,22
	- mitogenic actions in cultured smooth muscle cells	23-25
	- release of endothelium-derived relaxing factor	26
	- coronary arterial vasoconstriction, increased perfusion pressure	27
Heart	- increased contractility	28-30
	- increased heart rate	31
	- stimulation of ANP release	32
Nervous Tissue	- enhanced neurotransmitter release	33

Endothelin-1 and Related Peptides - Since the initial identification of ET-1, two other related peptides have been reported and designated endothelin-2 (ET-2) and endothelin-3 (ET-3), differing by 2 and 6 amino acid residues respectively (34,35). All three forms appear to be distinct gene products (36). Two endothelin related genes were identified by cloning and sequence analysis of the mouse genome (37). One encoded the peptide ET-1, while the other encoded a new peptide differing by three amino acid residues. The gene for this novel peptide is only expressed in the intestine and has been referred to as "vasoactive intestinal contractor" (VIC) (37).

In addition to the differences in species distribution, variations in the ability of the different endothelins to constrict isolated vascular muscle and depress cardiac output following intravenous administration have been reported (34,38). This response may be due to differences in the ability of the endothelin peptides to stimulate the release of EDRF (26). Taken together, these results suggest the existence of multiple receptors for endothelin with different physiological responsibilities (39).

The sarafotoxins (SRTX's) isolated from the venom of the Israeli burrowing asp Atractaspis engaddensis (40), show a remarkable sequence homology with the endothelin peptides, suggesting a common evolutionary origin (41,42).

Table 2. Sequence comparisons among the endothelins and sarafotoxins

DISULFIDE BONDS

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
ET-1 (porcine, mouse human)	Cys	Ser	Cys	Ser	Ser	Leu	Met	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
ET-2	Cys	Ser	Cys	Ser	Ser	Trp	Leu	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
ET-3 (rat)	Cys	Thr	Cys	Phe	Thr	Tyr	Lys	Asp	Lys	Glu	Cys	Val	Tyr	Tyr	Cys	His	Leu	Asp	Ile	Ile	Trp
Mouse VIC	Cys	Ser	Cys	Asn	Ser	Trp	Leu	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
SRTX-a	Cys	Ser	Cys	Lys	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Asn	Phe	Cys	His	Gln	Asp	Val	Ile	Trp
SRTX-b	Cys	Ser	Cys	Lys	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Tyr	Phe	Cys	His	Gln	Asp	Val	Ile	Trp
SFTX-c	Cys	Thr	Cys	Asp	Asn	Met	Thr	Asp	Glu	Glu	Cys	Leu	Asn	Phe	Cys	His	Gln	Asp	Val	Ile	Trp
SRTX-d	Cys	Thr	Cys	Lys	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Asn	Phe	Cys	His	Gln	Asp	Ile	Ile	Trp

Mechanism of Action - Although a direct involvement of endothelin with the slow calcium channel was originally postulated by Yanagisawa and co-workers (15), subsequent studies have demonstrated that the vasoconstrictor response to endothelin can be observed in calcium-free conditions, and that in certain tissues calcium channel blockers have little effect on the response to endothelin (43,44). In addition, endothelin binding sites can be disassociated from the binding sites of the calcium channel stimulant PN 200-110 by sucrose gradient ultracentrifugation, and the solubilized endothelin binding protein is not immunoprecipitated with a monoclonal antibody that recognizes the dihydropyridine-sensitive calcium channel complex (45). The response to endothelin is also not blocked by a variety of receptor antagonists and enzyme inhibitors, including phentolamine, nordihydroguaiaretic acid, atropine, methysergide, diphenhydramine, tetrodotoxin and indomethacin (15,46,47). The sodium-calcium exchange inhibitor dichlorobenzamil totally blocked the constrictor response to endothelin in isolated rat aortic rings, suggesting that endothelin plays a role in stimulating sodium movement into the cell (48). As a group, the sarafotoxins share many of the actions described previously for the endothelins, e.g., they cause coronary arterial vasoconstriction and induce phosphoinositide hydrolysis both in the presence and in the absence of extracellular Ca^{2+} (40).

Endothelin Processing - Many questions remain regarding the factors responsible for regulating the synthesis, release and degradation of endothelin. Active endothelin represents the product of two cleavage reactions, the first being dibasic endopeptidase-mediated hydrolysis of a 203 amino acid precursor known as preproendothelin to a 38 (human) or 39 (porcine) amino acid peptide referred to as big endothelin (49). Big endothelin is then converted to active endothelin by a putative endothelin converting enzyme (15,50,51). The dibasic endopeptidase has not been identified. Very

recently, however, it has been suggested that an aspartic protease may participate in the conversion of big ET-39 to ET-1 (52). Little information is currently available regarding specific peptidases which are responsible for clearing active endothelin from the circulation, although neutral endopeptidase 24.11 appears to contribute to its degradation (53).

Structure-activity Relationships - Initial attempts have been made to define the regions essential for binding, immunoreactivity and vasoconstrictor activity. The different biological potencies of the endothelins and sarafotoxins have largely been attributed to the sequence heterogeneity in the N-terminal region of the peptides, specifically between residues 4-7 (54-56). The four cysteine residues at 1,3,11,15, the carboxyl-terminal region 16-21 and the charged loop region Asp-Lys-Glu are highly conserved among the endothelins.

The contractile responses of porcine coronary artery strips in vitro have shown ET-1 to be the most potent constrictor, while ET-2 caused the greatest maximum contractile tension. ET-3 was the least potent of the three peptides (38,57). Studies with rat stomach smooth muscle have found ET-1 to be either equipotent or to show 5-10 times greater vasoconstrictor and ulcerogenic activity than ET-3, although the two peptides were found to be equipotent in producing gastric mucosal hemorrhage (58,59). ET-3 has been reported to be ten times less potent at contraction of guinea pig ileum and twenty times less potent in the rat aortic strip preparation than ET-1 (58).

The substitution of serine for threonine at position 2 in ET-3 is also shared by two weak constrictor peptides in the sarafotoxin family, SRTX-c and SRTX-d, suggesting its importance. It has been postulated that the acidic glutamic acid residue at position 9 of SRTX-c, instead of the basic lysine residue, might be responsible for this low potency, although this seems unlikely in view of the structure of SRTX-d (42,55). The most important contribution to this loss of activity appears to arise from the differences at positions 2,4 and 5. It has been proposed that the net charge within the Cys3-Cys11 loop may be important for biological activity, although SRTX-d, which possesses extremely low biological activity, calls this theory into question (54).

It has been suggested that the cyclic structure of these peptides is essential for binding to the putative receptors (60,61), although the outer disulfide bond Cys1-Cys15 would appear to be much more important than the inner Cys3-Cys11 bond (62). Removal of the C-terminal tryptophan residue reduces the vasoconstrictor potency by three orders of magnitude, although other aromatic residues appear to be tolerated (38,63). Chirality at this position is clearly important (63). Progressive deletion of the amino acid residues decreases receptor binding and vasoconstrictor activity with ET(1-16) being essentially inactive (60,63). ET(16-21) has been reported to be full agonist on guinea pig bronchial tissue with 33 times lower potency than ET-1 (64). In contrast, ET(16-21) was devoid of any agonist or antagonistic activity in rat thoracic artery and rat pulmonary artery rings (38,64). Several important residues for expression of biological activity have been reported (Asp 8, Glu 10, Phe 14 and the terminal NH_2 group) (38). The physiological importance of cleavage of ET(1-39) is indicated by the 140-fold increase in vasoconstrictor activity upon cleavage to ET-1 (65). Several models for the secondary structure of endothelin-1 in solution have recently been suggested (66-68).

Summary - Since its discovery, endothelin has attracted considerable interest because of its concerted actions on the heart, vascular smooth muscle, and the kidney, as well as its ability to alter the release of other hormones and neurotransmitter substances. However, although much information has been obtained regarding the inotropic, vasoconstrictor, and mitogenic actions of endothelin, its involvement in modulating the activity of the cardiovascular system under normal conditions has not been elucidated, nor has a role for endothelin in the pathogenesis of hypertension or heart failure been established. In addition, the recent identification of other endogenous substances structurally related to endothelin, which differ in their activities and tissue distribution, suggests that endothelin-type peptides may play an important role in regulating many diverse physiological and pathophysiological events (69-71). The discovery of pharmacological agents which block either the generation of endothelin from its protein precursor, or antagonize its binding to cellular receptors, should provide a means to assess the physiological role for endothelin, and also provide useful therapy for conditions associated with altered production or responsiveness to endothelin.

ATRIAL NATRIURETIC PEPTIDE

Atrial natriuretic peptide (ANP) is a 28 amino-acid peptide which is stored in secretory granules in atrial and ventricular tissue (72). Administration of exogenous ANP produces multiple effects on the cardiovascular system, including vasorelaxation, natriuresis and inhibition of the renin-

angiotensin-aldosterone system, indicating that ANP may play an important role in regulating fluid balance (73,74). Elevated plasma concentrations of ANP have been reported in a variety of diseases including hypertension, congestive heart failure (CHF) and renal failure (75-77). In addition, a correlation has been reported between decreases in ejection fraction in patients with heart failure and increases in plasma ANP (75).

As with endothelin, recent studies suggest that a family of natriuretic peptides may exist in different tissue compartments, including brain, heart and kidney (78-80). Human brain natriuretic peptide (BNP) demonstrates considerable amino acid sequence homology with circulating ANP, and appears to be processed from its own distinct precursor protein (78). Several investigators have described another 32 amino-acid form of ANP, urodilatin, which is localized to kidney tubules and collecting ducts (80). Initial results suggest that urodilatin is generated as a post-translational product of a different processing system from that of ANP (80). It has been suggested that urodilatin may act specifically in the renal collecting ducts, while ANP acts on renal vessels, proximal tubules and the juxtaglomerular apparatus, since only urodilatin has been detected in the urine (80).

Table 3. Amino acid Sequence of ANP, Urodilatin and BNP

Peptide	Amino acid residues
	100 105 110 115 120 125
Human ANP	S LRRSS CFGGR MDRIG AQSGL GCNSF RY
Urodilatin	TAPRS LRRSS CFGGR MDRIG AQSGL GCNSF RY
Brain natriuretic peptide	SPKM VQGSG CFGRK MDRIS SSSGL GCKVL RRH

Several approaches have been taken to develop therapeutic agents which produce the vasorelaxant/diuretic actions of ANP including (i) synthesis of ANP or ANP analogues, (ii) discovery of antagonists of the ANP clearing receptor (C-ANP receptor) which seems to play an important role in the degradation of circulating ANP, and (iii) discovery of inhibitors of endopeptidase 24.11 which hydrolyzes a variety of endogenous peptides including ANP.

Administration of ANP - Results of several studies in which ANP was administered in vivo have recently been reported (81). Some studies with synthetic ANP in heart failure patients have shown it produces favorable effects on cardiovascular hemodynamics, e.g. reduction in preload and afterload (82-84). However despite its natriuretic, diuretic, and vasorelaxant properties, the therapeutic utility of increasing circulating levels of ANP in patients with congestive heart failure remains to be conclusively established. The natriuretic response to ANP is attenuated in these patients and similarly in animal models of heart failure (85-87). The basis for diminished responsiveness to ANP is not known, but may be related to the very high circulating levels of ANP associated with CHF (85). The effect of increasing levels of ANP in the treatment of hypertension, in which circulating ANP levels are not elevated to the same degree as in heart failure, has only recently begun to be evaluated in a clinical setting (88-90).

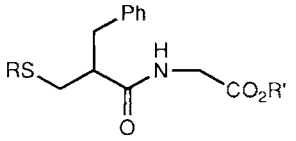
ANP was found to be a potent bronchodilator in a recently reported clinical study in asthmatic patients, which suggests important effects on airway tone in addition to peripheral renal and vascular actions (91). There have been several studies that indicate a role for brain ANP in central control of cardiovascular and body fluid regulation (92).

Analogues - Structure-activity relationships for binding of ANP and analogues to the guanylate cyclase coupled and non-cyclase coupled receptors have recently been reviewed (93,94). Although potent and stable analogues have been synthesized by several groups, none have been reported with oral activity (95-97). Isolation, sequence and expression of a cDNA clone encoding the membrane form of guanylate cyclase from rat brain has recently been described (98).

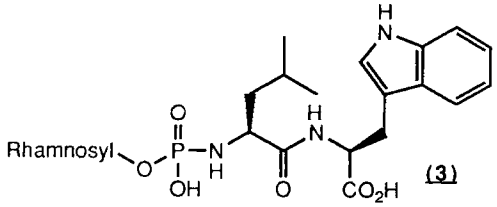
Clearing Receptor Antagonists - Biochemical and molecular biology studies investigating the C-receptor structure have been reported (99). It has been suggested that a single sequence of seven contiguous amino acids from the cyclic core of the hormone N-Ac-ANP[108-114]NH2 is responsible for recognition of ANP by the C-receptor (100). Blockade of the C-ANP receptors by the ANP analogue SC-46542 [des(Phe106,Gly107,Ala115,Glu116)] ANP[103-126] was found to cause increases in urinary flow and sodium excretion (101).

In anesthetized rats C-ANP antagonists increase circulating levels of ANP 2 to 4-fold, causing increases in urine flow and sodium excretion (102). Similar results were observed in conscious dogs (103). ANP clearance receptor antagonist activity has been claimed for peptide molecules containing a core pentapeptide sequence corresponding to the ANP[109-113] region, most commonly -Arg-Met-Asp-Arg-Ile- (104). However to date, no oral activity has been reported for any C-receptor antagonist, nor have their effects in animal models of hypertension or heart failure been described.

Atriopeptidase Inhibitors - Neutral endopeptidase 24.11, a zinc metallopeptidase homologous to thermolysin (105) (also known as enkephalinase, NEP or E.C.3.4.24.11) cleaves a variety of active peptides, including enkephalins, kinins, endothelin, chemotactic peptide, ANP and substance P (53,106-109). With the discovery that NEP degrades ANP to ANP(99-105/106-126) in human plasma, inhibitors of NEP may provide a valuable means for regulation of ANP levels (110). Indeed, the enkephalinase inhibitor thiorphan (1) has been shown to potentiate pharmacologic effects of ANP and to elevate ANP immunoreactivity when coadministered with ANP (111). Combination of SC-46542 and thiorphan have shown greater diuresis and natriuresis than with either agent separately (101). Sinorphan (2), which is a pro-drug of (S)-thiorphan is under clinical evaluation for the treatment of hypertension and CHF (112). The NEP inhibitor phosphoramidon (3) has been shown to prevent degradation of ANP in the proximal convoluted tubule and glomerulus suggesting these to be major sites of ANP degradation (113).

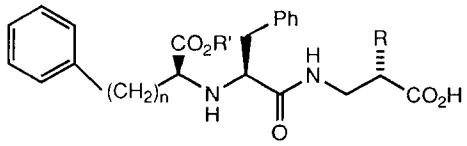


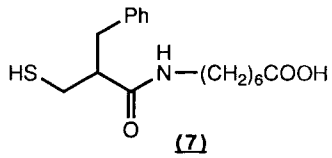
(1) R=H R'=H
(2) R=Ac R'=CH_2Ph



(3)

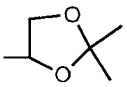
The carboxyalkyl dipeptide NEP inhibitor SCH-39370 (4) significantly delayed the disappearance of immunoreactive ANP from plasma in rats after an i.v. infusion of ANP (114,115). SQ 29,072 (7), a novel inhibitor of NEP derived from thiorphan has been shown to potentiate the depressor response to several ANP's (human 99-126,105-126; rat 99-126,103-126 and 103-123) suggesting that the exocyclic N-terminal and C-terminal residues do not influence ANP potentiation by (7) (116,117).



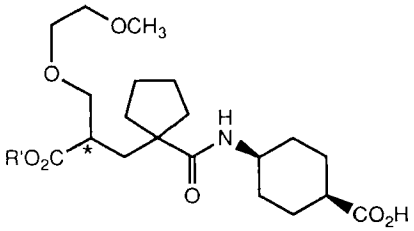


(7)

(4) n=2 R=OH R'=H
(5) n=1 R=H R'=H
(6) n=1 R=H R'=CH_2



Clinical trials of several atriopeptidase inhibitors (8,9,11) are currently underway (118,119). The most advanced is the orally active UK-79,300 (11), under clinical evaluation for the treatment of congestive heart failure and hypertension (119). Compound (11) is the active enantiomer and prodrug of UK-69,578 (8), from which it was developed. UK-73,967 (9), the i.v. form of (8), is also under evaluation in heart failure.



(8) (R,S) R'=H
(9) (S) R'=H
(10) (R,S) R'=
(11) (S) R'=

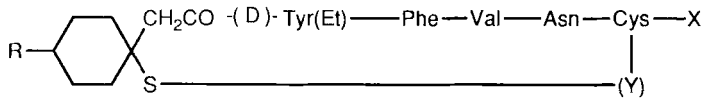
As mentioned previously, endopeptidase 24.11 degrades many other peptides including enkephalin, and analgesic activity has been reported for several closely related analogues of (4); SCH 32615 (5) and SCH 34826 (6) (121). There have been no reports describing the effects of (4), (7) or (8) on peptides other than ANP.

RENIN-ANGIOTENSIN SYSTEM

The involvement of the renin-angiotensin system in regulating blood pressure has been well documented (122,123). This involvement is mediated by the actions of the vasoactive peptide, angiotensin II, on key physiological processes involved in regulating vascular muscle contractile function, salt and water excretion, and neurotransmitter release (124). Recent reports suggest that angiotensin II may also play a role in promoting ventricular arrhythmias, and stimulating vascular and cardiac muscle hypertrophy (125,126). Interest in the renin-angiotensin system as a target for potential cardiovascular drugs remains high. For recent developments in renin and angiotensin converting inhibitors and in the novel non-peptide A II antagonists, see Chapter 6.

VASOPRESSIN

There have been conflicting reports regarding the role of vasopressin in hypertension and congestive heart failure (127,128). Several recent reviews on the subject are available (129,130). In severe hypertension and CHF, patients with increased plasma vasopressin levels, antagonists of the vasopressin-1 (V_1) receptor appear to offer some benefit (131). The vasodilation associated with the V_2-type vasopressin activity has recently been reviewed (132). The development of selective vasopressin antagonists has been hampered by the phenomenon of species differences (133-135). A new dog animal model has been developed that duplicates the agonist effects exhibited by the V_2 receptor antagonist SK&F 101926 (12) and that allows accurate assignment of the agonist/antagonist activity (136). Other recent results indicate that V_2 receptor antagonist activity requires a basic functional group extended an optimal distance from the hexapeptide ring while agonist activity requires the basic group in the tripeptide tail to be fixed in a more defined spatial orientation (137). The V_2 antagonist SK&F 105494 (14), an analogue of SK&F 104146 (13) (three times more potent) is currently in Phase 1 trials in hyponatremia (138). Recent evidence indicates the possible involvement of endothelin-1 in the regulation of vasopressin release (139).



(12)	Y=S	R=H	X=Pro-Arg-NH_2
(13)	Y=S	R=H	X=Pro-(D)-Arg-NH_2
(14)	Y=CH_2	R=H	X=Pro-(D)-Arg-NH_2

OTHER ENDOGENOUS VASOACTIVE PEPTIDES

Over the last decade many studies have demonstrated a relationship between endogenous opioid peptides and the cardiovascular system under both homeostatic and pathophysiological conditions (140,141). Further research is clearly required to define the role and mechanism by which endogenous opioids may modify cardiovascular effects.

Of the peptides identified to date in cardiovascular nerves the most widely distributed are neuropeptide Y, calcitonin gene-related peptide (CGRP), the tachykinins and vasoactive intestinal peptide (VIP). Neuropeptide Y (NPY) is a 36 amino-acid C-terminally amidated peptide that exerts direct vasoconstrictor effects on coronary vessels. It has been implicated in the pathogenesis of hypertension, myocardial infarction and renal failure (142,143).

Calcitonin gene-related peptide (CGRP), a 37 amino-acid peptide, is a potent vasodilator of cerebral coronary and other peripheral blood vessels (144), and also inhibits aldosterone secretion (145). Intravenous infusion of human CGRP causes a transient but significant increase in heart rate and a marked hypotensive effect (144). The mechanism of these cardiovascular effects is currently unclear. However it has recently been proposed that the vasodilation produced by CGRP is

mediated by the activation of K_{ATP} channels (146). Structure-activity studies indicate two populations of CGRP receptor subtypes in brain and peripheral tissues (147). A possible use for CGRP in subarachnoid hemorrhage has been suggested and the recombinant product is currently in clinical trials for severe Raynauld's disease and for patients who have undergone surgery following subarachnoid hemorrhage (148).

Substance P, a member of the tachykinin family has numerous pharmacological effects and its physiological role as a neuromodulator is well established (149). The intraganglionic release of substance P may contribute to the development of hypertension in genetically hypertensive rats (150). Structure-activity studies have indicated that the biological effects are mediated by the aminoterminal region (149).

Recent developments of specific bradykinin B2 receptor antagonists and their possible role in cardiovascular function, inflammation and pain have been reviewed (151). The bradykinin antagonist NPC-567 is currently undergoing evaluation for the treatment of lung inflammation and burn pain (152).

Vasoactive intestinal polypeptide (VIP), a 28 amino-acid peptide present in cardiac tissues is a potent vasodilator, inhibitor of smooth muscle activity and stimulator of secretion (153). VIP has been implicated in pathophysiological aspects of hemorrhagic shock, hypertension and heart failure (142,153). There is evidence that increased VIP receptor affinity and decreased receptor density occur in failing human ventricular myocardium (154).

The actions of transforming growth factor-ß (TGF-ß) have been described and although the physiological role of this peptide in normal cardiac function is unknown, it may accelerate repair if administered at the site of myocardial infarction (155). Recent results indicate that in human hepatocellular carcinoma, TGF-ß can induce the production of endothelin-1 (156).

Conclusion - The study of vasoactive peptides is of great interest and potential at the present time. It is anticipated that research in the next few years will provide answers to many of the key questions concerning mechanism of action, control of release, and significance of a wide range of endogenous peptides in cardiovascular regulation.

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Chapter 11. Thromboxane Synthase Inhibitors and Receptor Antagonists

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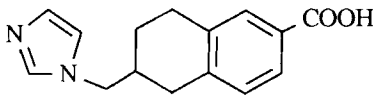
Introduction - The prevalence of occlusive vascular and platelet related diseases continues to stimulate research into new therapies for these conditions. The potential involvement of thromboxane A_2 (TxA_2) in the pathogenesis of myocardial ischaemia, coronary vasospasm and thrombosis (1) has led to continuing interest in identifying and developing appropriate agents which modulate its action. The research effort on thromboxane synthase inhibitors (TxSI) has reduced markedly during the past few years, probably due to the realization that the accumulated prostaglandin endoperoxides can directly activate the TxA_2 receptor, blunting the response to TxA_2 synthase inhibition. This is substantiated by clinical studies with TxSI which have generally failed to realise the beneficial effects anticipated (2). The emphasis has shifted towards the search for improved TxA_2 receptor (TP) antagonists, although as yet insufficient clinical data are available to enable a meaningful judgement about this class of compound (2). This chapter reviews the progress made since the last review (3).

THROMBOXANE SYNTHASE INHIBITORS

Despite declining effort in this area several interesting entities have recently been described, and animal studies with these and established TxSI have been further investigated.

As with earlier TxSI, the more recent analogues invariably include an imidazole or 3-pyridyl moiety, capable of binding to the haem iron of the enzyme, together with a carboxylic acid group. From a series of tetrahydronaphthalene and indan derivatives, the imidazole DP-1904, **1** was preferred because of its potency, duration of action and safety profile (4). Compound **1** inhibited *ex vivo* thromboxane B_2 (TxB_2) production in rats by 98 and 88% at 1 and 6hr respectively following a single oral dose of 1 mg/kg.



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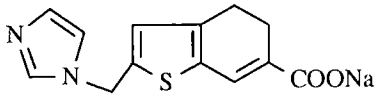

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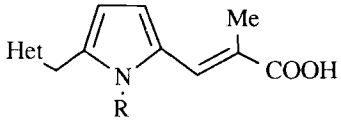
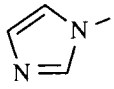
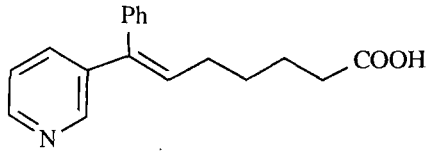


3 **Het =**  **R = Me**

4 **Het =**  **R = Et**

Structure-activity relationships (SAR) have also been reported for a series of di- and tetrahydrobenzo[b]thiophene carboxylic acids (5). The most potent compound was RS-5186 (2), which inhibited the human platelet microsomal Tx-synthase enzyme with an IC_{50} of 6 nM (cf. dazoxiben IC_{50}= 620 nM). Compound 2 inhibited TxB_2 production in dogs by >90% for 8 hr and up to 70-80% for 24 hr (6). The mechanism of its long duration of action appears to be due to a specific platelet uptake of 2, with a several fold higher concentration of drug being found in the platelet rather than the plasma or red blood cells. In a series of compounds that utilise a pyrrole moiety to separate a 3-pyridyl or imidazole group from the acidic function, the pyridine 3 was the most potent at inhibiting ADP-induced aggregation of human platelet rich plasma (PRP) in the presence of pig aortal microsomes (IC_{50}=0.042 nM) (7). However, there was no correlation between in vitro potency and activity in vivo, and the related imidazole 4 (in vitro IC_{50}=1 μM) was more potent in a model of pulmonary thromboembolism in mice (7).

The role of TxA_2 in myocardial ischaemia/reperfusion injury has been further studied. Invading polymorphonuclear leukocytes (PMN's) may play an important role in the development of myocardial ischaemic injury (8,9). Compound 2 reduces infarct size and gross myocardial haemorrhage in a canine coronary occlusion-reperfusion model, and also reduces the intensity of PMN infiltration into the infarcted area suggesting that TxA_2 inhibition may modulate neutrophil migration (10). This is further substantiated by studies with 5 (CV4151) (11) and 6 (furegrelate) (12). In contrast, 1 failed to have any beneficial effects on infarct size in a rabbit ischaemia/reperfusion model (13). Neither 2 nor 6 protected against ventricular fibrillation (VF) during myocardial ischaemia in dogs and cats, respectively (1,14). Interestingly, 2 does reduce reperfusion arrhythmias in isolated hearts and this may be related to an elevation in prostacyclin (PGI_2) levels (15).



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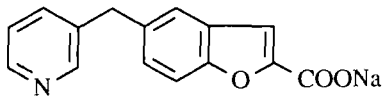

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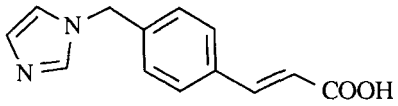

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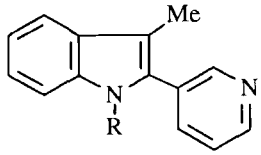
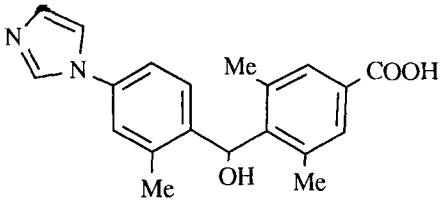


R= $(CH_2)_7COOH$ 8



9

Percutaneous transluminal coronary angioplasty (PTCA) is widely used for the non-surgical treatment of coronary atherosclerotic disease. However, subsequent coronary occlusion often leads to myocardial infarction (MI), emergency surgery or death. Suppression of the formation of arterial thrombi after balloon injury has been observed with **5** (2 mg/kg, i.v.) and suggests that Tx-synthase inhibition may be useful in PTCA (16).

Several studies have addressed the role of TxA_2 in cerebral ischaemia and protective effects have been demonstrated with OKY 046 **7** (17) and 1-benzylimidazole (18).

Both **7** and CGS-12970 **8** have been shown to be effective in preserving the function of rejecting rat renal allografts by promoting renal blood flow and glomerular filtration rate (GFR). Investigations with **6** have demonstrated a protective effect on renal function during septic shock in sheep, and support a role for TxA_2 in the pathogenesis of acute renal failure in systemic sepsis (19-22).

Relatively few clinical studies with TxSI have been reported recently (2). In single oral doses of 200 to 1600mg to normal males, **6** significantly inhibited platelet aggregation but the effect was variable and measurements of Tx-synthase inhibition did not predict the impact on platelet aggregation (23). Compound **7** at 80 mg b.i.d. has been shown to be clinically useful in treating the cerebral vasospasm and ischaemic symptoms in patients with a subarachnoid haemorrhage due to aneurysmal rupture (24). This suggests that the vasoconstrictor actions of TxA_2 could be responsible for these symptoms.

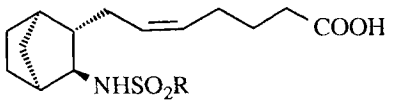
Oral dosing of **1** to patients with exercise-induced angina and post MI angina reduced the 3-month restenosis rate, as defined by coronary angiography, following PTCA (25). Chronic administration of **8** to renal allograft recipients maintains allograft function by improving renal blood flow and GFR (26). Finally, Y-20811 (**9**) has undergone Phase I evaluation and is a long-acting TxSI by either the i.v. (27) or oral route (28).

THROMBOXANE RECEPTOR ANTAGONISTS

The development of agents that block the action of TxA_2 at its receptor site continues to be an active area of research. Since the endoperoxide PGH_2 possesses a pharmacological profile similar to TxA_2 and appears to share a common receptor, at least on platelets, new stable bicyclic analogues derived from PGH_2 have been reported as antagonists of TxA_2. SAR for a series of novel sulphonyl derivatives **10** designed as U-shaped analogues of sulotroban (BM13.177, **11**) have been reported (29,30). TP blocking activity was dependent on lipophilicity of the sulphonylamino residue and location of the phenyl group in the ω-side chain. S-145 (**10**, R=Ph) and its d-isomer were chosen for detailed study and the former inhibited U-46619 and collagen-induced aggregation of human washed platelets (WP) with IC_{50}'s of 7.7 and 4.5 nM respectively (29). S-145 ($3x10^{-10}$ - $3x10^{-9}$ M) attenuated the contraction induced by U-46619 in monkey cerebral, coronary and mesenteric arteries, and cat cerebral arteries (31). However, antagonism appeared to be non-competitive, and in some isolated preparations produced transient contractions indicative of TP partial agonistic activity. At 10 mg/kg, p.o. S-145 produced both a 50-60% inhibition of collagen-induced thrombocytopenia in mice, and in a model of myocardial ischaemia showed a

marked improvement in the collagen-induced ECG changes in rats, with a duration of action greater than 4 hr (32).

Efforts to remove undesirable TP agonist activity by moving to the related sulphonamido antagonists **12** or **13** were unsuccessful (33,34). However, within the 6,6-dimethylbicyclo[3.1.1]heptane series, **14** had significant inhibitory activity and did not act as a partial agonist or cause platelet shape change (35). In the search for more potent analogues, the regioisomer **15** has been claimed as a pure antagonist with an IC_{50} of 0.97 μM against collagen-induced aggregation on rat WP (36).



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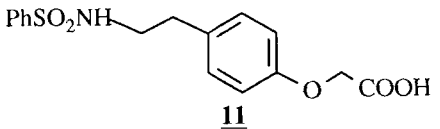

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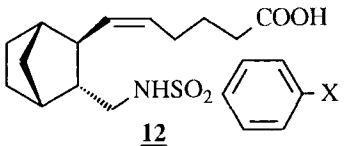

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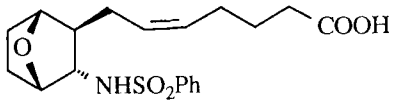

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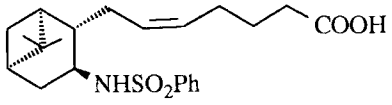

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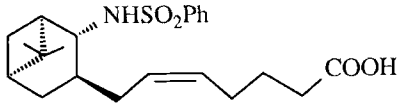
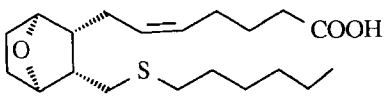


15

Further side chain modification in the 7-oxabicyclo [2.2.1]heptane series of compounds has led to some novel thioether analogues with TxA_2 antagonist activity (37). Although it was noted that these thioethers had some level of direct TxA_2 agonist activity, compound **16** was 30-40 fold more potent than **11** in inhibiting arachidonic acid (AA)-induced aggregation of human PRP, and at 1 mg/kg i.v. inhibited bronchoconstriction induced by AA in anaesthetised guinea-pigs. It would appear that a growing number of TxA_2/PGH_2 analogues, despite being potent antagonists, display varying degrees of agonist activity which may limit their development. Other related compounds under investigation are SQ 33261 (**17**) and SQ 33552 (**18**); against U-46619-induced human platelet aggregation and rat aortic strip contraction they had IC_{50}'s of 200 and 70 nM, and pA_2's of 9.0 and 10.1, respectively (38).



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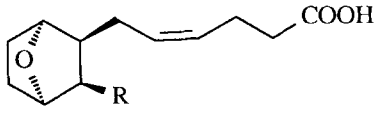
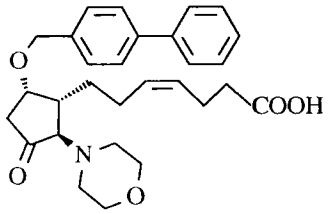


17 R=NHNHCONHPh

18 R=CH_2NHNHCONHC$_6$H$_4$-4-Cl

The promising 13-azaprostanoid antagonist AH 23848 (19) has been found to be teratogenic and its development discontinued (39). A close analogue replacement, GR 32191 (20), is a more potent and specific TP-receptor blocking drug than 19 (40). In human PRP, 20 potently and selectively inhibited aggregation induced by TxA_2, PGH_2, collagen, AA and U-46619 (41). Against U-46619-induced aggregation of human whole blood (HWB) and contraction of rat aortic strip it had pA_2's of 8.2 and 7.9, respectively (42). Compound 20 also antagonised U-46619-induced contractions of airway smooth muscle from rat, dog, guinea-pig and rabbit with varying degrees of potency. The profile of action of 20 upon vascular and airway smooth muscle appears to be of a competitive, surmountable type, but on platelets competitive antagonism was only observed at low concentrations (41,43). In the Baumgartner chamber 20 inhibited the deposition of platelets from flowing whole blood onto damaged blood vessels *in vitro*, and was slightly more potent than PGI_2 (43). The compound partially inhibits platelet deposition onto damaged arteries and artificial surfaces *in vivo* (44, 45), and reduces luminal stenosis of prosthetic grafts (46).

In man, single oral doses of 20 inhibited *ex vivo* platelet aggregation induced by U-46619 for 8 to 24 hr, and repeat dosing for 10 days resulted in a large continuous blockade of aggregation (47). Chronic oral dosing with 20 also effectively blocks vascular smooth muscle TP-receptors in man (48). In atopic asthmatic patients in whom bronchoconstriction was provoked by inhaled PGD_2, compound 20 produced a significant improvement in airway resistance following a single 80 mg oral dose (49). The same dose inhibited to varying degrees the immediate airway response to inhaled allergen.



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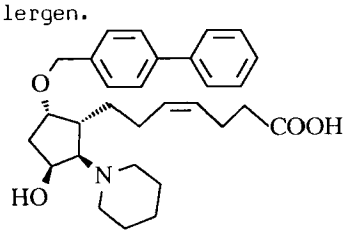

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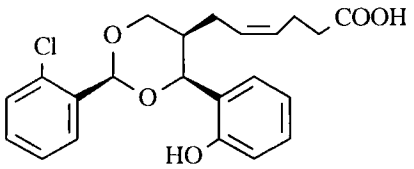
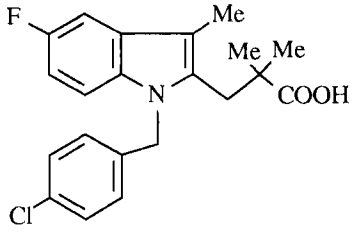


21

Modification to the 1,3-dioxane series of TxA_2 antagonists has given the chemically more stable ICI 192605 (21). Compound 21 was competitive and selective as a TP-antagonist, with pA_2's of 8.0, 8.4 and 8.16 against U-46619 on rabbit and rat aorta, and human platelets (50, 51). In man *ex vivo*, 21 has a minimum effective oral dose of 0.03 mg/kg against U-46619-induced platelet aggregation (52).

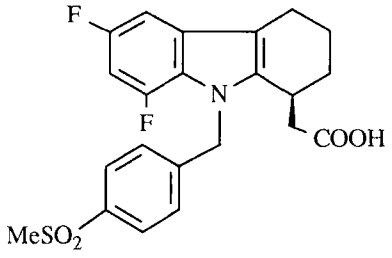
Further TxA_2 antagonists structurally unrelated to the prostanoid skeleton continue to be described. L-655,240 (22) is representative of a novel class of TxA_2 antagonist derived from indole-2-propanoic acids (53). Compound 22 inhibited aggregation of human PRP induced by

U-46619, TxA_2 and collagen, and competitively antagonised contraction of human trachea by U-44069 with a pA_2 of 8.0 (54). Oral activity of **22** has been demonstrated in the rhesus monkey at 3 mg/kg. At 0.3 mg/kg i.v., it was also effective at reducing the incidence and severity of ischaemia, especially reperfusion-induced arrhythmias, in a canine model of coronary artery occlusion (55). This series also possesses leukotriene biosynthesis inhibitory activity, and attempts to reduce this action led to the discovery of the related tetrahydrocarbazolyl acetic acids of which L-670,596 (**23**) is the most potent and selective (56).



22

From a study of SAR in a novel series of ω-phenyl-ω-quinonylalkanoic acids, AA-2414 (**24**) was one of the most potent, orally active, and long acting TxA_2 antagonists (57). It inhibited aggregation of guinea-pig platelets by U-44069 and showed pA_2's of 8.29 and 8.28 against contraction of guinea-pig lung and rabbit aorta induced by U-46619 and U-44069 respectively. It was also effective at inhibiting (MED 0.3 mg/kg p.o.) bronchoconstriction in guinea-pigs induced by U-46619, LTD_4 and PAF with a long duration of action (58).



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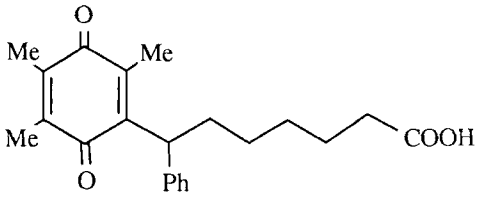
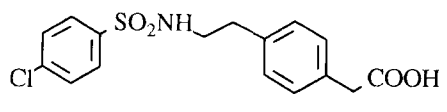


24

Further evidence supporting the involvement of TxA_2 in the pathogenesis of ischaemia-induced myocardial injury comes from a number of studies (60-62). Moreover, TxA_2 antagonists have been found to be particularly effective in reducing the severity of such injury (63, 64). In a rat model of ischaemic damage daltroban, BM 13505 (**25**), although not improving survival rate did produce a significant reduction in myocardial injury (65). The compound has also been reported to have an anti-ischaemic effect in related models of reperfusion-induced ischaemic damage in cat (66) and rabbit (67). A brief review of the pharmacological properties of **25** has appeared (68). Although myocardial protection is greater when TxA_2 receptor blockade is present during both occlusion and reperfusion, in anaesthetised dogs SQ 30741 (**26**) is the first antagonist to be reported to have a beneficial effect on infarct size when given during reperfusion alone (69).

Studies with ONO 3708 (**27**) have confirmed and extended previous observations that TxA_2 antagonists significantly improve survival rate in circulatory shock (70).



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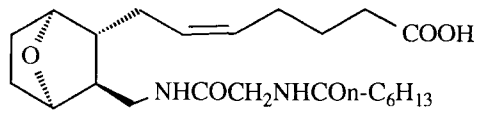

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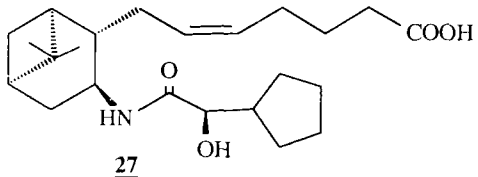


27

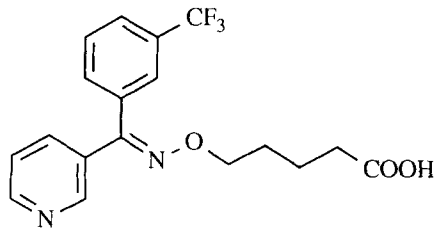
Thromboxane A_2 has been implicated as a possible mediator of platelet activation in patients undergoing coronary thrombolysis following an acute MI (71), and in experimental models of coronary thrombosis TxA_2 limits the response to i.v. thrombolytic therapy (72). While the effect of inhibiting TxA_2 with **26** on streptokinase-induced thrombolysis in the monkey did not accelerate thrombolysis, it did increase and sustain reflow once thrombolysis had been established (73). However, a beneficial effect on the time to reperfusion has been reported (74) in patients undergoing thrombolysis with streptokinase in combination with **11**, and in anaesthetised dogs **11** significantly enhanced the thrombolytic efficacy of a minimally effective dose of streptokinase (75). Compared with aspirin alone, a combination of a TP-receptor antagonist with aspirin has been found to accelerate reperfusion in response to tissue-type plasminogen activator in the anaesthetised dog, suggesting that a very marked degree of TxA_2/PGH_2 suppression may be required to achieve a biological effect during thrombolysis (72).

In spite of a number of studies over the years, the possible heterogeneity of platelet and vascular thromboxane receptors remains controversial. Studies using platelets and blood vessels from different species have raised the possibility that differences may be species-selective rather than receptor-selective. However, some reports strongly support the existence of receptor subtypes (76-78). In contrast, in a series of experiments using several structurally dissimilar TxA_2 antagonists with a wide potency range both *in vitro* and *in vivo*, it was concluded that subclassification of the TP receptor is not warranted at this time (79). The use of [^{3}H]S-145 as a radioligand and **10** (R=Ph) as an immobilised ligand for affinity chromatography, has allowed purification of the TxA_2/PGH_2 platelet receptor (80). This should enable determination of its structural features and henceforth help to clarify the issue of molecular heterogeneity of receptor subtypes found in platelets and smooth muscle.

COMBINED THROMBOXANE ANTAGONISTS/SYNTHASE INHIBITORS

Theoretical arguments have been made to support the potentially superior antithrombotic efficacy of using a combined TxA_2/PGH_2 receptor blocker/TxA_2 synthase inhibitor over either class of agent alone. Such activity in one molecule has recently been described for R68070 (**28**) although similar claims have also appeared for **5** (81,82). Compound

28 has progressed to man, where a single oral 400 mg dose produces ≥90% inhibition of serum TxB_2 production for 48 hr and >70% inhibition of collagen-induced platelet aggregation in PRP for 8 hr (83). In dogs, 28 reduces coronary thrombosis induced by electrical damage and prevents the progression of arrhythmias into VF upon reperfusion. Despite their dual action, 28 and 5 are no more effective as inhibitors of platelet aggregation than the TxA_2 antagonist 20, and this probably reflects their relatively weak receptor blocking action (pA_2 values for U-46619-induced platelet aggregation in HWB of 5.4, 4.7 and 8.2 for 28, 5 and 20, respectively) (84). It is likely that analogues with enhanced levels of antagonist activity will be required before the full benefits of this novel pharmacological approach can be demonstrated (84).



28

Conclusion - Considerable progress has been made in the development of TxA_2 receptor antagonists. However, while such compounds have provided insight into the role of TxA_2 in vitro and in animal models, it is only relatively recently that potent, specific and long acting candidates have become available which will allow the clinical utility of TxA_2 receptor blockade to be established conclusively. It is more widely accepted that TxA_2 antagonists have greater clinical potential than TxA_2 synthase inhibitors, but the emerging effectiveness of aspirin, e.g. against vascular occlusion, will necessitate that TxA_2 antagonists show superior efficacy if they are to be widely utilized (2).

The greater clinical potential of combined Tx synthase inhibition/ receptor blockade has been recognized, but the full beneficial action of this class of drug has yet to be realized (85).

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Section III - Chemotherapeutic Agents

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Chapter 12. Antibacterial Agents

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Introduction - Major areas of antibiotic research reported in 1989 include the quinolones, β-lactams, glycopeptides and a variety of other compounds which are active against methicillin-resistant *S. aureus* (MRSA). Macrolides are discussed in Chapter 13.

Quinolones - The proceedings of the Second International Symposium on New Quinolones and the proceedings of the International Telesymposium on Quinolones were recently published (1, 2). A recent review highlights advances including improved Gram-positive, anaerobe, *Chlamydia* activity and in some cases improved pharmacokinetics in comparison with ciprofloxacin (1) (3). Other reviews have been published covering the bacteriology, pharmacokinetics and clinical experience of the newer quinolones (4-6). Papers covering the clinical utility of quinolones as a class have also been published (7-9).

An updated review on structure-activity relationships was published, pointing out that with the exception of the C-4 position, every position of the quinolone has now been successfully modified (10). A new thiazetoquinolone, NAD-394 (2), and its prodrug NAD-441A [N-(5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl NAD-394] were disclosed. NAD-394 (2) has broad spectrum activity in vitro and its prodrug NAD-441A is 2-3x more potent than ofloxacin (3) in vivo (11). The difluorinated benzothiazine, MF-961 (4), is roughly as active as ofloxacin (12). A series of benzo [i, j] quinolizine 2-carboxylic acids was prepared in which the 8-methylamino and 8-ethylamino analogs (5) showed significant activity vs. *S. aureus* and *Acinetobacter* strains compared to ciprofloxacin, but otherwise were generally less active (13). A study has shown that the N-1-(4'-difluoromethoxyphenyl) residue was halogen-like when compared to 4'-Br or 4'-Cl, but did not compare with 4'-F for overall activity (14).

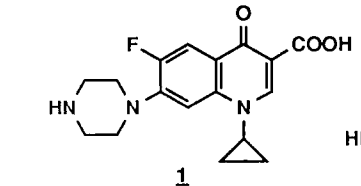
Studies were conducted to optimize the activity of the previously reported 1-*t*-butyl-substituted quinolones and naphthyridines (15) by altering the C-7 piperazine residue. The 2-methylpiperazine derivative (6) has the best activity; the 2(S)-methylpiperazine in the naphthyridine series is more active than the (R)-isomer (16). Danofloxacin (CP-76,136) (7), a 7-diazabicycloquinolone, is being evaluated for diseases in food-producing animals because of its good pharmacokinetics, activity, and solubility (17-20).

The effect of absolute and relative stereochemistry of C-7 pyrrolidinyl substituents was studied (21). The most active compound in this series is the 3R, 1'S isomer DS-4524 (8). WIN-57273 (9), a 7-pyridinyl quinolone, is more active than ciprofloxacin vs. Gram-positive but less active vs. Gram-negative bacteria. WIN-57273 showed excellent activity against certain methicillin, gentamicin and even some ciprofloxacin resistant organisms. It is also more active than ciprofloxacin vs. AIDS related *Legionella* sp. and *Mycobacterium avium* (22-29).

A series of enantiomeric tetracyclic quinolones in which the basic C-7 substituent is linked to C-8 by means of an oxygen atom are all comparable or slightly better than ofloxacin. The enantiomer (10) appears to be the best compound in vivo (30).

In a study of norfloxacin prodrugs, it was found that N-masked congeners having acidic hydrogens on the carbon α to the nitrogen atom liberate norfloxacin in vivo (31). In another study, it was found that the rearrangement product (**11**) obtained by the chemical oxidation of N-[(5-methyl-2-oxo-1,3-dioxol-4-yl)methyl] norfloxacin also acts as a prodrug of norfloxacin (32).

A recent study determined that the 6-fluoro-7-(4-hydroxypiperazin-1-yl) quinolone derivatives were superior to their corresponding deoxy analogs, norfloxacin, ciprofloxacin and enoxacin. Subsequently **12** was found to be >5x more potent than ciprofloxacin in vivo vs. *S. aureus* and *E. coli* (33).



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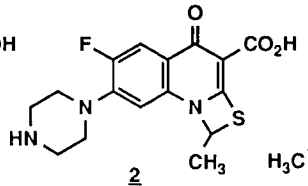

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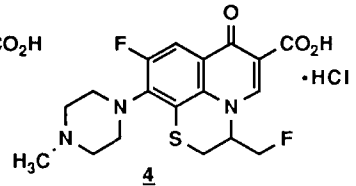

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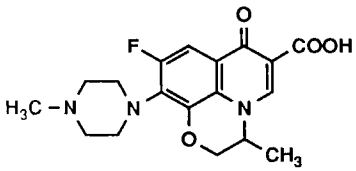

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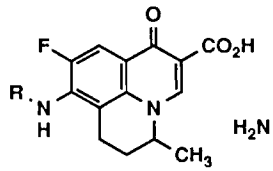
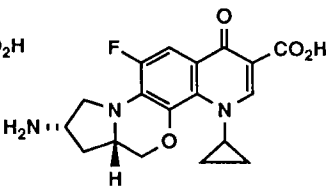


5 R=Me or Et



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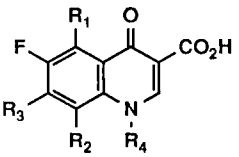
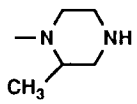
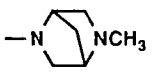
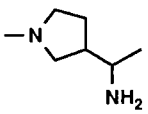
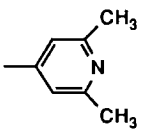
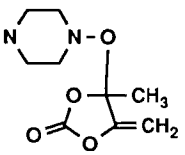


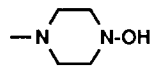
6 $R_1=R_2=H$, $R_3=$  $R_4=$ 

7 $R_1=R_2=H$, $R_3=$  $R_4=$ 

8 $R_1=H$, $R_2=F$, $R_3=$  R_4 = Et

9 $R_1=R_2=H$, $R_3=$  $R_4=$ 

11 $R_1=R_2=H$, $R_3=$  R_4 = Et

12 $R_1=H$, $R_2=F$, $R_3=$  $R_4=$ 

The mechanism of resistance to quinolones is still undergoing active investigation. A recent review suggests resistance is due to mutations affecting DNA gyrase and/or drug permeability (34). A combination of decreased permeability and DNA susceptibility was seen in experimental endocarditis with *Pseudomonas aeruginosa* (35) and in experimental peritonitis with *Enterobacter cloacae* (36). Decreased permeability was seen as the mechanism of resistance in *E. coli* mutants (37, 38), while changes in DNA gyrase were cited in studies on *E. coli* (39, 40) and *Serratia marcescens* (41). Widespread resistance among MRSA was found in a Tel-Aviv hospital (42).

A proposed cooperative quinolone-DNA binding model for the inhibition of DNA gyrase has been published (43), along with supporting experimental evidence. In this model

the quinolone molecules bind to a gyrase-induced DNA site during the "gate-opening" step of the supercoiling process via hydrogen bonds to the unpaired bases. The formation of tyrosine-quinolone binary complexes was also determined in a UV photometric study (44).

The use of calf thymus topoisomerase II inhibition assays as a predictor of cytotoxicity is being investigated by several groups with varying results (45-47). There is a large difference among the quinolones in their selectivities between the bacterial enzyme and its eucaryotic counterpart.

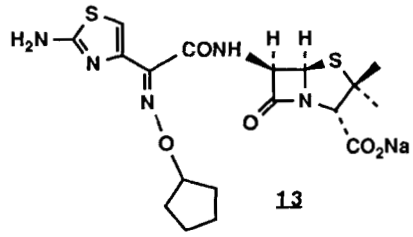
A recent paper points out that quinolones administered intravenously to mice concomitantly with oral biphenyl acetic acid, a metabolite of fenbufen, provoked clonic convulsions and subsequent death at doses of 6.25 mg/kg or more when the compound has a non-substituted piperazine moiety at the 7 position. There was a close correlation between the epileptogenic activities of quinolones and their inhibitory potencies for [^{3}H] muscimol binding to GABA receptor sites (48). Another study linked CNS side-effects of quinolones to their induction of increased interleuken-2 levels in phytohemagglutinin (PHA)-stimulated human lyphocytes (49) at clinically achievable concentrations (5 μg/ml).

Following previous reports of in vitro activity vs. *Plasmodium falciparum*, norfloxacin was found to be effective in humans with falciparum malaria (50). Ciprofloxacin, difloxacin and ofloxacin were found to have good activity (MIC_{50} 1 μg/ml) vs. *Mycoplasma hominis* (51). Quinolones were also found effective in experimental *Leishmania donovani* (52).

Additional information was presented on KB-5246. Tissue levels were 4-40x plasma levels while CSF levels were ~10-20% of plasma levels (53). AT-4140 was the most active quinolone tested in vitro vs. *C. trachomatis* (54) and *Legionella* sp. (55). In experimental bacterial prostatitis (*E. coli* or *E. faecalis*) AT-4140 was the most effective quinolone tested (56). More detailed in vitro and in vivo information on BMY-40062 has appeared, showing that this agent compares favorably with ciprofloxacin (57, 58).

β-Lactams - Research has focused on structures having stability to lactamase enzymes and improved pharmacological properties. BRL 44154 (**13**) is the preferred compound in a series of alkoxyimino penicillins synthesized (59) due to its activity vs. MRSA, stability to staphylococcal β-lactamase (60) and ease of synthesis. BRL 44154 is effective in a series of experimental infections in the mouse produced by both Gram-positive and Gram-negative organisms including β-lactamase producing staphylococci (61). A new penem with high oral availability, FCE 25199 (**14**), was disclosed. It is generally comparable to imipenem in vitro (62, 63). A novel β-methyl carbapenem LJC 10627 (**15**), was shown to be about two times as active as imipenem vs. Gram-negative bacteria in vitro and in vivo. LJC 10627 is stable to renal dihydropeptidase and a pharmacokinetic study indicated 95% urinary recovery in monkeys (64-66). Additional information on the synthesis and β-lactamase activity of BRL-42715 (**16**) has been published (67). BRL-42715 inhibits a broad range of plasmid-mediated β-lactamases, including the class V OXA group of enzymes and all of the class I cephalosporinase enzymes (68). A synthesis of γ-lactam analogs of oxa-penams (**17**) was undertaken and the compounds were found to be inactive (69).

A new 1-carba-1-dethia-cephalosporin, LY-249902 (**18**), showed enhanced bioavailability over cefetamet in the monkey, but not in the dog (70). CP 6162 (**19**), a new parenteral cephalosporin, was reported to have potent in vitro and in vivo activity against β-lactamase producing Gram-negative bacteria including *Ps. aeruginosa* (71). A new broad spectrum cephalosporin, GR-69153 (**20**), with an extended half-life (t 1/2 = 3 1/2 hr, man) was also presented. A 1 g dose gives plasma levels exceeding the MIC_{90} for 24 hr for many organisms (72, 73). A series of C(3)-cyclopropyl cephalosporins and carbacephalosporins was synthesized. The phenylglycyl analogs (**21**) showed better Gram-positive activity than cefaclor while the *m*-methylsulfonamidophenylglycyl cephalorporin (**22**) had a markedly longer half-life than (**21**) (74, 75). KP-736 (**23**) is a new parenteral cephalosporin with high activity vs. *Pseudomonas* sp. including multiple drug-resistant strains. In vivo, KP-736 is comparable to ceftazidime vs. Gram-positive infections while it is ~6x as effective vs. *Pseudomonas* (76-80). SCE-



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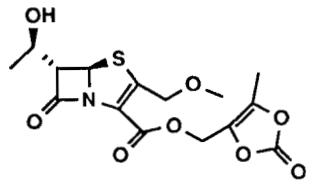

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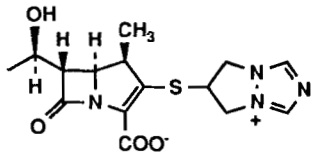

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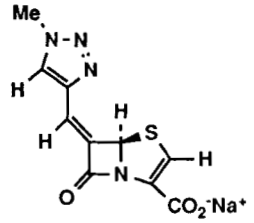

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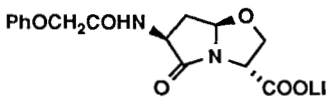

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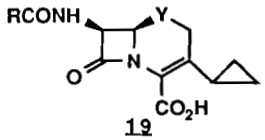
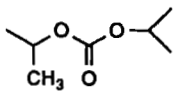
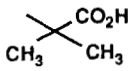
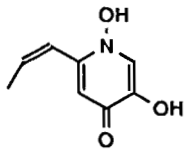
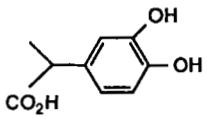
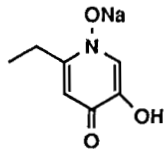
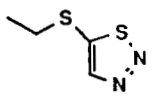


19

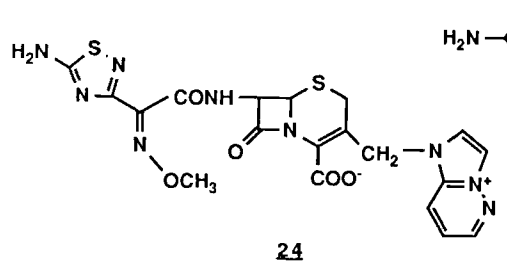
	R	Y
21	PhCH(NH_2)	S or CH_2
22	m-(CH_3SO_2NH)PhCH(NH_2)	S

	R_1	R_2	R_3	X
18	CH_3		$CO_2C_2H_5$	CH_2
19		H		S
20		H	H	S
23		Na^+		S

2787 (24) is a new β-lactamase-stable parenteral cephalosporin which is active vs. *S. aureus* and *Ps. aeruginosa* both in vitro and in vivo. It has been selected as a candidate for clinical evaluation (81-86). KP-736, as well as CP-6162 and GR-69153, are noteworthy examples of structures which exploit the ton B-dependent iron transport pathway. An extensive review of the chemistry of therapeutically important, siderophore containing molecules has appeared (87).

A structure activity study to improve the in vivo efficacy and pharmacokinetics of SQ 83,325 resulted in the synthesis of SQ 83,989 (25). The protonated aminomethyl substituent on the 5-hydroxy-4-pyridone ring is the optimal substituent for improving both in vivo efficacy and urinary excretion (88).

An acyclic phosphonate monoester, m-carboxyphenyl phenylacetamidomethyl-phosphonate, has been found to give rapid inhibition of a class C β-lactamase (89).



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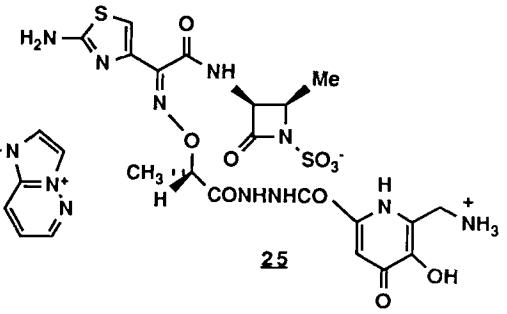
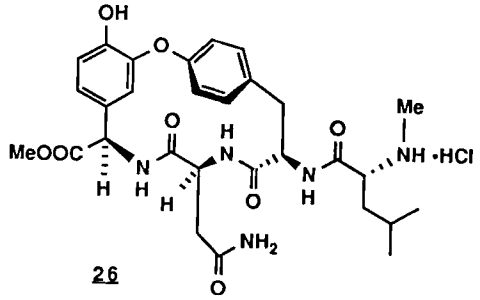


25

Glycopeptides - A proposal has been made to name the vancomycin-glycopeptides "dalbaheptides" from DAL (D-alanyl-D-alanine) B (binding) A (antibiotics) with a HEptapeptidic structure (90). A further distinction would distinguish the glycodalbaheptides which have only sugars attached to the peptide core (vancomycins, ristocetins, avoparcins) and lipoglycodalbaheptides which have a fatty acid chain attached to the core (teicoplanins, kibdelins, parvodicins).

A recent approach to vancomycin derivatives involved the biotransformation of vancomycin by *Actinomadura citrea*, leading to its biologically inactive hexapeptide core. This was the first bioconversion of a glycopeptide antibiotic by a non-glycopeptide-producing culture (91). Another approach to vancomycin derivatives involved oxidative phenolic coupling with thallium nitrate to yield tetrapeptide (26) which is expected to bind to N-acyl-D-Ala-D-alanine (92). Arene-manganese tricarbonyl complexes were used in the synthesis of arylglycines as potential sub-units in the synthesis of ristocetin and vancomycin (93).

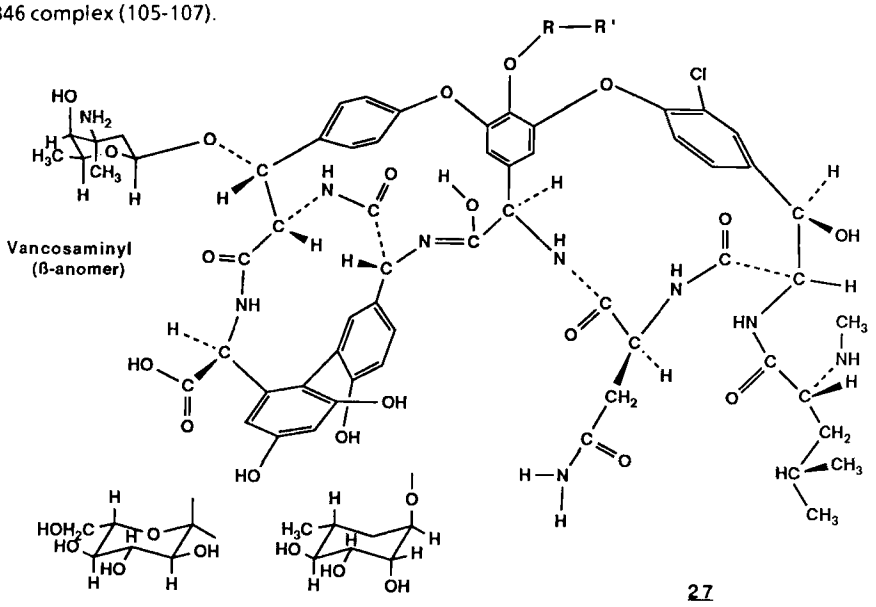


26

Studies have probed the influence of thioureas and isothiouronium salts on teicoplanin aglycone (94), and the intramolecular cyclization of the isothioronium derivative was investigated (95). Sequential removal of chlorine from teicoplanin gave decreased binding affinity and decreasing antibacterial activity (96). Base catalyzed elimination of N-acetyl-β-D-glucosamine from teicoplanin gave unsaturated products. The 35,52-unstaturated compound maintained binding affinity and antibacterial activity while the 34,35-unsaturated compound

did not, due to changes in molecular configuration (97). N^{63}-carboxamide and N^{63}-carboxypeptides of teicoplanin showed some improved activity which was dependent on ionic and lipophilic character and on the type and number of sugars present (98, 99). Deamination of teicoplanin resulted in loss of activity (100).

New minor components of the teicoplanin complex have been isolated from large scale fermentations of *Actinoplanes teichomyceticus* (101) and designated as related substances RS-1 through RS-4. Biological activity was not disclosed. A-42867 (**27**) is a new glycopeptide isolated from a Nocardia nov. sp. ATCC 53492 which has biological activity comparable to the other members of the group (102). UK-68,597 (**28**) is another new member of this class which was isolated from *Actinoplanes* sp. ATCC 53533 (103, 104). Several papers were published exploring the structural relationships between the recently reported orienticins, chloroorienticins and A-82846 complex (105-107).



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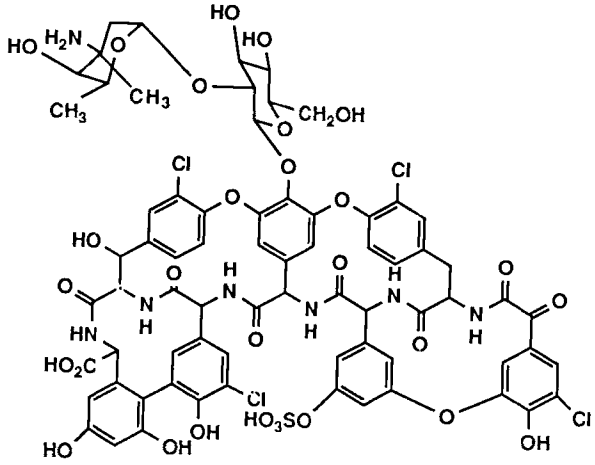
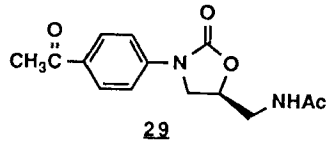


28

Miscellaneous - The antituberculosis activity of DUP-721 (**29**) was compared to a nitroimidazole CGI-17341 (**30**). In vitro both were active vs. *Mycobacteria tuberculosis* (DUP 1.25-4 µg/mL, CGI 0.06-0.3 µg/mL) while both were inactive vs. *M. avium intracellulare* (108). A short, chiral synthesis of the oxazolidinone DUP-721 in 60% yield has been published (109) and many other analogs were synthesized using this synthetic sequence in a structure-activity report (110). Coumermycin analogs have come under renewed interest due to their activity vs. MRSA and their mechanism of action as an inhibitor of DNA gyrase. The most promising analog of the series was (**31**) although it was less active than coumermycin A1 (111).

Cyclic Peptides - The structure of the ramoplanin complex (A-16686) was published (112,113). This complex is active vs. aerobic and anaerobic Gram-positive bacteria including MRSA. Hypeptin is a cyclic peptide isolated from a *Pseudomonas* sp. with activity mainly vs. aerobic and anaerobic Gram-positive bacteria (114). The janthinomycins (**32**) are a complex of macrocyclic peptide lactones produced by *Janthinobacterium lividum* which were 2-4 times more active than vancomycin in vitro (including MRSA) and were roughly equivalent in vivo vs. *S. aureus* (ED_{50} <1.6 for J, 2.0 for vanco.). Janthinomycin A and B are unique in that β-hydroxytryptophan and β-ketotryptophan have not previously been isolated as natural products. (115, 116). UK-63052 complex is a group of quinomycin-like peptides isolated from *Streptomyces braegensis* ss *japonicus* having activity vs. *S. aureus* (MIC <0.20-1.56 µg/mL) (117). Mersacidin is a sulfur-containing peptide produced by a *Bacillus* sp. which was ~2x more active in vivo than vancomycin vs. *S. aureus* including MRSA (118). Thioxamycin is a thiopeptide isolated from a *Streptomyces* sp. and is mainly active vs. anaerobes. It is also active vs. *S. aureus* (MIC 3.13 µg/ml) (119). A-10255B is quite similar in structure and spectrum to thioxamycin and may be targeted as a growth promotant in animals. The A-10255 complex was produced by *Streptomyces gardneri* (120-123).

Other Anti-staphylococci Antibiotics - The structure of the previously reported ficellomycin was elucidated (124). The altromycins are a group of pluramycin-like antibiotics isolated from a nocardioform actinomycete using a super-sensitive *Pseudomonas aeruginosa* strain (125,126). A-82810 is a new polyether antibiotic produced by *Actinomadura fibrosa* which is also an anthelmintic and insecticidal compound (127). CP-73064 is another new polyether isolated from *Streptomyces* sp. ATCC 53523 (128).



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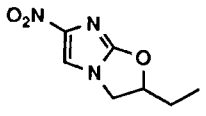

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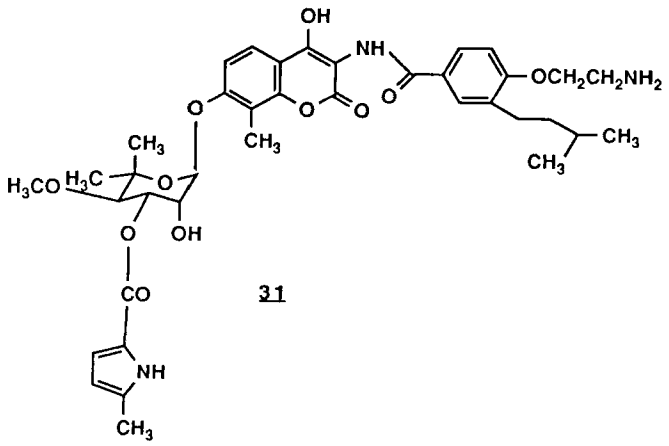

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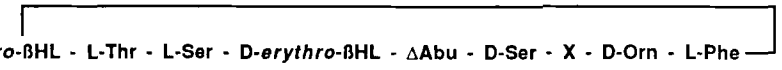

Janthinomycin A, X = *threo*-ß-hydroxytryptophan
Janthinomycin B, X = ß-ketotryptophan
Janthinomycin C, X = dehydrotryptophan
ΔAbu = 2,3-dehydro-α-aminobutyric acid
ßHL=ß-hydroxyleucine

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Chapter 13. Macrolide Antibiotics

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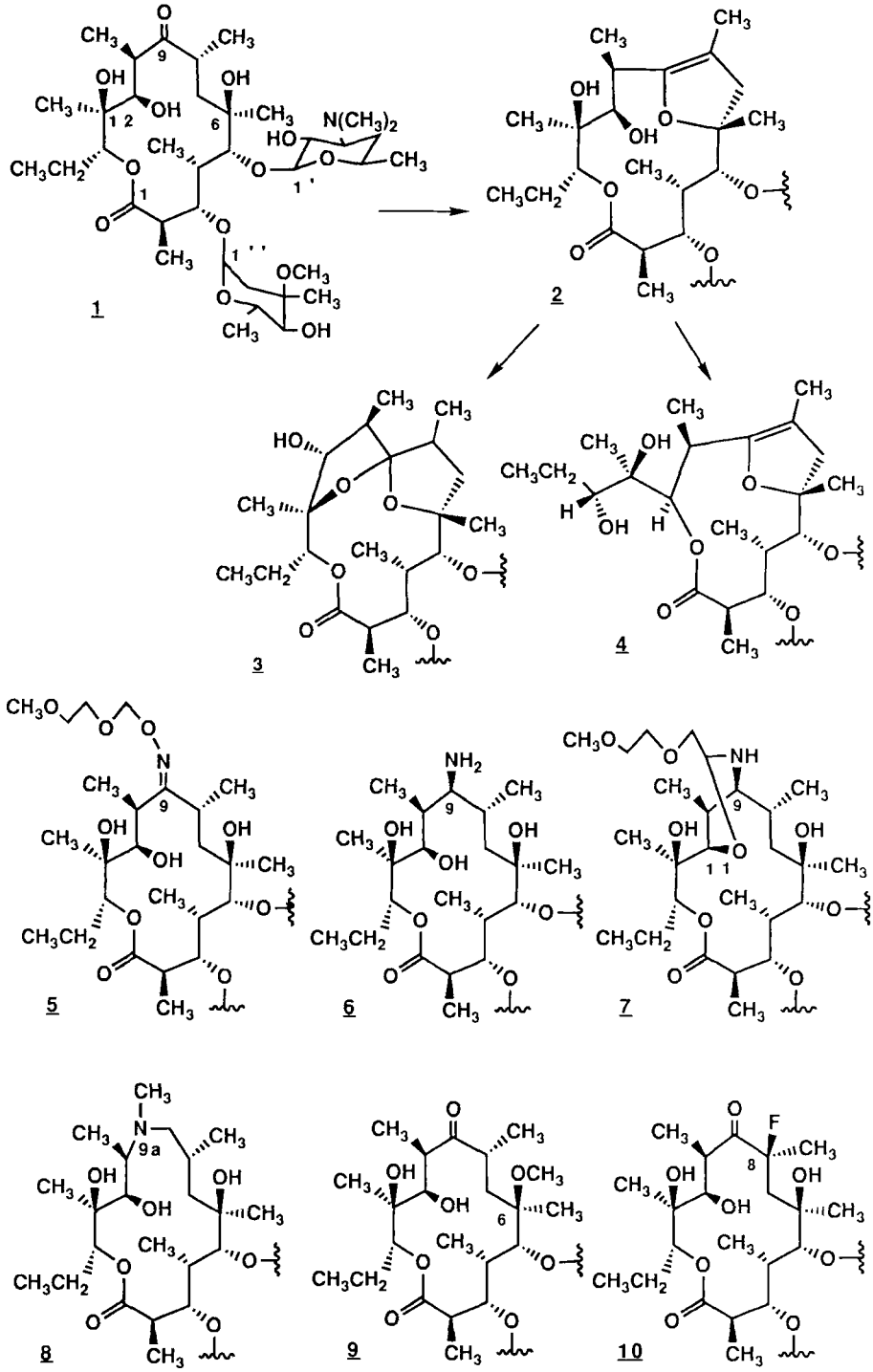
Introduction - The therapeutic utility of macrolide antibiotics is well established in both clinical and veterinary medicine; erythromycin, spiramycin, and tylosin remain important antimicrobial agents (1-6). An excellent book covers work prior to 1984 (7). Over the past decade, several newer macrolides possessing advantageous features have been prepared and evaluated in preclinical tests (8-13). Results are now emerging from clinical trials, which will determine the therapeutic value of these products. In addition to those cited above, other reviews have focused on various aspects of the chemistry and biology of macrolides (14-17). This chapter will summarize some of the more important trends within the class of traditional 14- and 16-membered macrolide antibiotics.

Newer Derivatives of Erythromycin - Decomposition of erythromycin (**1**) under acidic conditions has been thought to proceed via the 8,9-anhydro-6,9-hemiketal **2**, which is subsequently converted into the 6,9;9,12-spiroketal **3**. A different mechanism, in which **2** and **3** arise from **1** independently rather than sequentially, has been recently proposed from kinetic studies (18,19). Trans-lactonization of **2** into the ring-contracted 8,9-anhydro-6,9-hemiketal **4** has also been reported (20,21). Since the intramolecular cyclization of erythromycin destroys its antibiotic activity, much effort has been spent to diminish or completely block this degradative pathway. One approach to preventing this decomposition has utilized water-insoluble, acid-stable salts, esters, and/or formulations to protect erythromycin during its passage through the stomach. The synthesis of new ester derivatives to improve the bioavailability of erythromycin is still being pursued (22,23). One recent example, 2'-O-acetylerythromycin stearate (erythromycin acistrate), is reported to exhibit less liver toxicity and is now in clinical trial (24). In a different approach, two salts of 2'-O-propionylerythromycin (N-acetylcysteinate and mercaptosuccinate), which attempt to combine antibiotic and mucolytic activities into a single agent, are also under clinical investigation (25,26).

Another successful strategy for inhibiting intramolecular cyclization of erythromycin has been directed toward modification of the functional groups on carbon atoms 6-12 of the macrocyclic ring (erythronolide). Many of the derivatives prepared from this approach have exhibited desirable features such as increased stability to acid, greater oral bioavailability, higher serum concentrations, better tissue penetration, and longer body half-life than erythromycin. The most advanced member of this next generation of macrolides is roxithromycin (**5**); its 9-oxime is less prone to participate in intramolecular cyclization than is the 9-ketone of erythromycin (27). Roxithromycin has been launched in France and is undergoing clinical trial in other countries (28). 9(*S*)-Erythromycylamine (**6**) is an older derivative of erythromycin in which the 9-ketone had been converted into an amino group. Dirithromycin (**7**), a more recent oxazine derivative of erythromycylamine, is now in clinical trial; it is a pro-drug which improves the oral bioavailability of erythromycylamine (29). Azithromycin (**8**) is the result of a third route to modification of the 9-ketone of erythromycin; a Beckmann rearrangement of erythromycin-9-oxime expanded the lactone to a 15-membered-ring intermediate, which was subsequently reduced and N-methylated (30,31). Azithromycin has recently been launched in Yugoslavia and is undergoing clinical trial elsewhere (28).

Structures of Erythromycin and its Derivatives



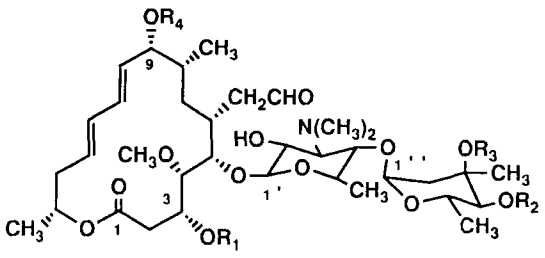
Structural modification of erythromycin at sites other than its 9-ketone has also proven successful in providing new derivatives with improved biological features. The 6-O-methyl derivative of erythromycin, clarithromycin (**9**), has demonstrated higher stability to acidic conditions and superior pharmacokinetic properties when compared with erythromycin (32). Clinical trials of clarithromycin are currently being conducted. Still another approach to blocking the degradation of erythromycin to **2** has involved replacement of the C-8 proton of erythromycin by fluorine; this modification has been achieved by both bioconversion and chemical methods to produce flurithromycin (**10**) (33).

A variety of more recent derivatives of erythromycin have been reported which are less advanced in their development than those described in the preceding paragraphs. Building upon the previously known 11,12-carbonate derivative of erythromycin, a series of 11,12-cyclic carbamates were synthesized which had improved properties in vivo when compared with erythromycin (34). An 11,12-methylene acetal had good in vitro activity, but lacked superior efficacy against infections in mice (35). A series of new 9-N-alkyl derivatives of 9(*S*)-erythromycylamine (**6**) showed better efficacy and bioavailability than erythromycin when administered orally in animal infection models (36). A series of azacyclic derivatives prepared from 9(*R*)-erythromycylamine showed similar in vivo advantages over erythromycin (37). Modifications of the 9-keto and 11-hydroxyl groups of erythromycin inhibited hydrolysis of the lactone by an esterase from *Escherichia coli* which may be involved in bacterial resistance to macrolides (38). From this series of derivatives of erythromycin, its 9-methoxime-11-[(2-dimethylaminoethyl)oxymethyl] derivative (*ER* 42859) was selected on the basis of good antibiotic activity and pharmacokinetics in animals; however, it gave lower blood levels than erythromycin when administered to humans (39). Work in this area has continued with 9-oxime-11,12-carbonate and 9-dihydro-9,11-cyclic acetal derivatives, but no clinical candidate has been reported (40,41). A novel series of 9,12-epoxy derivatives has shown unexpectedly good oral efficacy and bioavailability; such activity was not expected since these compounds resulted from further chemical modification of the intramolecular cyclization product **2** (42). Attempts to correlate the conformation of a variety of derivatives of erythromycin with their antibiotic activity have met with only limited success (43,44).

Newer Derivatives of 16-Membered Macrolides - Among the 16-membered macrolides, josamycin (**11**), spiramycin (**16**) and tylosin (**17**) are well established antibiotics. The most advanced of the newer 16-membered macrolide derivatives are rokitamycin (**13**) and miokamycin (**15**), which have been launched for clinical use in Japan (28). They are, respectively, a 3"-O-acylated derivative of leucomycin A5 (**12**) and a 3",9-di-O-acylated derivative of midecamycin (**14**). Products derived from 3"-O-acylations of the neutral sugar (mycarose) in these 16-membered macrolides have demonstrated pharmacokinetic advantages such as a longer in vivo half-life (45,46).

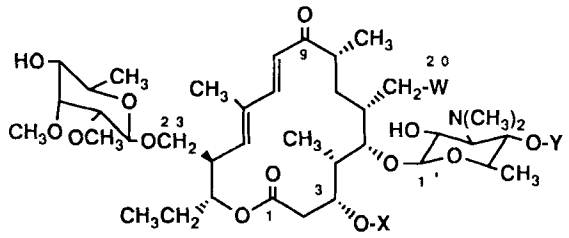
Two new derivatives of tylosin are now in field trials for veterinary applications. The first of these, 3-O-acetyl-4"-O-isovaleryltylosin (**18**), has demonstrated activity against tylosin-resistant organisms and greater oral bioavailability in animals (47,48); a clinically useful derivative of tylosin which exploits these features is also being sought (49-51). The second derivative, tilmicosin (**20**), has expanded the spectrum of tylosin to cover *Pasteurella* species responsible for pneumonia in pigs and cattle (52-54); since modification of the aldehyde of desmycosin (**19**) improved its in vivo activity, a clinically useful derivative within this series is also being sought (55,56). Another related 16-membered macrolide is miporamicin (mycinamicin II), which is being investigated for its potential utility (57). 9-Oxime and 3-O-cladinosyl derivatives of tylosin have been prepared, which represent structural hybrids between tylosin and either roxithromycin or erythromycin (58,59). The beneficial effects of dialkylamino groups, which have been previously observed in other series, have now been further explored with modifications of the 9-keto group of niddamycin (60).

Structures of 16-Membered Macrolides



	R_1	R_2	R_3	R_4
11	Ac	iVal	H	H
12	H	Bu	H	H
13	H	Bu	Pr	H
14	Pr	Pr	H	H
15	Pr	Pr	Ac	Ac
16	H	H	H	For

Ac = acetyl
Pr = propionyl
Bu = butyryl
iVal = isovaleryl
For = forosaminyl



	W	X	Y
17	CHO	H	Myc
18	CHO	Ac	iVal-Myc
19	CHO	H	H
20	CH_2NR_2	H	H

Myc = mycarosyl,
iVal-Myc = 4-O-isovaleryl-mycarosyl,
NR_2 = 3,5-dimethylpiperidinyl

Antimicrobial Activity - In vitro studies of the newer macrolides are too numerous to completely cover in this review; some have been previously summarized (8). Results are now available from extensive in vitro side-by-side comparisons of many of the macrolides which are currently in clinical trial (61-65). While all of these were potent antibiotics against bacteria traditionally covered within the macrolide spectrum, their activity relative to each other was dependent upon the particular bacterium and test conditions (such as presence of serum, etc.) (61,62,66). Azithromycin has achieved the largest expansion in antimicrobial spectrum by increasing in vitro activity against Gram-negative bacteria (66,67); its activity against *Haemophilus influenzae*, *Branhamella catarrhalis* and *Neisseria gonorrhoeae* has increased 2-8 fold over that of erythromycin (67-69). It was orally effective against a middle ear infection in gerbils caused by *H. influenzae* (70). Roxithromycin and clarithromycin have shown efficacy superior to that of erythromycin against experimental infections, including *Legionella pneumophila* infections in guinea pigs (27,71-73). Given once a day subcutaneously, dirithromycin was more efficacious than erythromycin against experimental infections in mice (74). A high proportion of bacteria which have been implicated in dental diseases have proven susceptible to spiramycin (75).

Despite their well established, traditional antibiotic spectrum, opportunities still exist to expand the therapeutic utility of macrolides. *Helicobacter pylori* (formerly *Campylobacter*) is highly susceptible to macrolides in vitro, but treatment of gastric disorders due to this organism has not yet been established (63,76). Azithromycin was effective against infections in rodents caused by *Borrelia burgdorferi*, the causative agent of Lyme disease (77,78). The activity of macrolides against *Mycobacterium avium* complex is being pursued by several groups (79-82); other mycobacteria are susceptible to macrolides in vitro (83,84), and efficacy against *M. leprae* in a mouse leprosy model has been reported (85). Although spiramycin is used to treat toxoplasmosis, more effective agents are being sought (86,87). Toward that end, several groups have reported activity by newer macrolides against acute toxoplasmosis or toxoplasmic encephalitis in mice (88-90). Spiramycin was tried but was found to be ineffective against an acute

diarrhea due to *Cryptosporidium* in pediatric patients (91,92). Roxithromycin was active in a mouse model of chlamydial salpingitis and a rabbit model of syphilis (93,94); it had in vitro activity superior to erythromycin against *Rickettsia* sp. (95). Azithromycin inhibited *Entamoeba histolytica* and organisms responsible for bacterial vaginosis and chancroid (96,97). These results illustrate current efforts to expand the clinical use of macrolides; however, most of these studies concluded that despite in vitro activity and efficacy in animal models, effectiveness in clinical trials must be proven before clinical utility can be claimed for any of these indications.

Macrolides are believed to inhibit bacterial growth by inhibition of protein synthesis; however, this process is complex and full details are unknown (16,98). One approach to better understanding of the macrolide-ribosome interaction has used photoaffinity labeling (99). Macrolides are lipophilic compounds which can penetrate many biological barriers; their accumulation in *Bacteroides fragilis* has been correlated with their antibiotic activity and hydrophobicity (100).

Bacterial resistance, always a concern in medicine, is usually due to modification of an antibiotic target site, enzymatic inactivation of the antibiotic, or reduced uptake into cells. Modification of the ribosomal binding site of macrolides involves methylation of 23S rRNA by an inducibly or constitutively derived methylase (101); some of the structural modifications of 23S rRNA which are correlated with macrolide resistance have been proposed (102,103). One advantage of 16-membered macrolides is their activity against bacteria which are inducibly resistant to erythromycin, although they lack activity against strains which are constitutively resistant to erythromycin (61,62). Recently, derivatives of erythromycin and clarithromycin which were substituted on their 11,12- and/or 4"-hydroxyl groups have shown activity against both types of MLS-resistant bacteria (104,105). Enzymatic inactivation of macrolides has now been found with esterases (106), phosphorylases (107,108), and glycosidases (109). The possible origin of these bacterial enzymes in macrolide-producing organisms is a subject of current debate (110). Resistance due to reduced uptake into bacterial cells has been proposed (111,112).

Pharmacokinetics and Pharmacology - Although expanding the antibiotic spectrum of macrolides is desirable, improvements in their pharmacokinetic properties, such as greater oral bioavailability and higher and more persistent concentrations in fluids and tissues than erythromycin, will also contribute significantly toward the realization of new therapeutic applications. Such improvements have already been partially achieved with the newer macrolides. Although details of their individual pharmacokinetic parameters are beyond the scope of this chapter, monographs for individual compounds (5,10-13,24) and reviews comparing several macrolides are available (9,113-115). The difficulty with animal models as predictors of human pharmacokinetics has also been discussed (116).

One beneficial feature of macrolides is their capability for intracellular penetration, which is important but not sufficient for killing intracellular pathogens (117-119). Several new macrolides have achieved higher concentrations and greater antibacterial activity within macrophages than erythromycin (120-125). Understanding the interactions between macrolides and components of the host's defense system is the subject of much current research (117-127). Treatment of infections associated with inflammation has been suggested (128,129), and reversal of drug resistance in multidrug-resistant (MDR) cells has been reported (130). Macrolides achieve high concentrations in certain tissues such as pulmonary and prostate that exceed concentrations in serum (113,114); this feature may permit therapeutic concentrations of antibiotic to be achieved at the site of localized infections .

Interactions between erythromycin and other drugs have been reviewed (131). The principal route of excretion is via the liver; consequently, the effects of macrolides on hepatic metabolic enzymes, especially cytochrome P-450, have been studied in order to reduce interference with

the metabolism of other drugs (132-134). Some of the newer macrolides are metabolized to products which retain antibiotic activity, thereby giving more persistent antibiotic levels in vivo. Clarithromycin is oxidized to its 14(*R*)- and 14(*S*)-hydroxy derivatives; the former has antimicrobial activity comparable to that of its parent (135). Dirithromycin was previously mentioned as a pro-drug of erythromycylamine (29). Both rokitamycin and miokamycin undergo deacylations, yielding various factors of the leucomycin family (136,137).

The principal side effects associated with erythromycin are gastrointestinal problems. Erythromycin has been recently identified as an agonist of motilin, a natural peptide responsible for stimulating contractility in the gastrointestinal tract (138). Although reduced promotion of gastrointestinal contractility in dogs has been used to select for macrolides with reduced potential to cause gastrointestinal problems, proof of this approach has not yet emerged from clincial studies. A different application of this work has been the development of macrolides related to **2** for the treatment of gastrointestinal motility disorders (139,140).

Clinical Results - Most clinical experience with the newer macrolides has been with miokamycin, rokitamycin and roxithromycin (11-13); many of the early results from the former two compounds and clarithromycin have been published by Japanese investigators (141-143). A monograph on azithromycin has just appeared (10). Most of the initial trials with the newer macrolides have focused on the same indications for which erythromycin has been used. Because of its increased in vitro activity against *H. influenzae*, an initial failure of azithromycin to eradicate that organism in a chronic bronchitis study was noteworthy (144). A review of erythromycin-induced hearing loss has recently been published (145).

Conclusion - Modification of macrolides by both chemical and bioconversion methods continues to generate new antibiotics with potentially useful properties (6,15). Fermentation remains an invaluable source of new macrolides, aided by new screening methods such as ELISA-based assays (146) and studies of biosynthetic pathways (147-149). Molecular biology is now employing its tools to create hybrid and genetically-engineered products (150-154). New therapeutic applications of macrolides are being sought in both clinical and veterinary medicine, and discoveries are being made on the mechanisms by which these agents kill microorganisms and interact with the host's defense systems. It certainly appears that macrolide research is alive and well, offering potential scientific and medical rewards (155).

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Chapter 14: Mechanism-Based Discovery of Anticancer Agents

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Introduction - To achieve increased efficacy and greater selectivity in the chemotherapy of cancer, it is essential to adopt approaches to drug discovery that are more specific and rational than those which have heretofore been used for the detection of lead compounds. In most areas of pharmacology, as exemplified in this and recent volumes of Annual Reports in Medicinal Chemistry, drug discovery has evolved to the use of specific assays with target receptors and enzymes involved in pathogenesis of disease rather than cellular or tissue assays. Such mechanism-based screens are used both for initial detection of leads and for evaluation of lead compounds. Despite rapid advances in our understanding of tumor biology and biochemistry during the past few years, the search for new drugs for the treatment of cancer remains largely dependent on the use of assays for cytotoxicity in cell culture rather than on activity against specific biochemical targets relevant to neoplastic growth. Although efforts are underway, as discussed below, to introduce selectivity criteria into in vitro cytotoxicity assays, the problem remains that: (a) compounds capable of inhibiting cell proliferation in tissue culture are quite common, (b) only a small proportion of cytotoxic compounds demonstrate any degree of antitumor activity in vivo in even the most chemosensitive animal tumor models, and (c) only a small proportion of in vivo "active" antitumor agents demonstrate a spectrum of activity and adequate selectivity in animal solid tumor models to warrant development to clinical trial. Many drugs with minimal efficacy in animal tumor models, often in a narrow spectrum of tumors, have been advanced to clinical trial. The failure of such agents to produce responses in patients with advanced cancer has led to the perception that animal tumor models have little or no predictive value for activity against human tumors. However, drugs with a high degree of efficacy and a broad spectrum of activity in animal tumor models have proven to be useful in the treatment of human cancer. Examples are cyclophosphamide, cisplatin, doxorubicin, etoposide, and the Vinca alkaloids.

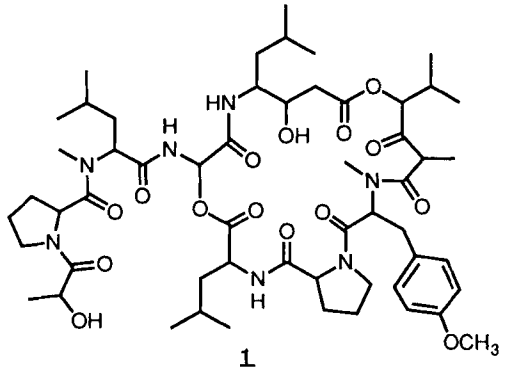
This chapter will focus on the use of mechanism-based assays to detect potential antitumor drugs that interfere with processes relevant to neoplastic growth and will cite examples of compounds identified by such approaches. Agents in preclinical development discovered by more traditional approaches are also included, with examples limited to those drugs that demonstrate a degree of preclinical efficacy which would appear to warrant development to clinical trial. Lastly, compounds that have recently entered Phase I clinical trial are included. Many of these clinical candidates have demonstrated only modest activity in preclinical tumor models as has been the case for the majority of drugs developed for cancer in the past. Several clinical candidates, however, have shown a spectrum and degree of preclinical efficacy comparable or superior to that of the most effective established antitumor agents.

ANTICANCER DRUG DISCOVERY BASED ON BIOLOGICAL ENDPOINTS

Cytotoxicity Assays - Several fermentation-derived compounds with significant antitumor activity were initially identified and isolated in antimicrobial screening programs with subsequent demonstration of mammalian cell toxicity. Notable examples are actinomycins, anthracyclines, bleomycins, and mitosanes. Unfortunately, the number of antimicrobial agents with cytotoxic activity but little or no significant antitumor activity is extensive, and more sophisticated assay methodology is required to select natural products with potential antitumor activity. Nevertheless, novel cytotoxic antibiotics have been isolated and characterized based on antimicrobial activity during the past year. These include 9-methyl-8,9-epoxystreptimidone (1), hataomycin (2), UK-63,052 (3), and spirocardin A and B (4), none of which has been reported to have significant in vivo antitumor activity.

Straightforward cytotoxicity assays with established tumor cell lines continue to be utilized to identify many novel natural products and synthetic agents with potential antitumor activity. It is beyond the scope of this chapter to cite the vast number of novel cytotoxic compounds identified

during the past year for which antitumor evaluation has not as yet been reported. However, a number of natural products identified by their cytotoxic activity have recently entered clinical trial. These include the first marine natural product, didemnin B (1), to be evaluated extensively in man (5). This cyclic depsipeptide may be of greater interest as an immunosuppressive agent in transplantation as its preclinical profile in tumor models is not outstanding (6, 7). Mechanistic studies suggest that didemnin B may act as an inhibitor of protein biosynthesis (8). Another natural product of interest both as an antitumor agent and immunosuppressant is deoxyspergualin (2) (9, 10). An analog of 2 was initially discovered in a screen for inhibition of focus formation in Rous sarcoma virus infected chicken fibroblasts (11). Compound 2 is in Phase I clinical trial in cancer patients (12) despite the finding that its preclinical antitumor activity is limited to leukemia models (9); its mechanism of cytotoxic and immunosuppressive activity has not been elucidated. A novel cytotoxic antibiotic recently introduced into clinical trial (13) is elsamicin A (3). This compound demonstrated activity in preclinical leukemia and solid tumor models and would appear to act by binding to DNA (14).



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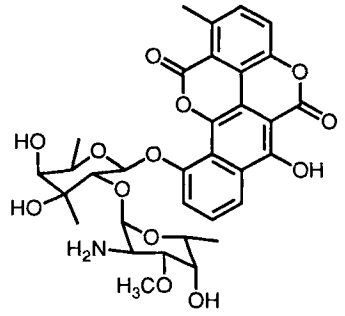

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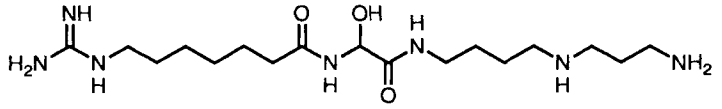

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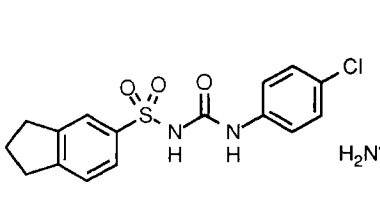
In Vivo Assays for Drug Discovery - Because of the poor translation of cytotoxicity to antitumor activity, a number of programs have utilized in vivo tumor models directly for drug discovery. A recent example of a lead compound from such a program is LY186641 (4), an indanylsulfonylurea derivative with broad-spectrum activity in preclinical solid tumor models (15). Compound 4 is currently in extensive clinical trials based on its impressive preclinical activity and apparent lack of toxicity to proliferating normal tissue (16,17). Other development candidates identified in screening against animal tumor models include girolline (5, RP49532A), NC-190 (6), batracyclin (7), mitoquidone (8), and penclomedine (9). Girolline was isolated from an aqueous extract of a sponge and has demonstrated activity in a spectrum of tumor models (18, 19); it is reported to act as an inhibitor of protein biosynthesis (19). NC-190 was shown to have a high degree of efficacy in both murine leukemias and solid tumors (20). Batracyclin is a water-insoluble quinazolinone derivative with antitumor activity on oral administration in murine colon tumor models (21). Marked species differences in the toxicity of 7 have been noted (22). Mitoquidone was advanced to Phase I clinical trial based on activity in murine solid tumors but only weak activity in the standard prescreens such as P388 leukemia (23). Pulmonary toxicities believed to be associated with the insolubility of 8 truncated clinical trials and led to the subsequent clinical evaluation of a water soluble analog, GR 63178A, with a similar preclinical activity profile (24-26). Penclomedine is under development by the National Cancer Institute (NCI) based on impressive activity in murine solid tumors and human tumor xenografts (27). The mechanisms of action of compounds 6-9 is unknown.



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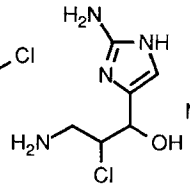

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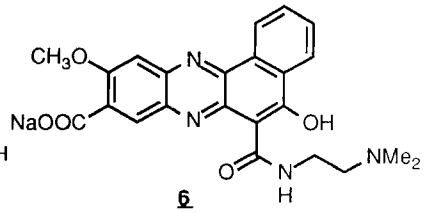

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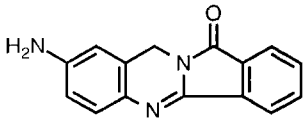

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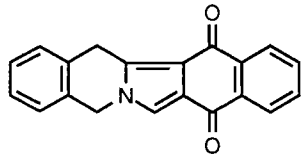

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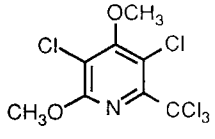

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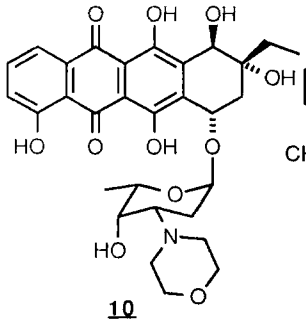
Analog Design - The design and synthesis of analogs of established antitumor drugs has remained an active area of antineoplastic drug development. Many novel anthracyclines have been developed during the past several years; three examples are cited for which initial clinical trials were reported in 1989 (28-30). KRN-8602 (**10**), 4'-iodo-4'-deshydroxydoxorubicin (**11**), and SM-5887 (**12**) are analogs with altered chromophores and/or sugars which demonstrate broad spectrum preclinical efficacy comparable or superior to that of doxorubicin.



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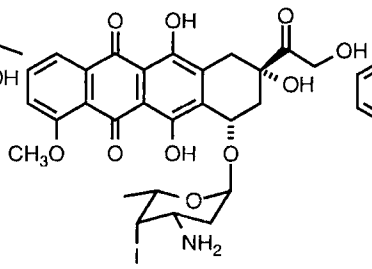

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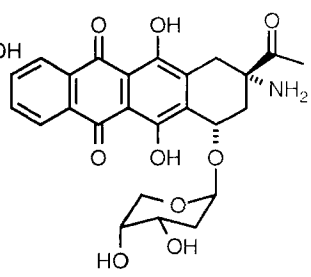
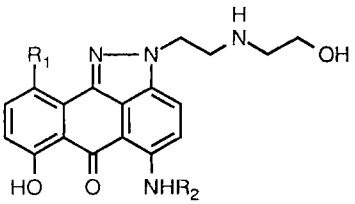
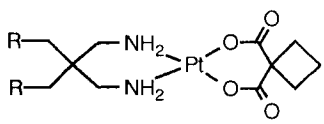


12

In an effort to produce a mitoxantrone derivative with reduced cardiotoxicity, compounds were designed lacking the potential bioreductively activated quinone moiety. A series of substituted anthrapyrazolones with excellent activity in preclinical tumor models was prepared, including oxanthrazole (**13**) and biantrazole (**14**), which have been introduced into clinical trial (31-34). Water-soluble platinum analogs with reduced potential for nephrotoxicity and ototoxicity have been targets for a number of medicinal chemists. Two new compounds with desirable preclinical efficacy and toxicity profiles, CL 287,110 (**15**) and CL 286,558 (**16**), were synthesized (35) and initial Phase I clinical trials reported no evidence of nephrotoxicity (36,37).



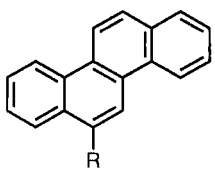
13 R_1 = OH, R_2 = $CH_2CH_2CH_2NH_2$
14 R_1 = H, R_2 = $CH_2CH_2NHCH_2CH_2OH$



15 R = OH
16 R = $-CH_2OCH_2-$

It is perhaps a greater challenge to design a new antitumor agent from first principles rather than modifying the structure of a known compound with activity. The realization that a number of

DNA intercalating agents with antitumor activity contain a planar electron-rich ring system tethered to a basic side-chain led to the design of a series of compounds in which a methylaminopropanediol was appended to diverse polycyclic hydrocarbons (38). The site of attachment and nature of the basic side-chain in these arylmethylaminopropanediols (AMAPs) proved critical to activity in preclinical models. Three AMAPs, crisnatol (**17**, BW770U), BW773U (**18**), and BW502U (**19**), have been advanced to clinical trial (39-41).



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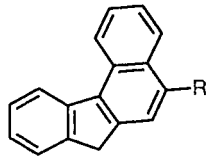

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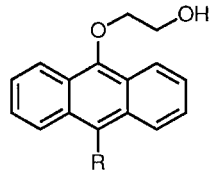

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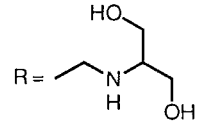
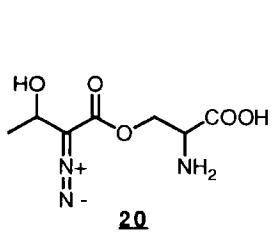


Selective Cytotoxicity Assays - The greatest emphasis on new methods for antitumor drug discovery during the past few years has been placed on the development of more sophisticated cytotoxicity assays to selectively detect those drugs which will have potential for efficacy in the major human solid tumors (42-44). The human tumor clonogenic assay, using primary explants of human solid tumors, has been employed extensively for evaluating the potential of lead compounds arising from drug discovery programs but is not amenable to primary screening of randomly selected compounds or natural product extracts (42). Established human solid tumor cell lines have been adopted as screens in place of primary explants (44). Such an approach might be expected to identify many of the same compounds or types of compounds identified in past screens. After all, human KB and HeLa cells have been mainstays of cytotoxicity screening for many years. One means to incorporate a selectivity criterion is to screen against an appropriate human solid tumor cell line in parallel with a murine tumor cell line to identify compounds which might be effective in human tumors, but would be missed by the in vivo animal tumor models used subsequently to establish antitumor activity. An interesting discovery from such a screen is FR 900840 (**20**), a close analog of azaserine, a cytotoxic antibiotic discovered in the 1950's (45). FR 900840 is cytotoxic to human A549 lung cancer cells, but minimally to murine P388 leukemia cells, and was shown to be effective in a number of human solid tumor xenografts, but not in P388 leukemia or other chemosensitive murine tumor models in vivo (46).

Another configuration of a selective cytotoxicity assay is to utilize a solid tumor and leukemia cell line to identify agents which may be selectively active against solid tumors (47,48). Ideally, such an approach would identify new mechanisms of cytotoxicity specifically applicable to solid tumors. An example of a compound active in such an assay is flavone-8-acetic acid (47). Although this compound was initially identified based on activity against murine leukemias in vivo (49), it has demonstrated selectivity against solid tumors in vitro and in vivo (47). Despite disappointing activity in clinical trials (50,51), flavone-8-acetic acid may prove useful in identifying new mechanisms for inhibiting tumors (52). An extension of this approach to identify selectively cytotoxic drugs is the establishment of a screening panel of histologically distinct human tumor cell lines. Such an approach, as currently being undertaken by the NCI (44), is predicated on the assumption that exploitable mechanisms for histiospecific cytotoxicity exist and that such differences will be detectable in long-term tissue culture-adapted cell lines. Clearly, histiospecific toxicity in normal cell populations exists, often as a result of cell type-specific activation (53). The question is whether these histiospecific toxicities will extend to malignant tissue. Supporting this concept is mitotane, which was developed for the treatment of adrenocortical carcinoma based on its adrenolytic properties (54). A current example of a histiospecific cytotoxic agent in Phase I clinical trials is 4-ipomeanol (**21**), a fungal toxin selectively activated by bronchial Clara cells (55, 56). It will be of interest to see whether **21** will show activity in lung cancer at doses tolerated by normal bronchial epithelium.

Multiple drug resistance is a major problem in cancer chemotherapy as discussed in detail in the previous two volumes of this series (57,58). Screens have been configured to identify compounds which retain cytotoxic potency (or demonstrate increased activity) against cells expressing the multidrug-resistant phenotype. Such a screen was used to identify a novel antibiotic, phenazinomycin (**22**), which is 8-fold more cytotoxic to doxorubicin-resistant P388 leukemia cells than to the parent cell line (59).



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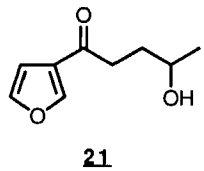

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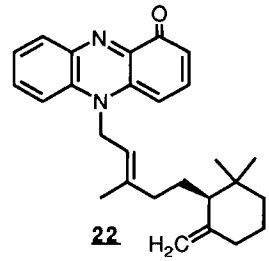
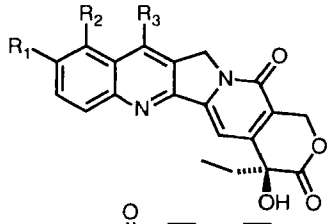


22

MECHANISM-BASED ANTICANCER DRUG DISCOVERY

Although cytotoxicity assays and animal tumor models still play a critical role in the evaluation of lead compounds and selection of development candidates, mechanism-based approaches are increasingly used for initial detection of leads and to guide synthetic efforts. Specific biochemical assays can be used to discover and develop cytotoxic drugs which work by mechanisms similar to those of established anticancer drugs; e.g., production of DNA damage, inhibition of nucleic acid biosynthesis, or interference with tubulin function. Such approaches are also being employed to identify lead compounds which selectively interfere with the expression of oncogenes or the function of oncogene products demonstrated to play a role in the malignant phenotype.

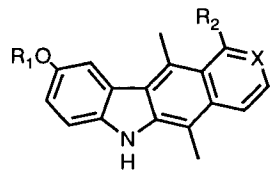
Topoisomerase Inhibitors - Among mechanism-based approaches to anticancer drug discovery, the design and synthesis of topoisomerase inhibitors has received a great deal of attention recently. Many of the clinically important anticancer agents marketed at present appear to work by inhibiting topoisomerase II, an enzyme linked to cellular proliferation (60). Camptothecin and its close analogs are the only known specific inhibitors of topoisomerase I, and derivatives are currently under development. Because toxicities of camptothecin could be related to its poor water-solubility, two groups have concentrated on introducing polar side groups to the alkaloid so as to facilitate solubility while maintaining inhibition of topoisomerase I. CPT-11 (**23**) appears to be a water-soluble prodrug that hydrolyzes to an active enzyme inhibitor in vivo; SK&F 104864 (**24**) is a water-soluble derivative of camptothecin that retains potent topoisomerase I inhibition. Both **23** and **24** demonstrate a broad spectrum and high degree of activity in murine and human tumor xenograft models and are in early clinical trial (61,62). The water-insoluble analog, 9-aminocamptothecin (**25**), has recently been reported to have striking activity in human colon cancer xenografts in mice (63).



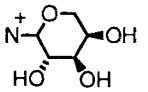
23 $R_1 = -O-C(=O)-N$ (piperazine) $-N$ (piperazine) $R_2 = H$, $R_3 = Et$

24 $R_1 = OH$, $R_2 = CH_2NMe_2$, $R_3 = H$

25 $R_1 = H$, $R_2 = NH_2$, $R_3 = H$



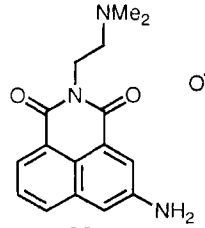
26 $R_1 = R_2 = H$, $X = N^+CH_2CH_2NEt_2$

27 $R_1 = R_2 = H$, X = 

28 $R_1 = CH_3$, $R_2 = NH(CH_2)_3NEt_2$, X = CH

Among topoisomerase II inhibitors, three ellipticine analogs have recently entered development. Datelliptium (**26**, SR95156B) appears to display fewer cardiovascular effects than elliptinium in preclinical models and is currently in Phase I clinical trials (64). The water-soluble ellipticine arabinoside, SUN4599 (**27**), has preclinical activity superior to that of elliptinium and has less hepatic and renal toxicity; it is in a Phase I clinical trial in Japan (65,66). SR95325A (**28**) is an ellipticine analog that, like **26**, contains a basic side chain off the pyridine ring; it was shown to be superior to elliptinium in several preclinical tumor models and is awaiting clinical trials (67).

Amonafide (**29**) is a DNA intercalator that was recently shown to be a potent inhibitor of topoisomerase II (68). Like most other anticancer agents that inhibit this enzyme, amonafide produces protein-linked DNA breaks in mammalian cells by stabilizing a covalent DNA-topoisomerase II complex; in addition, **29** seems to have unusual DNA site selectivity. Amonafide is currently undergoing Phase II clinical trials and has been found to have interpatient variability in metabolism (69). Fostriecin (**30**) is a topoisomerase II inhibitor with a mechanism that is markedly different from existing antineoplastic agents that inhibit this enzyme (70). Fostriecin inhibits the catalytic activity of topoisomerase II in an uncompetitive manner and does not stabilize the covalent enzyme-DNA complex or cause DNA strand breaks. Another antitumor agent that inhibits the catalytic activity of topoisomerase II without producing enzyme-linked DNA breaks is merbarone (**31**). This thiobarbituate derivative, which appears to act by binding to a non-covalent complex between DNA and topoisomerase II, inhibits the production of topoisomerase II-associated breaks by other antitumor agents (71). Merbarone is currently undergoing Phase I clinical trials (72).



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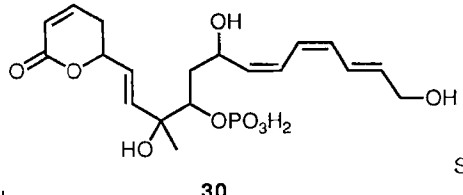

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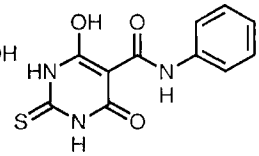
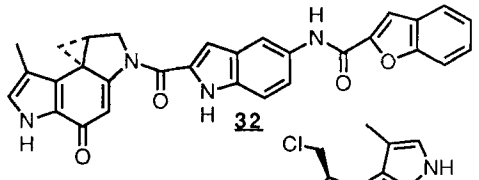


31

DNA-interactive Agents - Among the most useful anticancer drugs in the clinic are DNA-interactive agents, including alkylating agents and non-covalent binders. A novel group of DNA alkylating agents based on the structure of a very potent antibiotic, CC-1065, was recently advanced to development. To eliminate the delayed toxicity problem of CC-1065, chemists simplified its structure to U-73975 (**32**). This compound demonstrates broad spectrum activity, lacks delayed toxicity in animal tests, and is currently in Phase I clinical trials (73). Analogs containing two DNA alkylating subunits, such as U-77779 (**33**), are capable of forming interstrand DNA crosslinks and are more potent than **32** in vitro and in vivo (74,75). Another group of extraordinarily potent and novel DNA-interactive antitumor agents are the calicheamicins (76) and esperamicins (77). These interesting compounds, exemplified by esperamicin A_1 (**34**), produce single- and double-strand DNA breaks which are thought to be initiated by the formation of a phenylene diradical intermediate.



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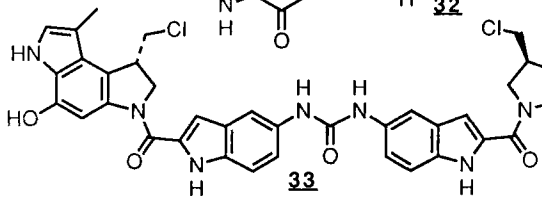

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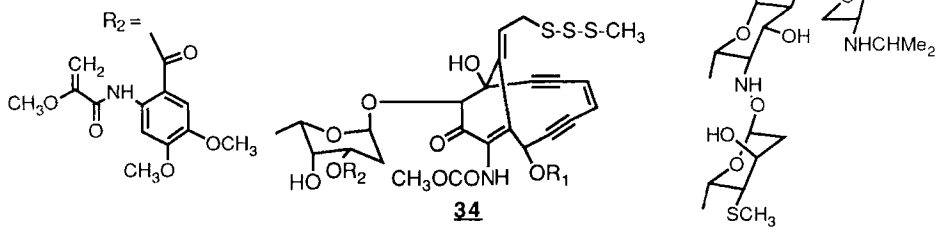
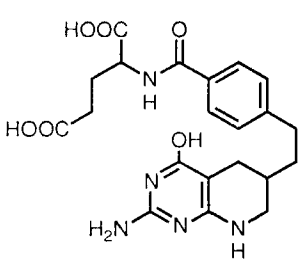


34

Inhibitors of Nucleotide Biosynthesis and Incorporation - Drugs affecting de novo purine or pyrimidine nucleotide biosynthesis and the incorporation of nucleoside triphosphates into nucleic acids are important in cancer chemotherapy. Medicinal chemists continue to explore analogs of known antimetabolites as well as novel targets within the nucleotide biosynthetic pathways. Four new antimetabolites reached clinical trials in 1989. 5,10-Dideaza-5,6,7,8-tetrahydrofolic acid (**35**, DDATHF) is a novel antifol which acts as an inhibitor of glycinamide ribonucleotide transformylase, an enzyme involved in de novo purine nucleotide biosynthesis (78). DDATHF, in contrast to methotrexate, demonstrates excellent activity in a spectrum of animal solid tumor models (79). 2',2'-Difluorodeoxycytidine (**36**, LY188011), a mechanistically novel pyrimidine antimetabolite (80), has demonstrated efficacy in a broad-spectrum of murine tumors and human tumor xenografts (81). Initial clinical trials on LY188011 have been reported (82,83) with some preliminary evidence of activity. Another pyrimidine analog which recently entered clinical trial (84) is fazarabine (**37**, 5-azacytosine arabinoside). Aphidicolin is a novel terpenoid fermentation product which is widely used as a research tool as it is a selective inhibitor of DNA polymerase α (85). A water-soluble prodrug, aphidicolin glycinate (**38**), has recently entered Phase I clinical trial (86) based on a modest profile of efficacy in animal tumor models (87).



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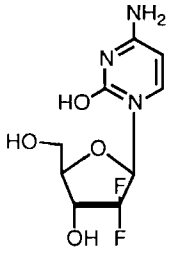

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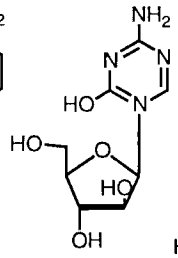

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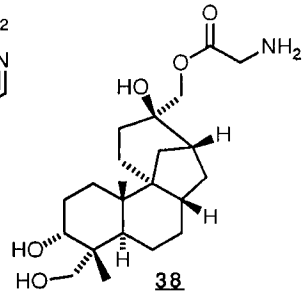
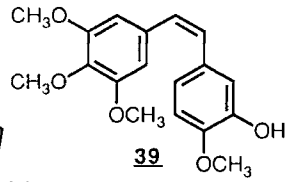


38

Antimitotic Agents - Several useful antitumor agents bind to tubulin and interfere with the assembly of microtubules, resulting in mitotic block. The most well-described antimitotic agents bind to the colchicine site on tubulin. Among these compounds are the combretastatins such as compound **39** which inhibits tubulin polymerization and demonstrates antineoplastic activity in animal models (88). The marine natural product dolastatin 10 (**40**) is a linear pentapeptide with potent cytotoxic activity that binds to tubulin at the Vinca alkaloid binding site and inhibits microtubule assembly (89). The most promising new antimitotic agents are taxol (**41**) and the recently described derivative, RP56976 (**42**). Taxol, a novel diterpenoid isolated from bark of the Western yew, has a novel mechanism. It enhances the rate of microtubule assembly and results in the formation of aberrant microtubule bundles, leading to mitotic arrest (90). Taxol is currently in Phase II clinical trials and has been shown to have activity in refractory ovarian cancer and melanoma (91). A major problem in the development of taxol has been the scarcity of material. The semi-synthetic compound, RP56976, is prepared from a more abundant plant taxane derivative and has an improved profile of activity in preclinical models (92).



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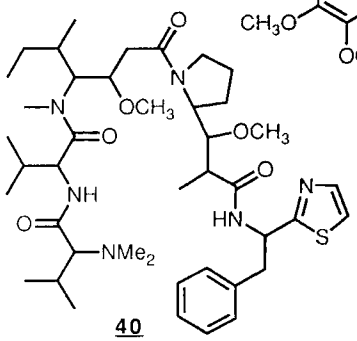

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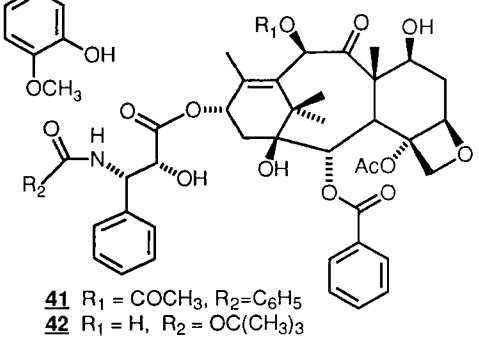
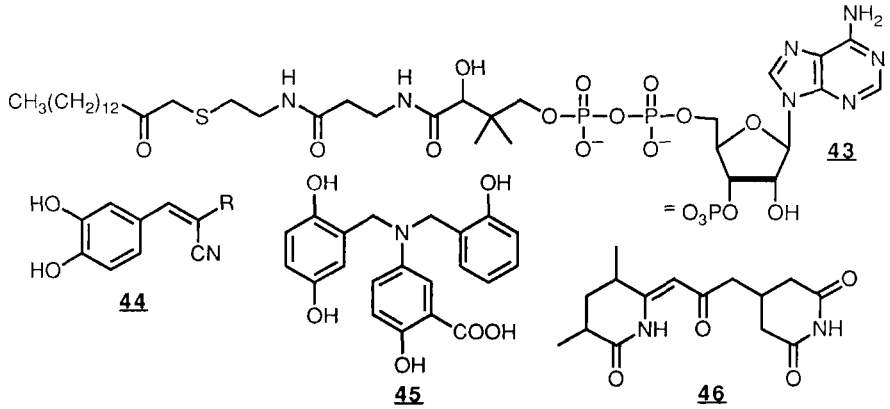


41 $R_1 = COCH_3$, $R_2 = C_6H_5$
42 $R_1 = H$, $R_2 = OC(CH_3)_3$

Inhibitors of Growth Factor Receptor Function and/or Protein Tyrosine Kinase Activity - Oncogenes encoding growth factor receptors or the protein tyrosine kinase domains of such receptors play an important role in malignant transformation and were reviewed in last year's volume (58). A number of leads have been discovered in the past year using specific assays to detect inhibitors of growth factor receptor function or activity. The post-translational modification of oncogene products such a $pp60^{v\text{-}src}$ by N-terminal myristoylation appears to play a role in the membrane translocation and cell transforming activity of these proteins and provides a target for therapeutic intervention (93). The design of an inhibitor of myristoyl CoA:protein N-myristoyl transferase (NMT) has been described; S-(2-oxopentadecyl)-coenzyme A (**43**) inhibits mouse brain NMT with an IC_{50} of 100 nM (94). Compounds derived from the benzylidenemalononitrile nucleus have been extensively investigated as inhibitors of EGF receptor tyrosine kinase (EGFRTK) (95). These compounds, named tyrphostins (**44**), have been shown to be effective inhibitors of EGFRTK and are orders of magnitude less potent as inhibitors of insulin receptor tyrosine kinase activity. The SAR for tyrphostins has been described and compounds **44** with trans substitution, R, in the cis-cinnamonitrile nucleus show improvement in activity as follows: COOH < CN < $CONH_2$ < $CSNH_2$ ≈ $C(NH_2)=C(CN)_2$. The most potent tyrosine kinase inhibitor described to date is lavendustin A (**45**) with an IC_{50} of 12 nM (96). This microbial metabolite was discovered in a screen using A431 cell membranes and a synthetic peptide substrate. The discovery of a novel microbial metabolite was described using an assay for inhibition of EGF-induced mitogenesis (97). Epiderstatin (**46**) is an unusual glutarimide antibiotic that inhibits EGF-induced thymidine incorporation in quiescent A431 cells at 17 nM; in proliferating cells, epiderstatin has little effect on DNA synthesis at much higher concentrations. Epiderstatin does not inhibit EGFRTK activity.



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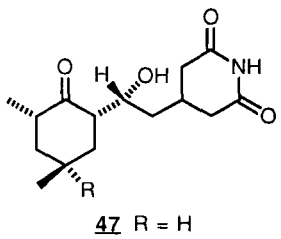
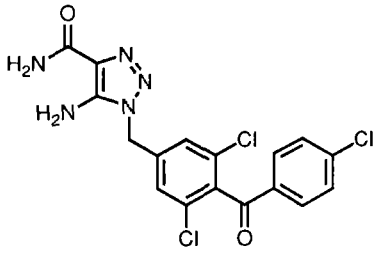
Signal Transduction Pathways - Interference with the transduction of signals from cell membrane receptors to the nucleus is another potential target for therapeutic intervention in malignancy. Oncogene products, notably $p21^{ras}$, play a role in signal transduction. Other targets in signal transduction such as phospholipase c, phosphatidylinositol kinase (PIK), and protein kinase c (PKC) can be targeted in antitumor drug discovery. In a screen for compounds which would induce reversion of Ha-ras-transfected NRK cells to a normal phenotype, the well-known glutarimide antibiotics, cycloheximide (**47**) and acetoxycycloheximide (**48**), were rediscovered (98). Interestingly, **48** is active in this assay at 72 nM and is specific for Ha-ras transformation; cells



47 R = H
48 R = $OCOCH_3$



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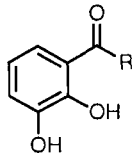

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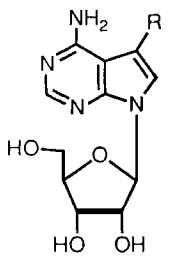
transformed with abl, Ki-ras, Rous sarcoma virus or adenovirus are unaffected. Other eukaryotic protein synthesis inhibitors are negative in the assay, and it was shown that **47** and **48** selectively inhibit synthesis of the p21 Ha-ras gene product compared to other cellular proteins. L651582 (**49**) was reported to inhibit tumor cell motility and adhesion as well as cell growth in culture and in nude mice (99). The proposed site of action of **49** is a pertussis toxin-sensitive G-protein involved in autocrine regulation of growth, adhesion and motility. In a screen for inhibitors of PIK (100), 2,3-dihydroxybenzoic acid (**50**, R=OH) was isolated from a Streptomyces and found to be a potent (IC_{50} = 0.7 μM) inhibitor of PIK; the benzaldehyde (**50**, R=H) was also effective (IC_{50} = 0.45 μM). Other hydroxy substitution patterns in this simple molecule abolished activity.

Novel and potent inhibitors of PKC have been recently described as potential antitumor agents. Sangivamycin (**51**, R=$CONH_2$), previously identified as an inhibitor of PKC (101), was rediscovered in a screening program to detect new PKC inhibitors using inhibition of phorbol ester-induced blebbing of cells (102). Notably, the related 7-deazaadenosine antibiotics tubercidin (**51**, R=H) and toyocamycin (**51**, R=CN) are inactive as PKC inhibitors. The 7-hydroxy derivative (**52**, UCN-01) of the potent PKC inhibitor, staurosporine, was discovered using an in vitro PKC assay (103). UCN-01 proved to be as potent as staurosporine but more selective as a PKC inhibitor (IC_{50} of 4.1 nM as opposed to 42 nM for protein kinase A). UCN-01 is a potent cytotoxic agent in HeLa cells (IC_{50} of 4.1 nM). Another class of PKC inhibitors was discovered by the same assay (104). The most potent compound in this series is calphostin c (**53**), which inhibits PKC at 50 nM and is cytotoxic to HeLa cells at 230 nM. Although less potent than staurosporine analogs, **53** is a highly selective PKC inhibitor (no inhibition of PKA at 50 μM).

Nuclear Regulatory Proteins - An important class of oncogenes are those which encode nuclear regulatory proteins — e.g., myc, jun, fos. Efforts to discover agents that interfere with the expression or function of these oncogene products could lead to novel antitumor drugs. It was recently demonstrated that the ansamycin antibiotics, geldanamycin (**54**) and herbimycin A (**55**), inhibit expression of c-myc in L51784 leukemia cells with a coordinate inhibition of the initiation of DNA synthesis (105). Inhibition by **54** and **55** is evident at submicromolar concentrations and is not related to a general inhibition of transcription. These effects of ansamycins may be related to an indirect action on gene expression. Herbimycin A is known to inhibit transformation by src (106) and to inhibit $pp60^{src}$ tyrosine kinase activity (107).



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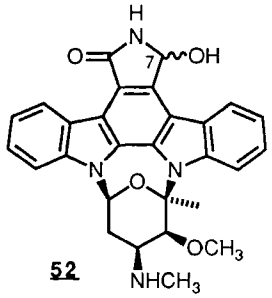

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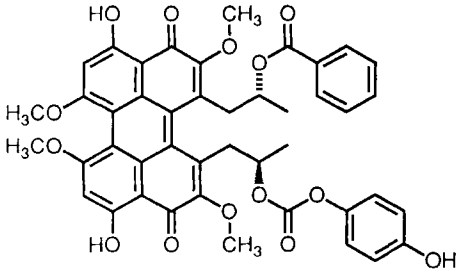

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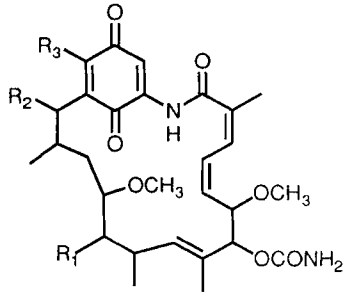

54 R_1 = OH, R_2 = H, R_3 = OCH_3
55 R_1 = R_2 = OCH_3, R_3 = H

CONCLUSION

Significant progress has been made in adopting new strategies for discovery of novel antitumor drugs using mechanism-based assays. Such approaches apply to targets of the cytotoxic drugs with established selectivity for tumors as well as to targets evolving from the study of oncogenes and tumor cell biology. A number of other targets beside those mentioned in this brief chapter can be exploited. Examples are: recombination pathways and DNA damage repair, proteases and adhesion molecules involved in invasion and metastasis, endothelial receptors for tumor-derived angiogenesis factors, and protein kinases and phosphatases involved in the control of cell division. Major advances in the understanding of each of these areas will provide further opportunities for drug discovery. It should be noted, however, that the less selective biological screens for new antitumor agents discussed above continue to identify novel compounds with interesting activity and selectivity in animal tumor models (and, hopefully, in cancer patients). These novel compounds could lead to the identification of new targets relevant to cancer therapy.

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Chapter 15. Targets for Antifungal Drug Discovery

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Introduction - During the last few years, it has been observed that the incidence of fungal infections has increased, and that a broader spectrum of fungal infections has emerged (1). These changes have been attributed to the intensive use of chemotherapy for bacterial infections, wider usage of catheterization, and immune suppression due to organ transplantation and other medical procedures. Many reports describe infections by fungi not previously recognized as human pathogens, as well as previously unknown modes of evasion of immune surveillance used by fungi (2-7). The advent of AIDS has introduced an additional group of immunocompromised patients who are predisposed to fungal infections with a unique pattern of infection. For example, in this patient population oral and esophageal *Candida* infections are prevalent, while *Cryptococcus* has emerged as a major pathogen. As a result of these developments, the relative importance of the fungal pathogens has changed in recent years (8-10).

In view of the scarcity of agents currently available to the physician, there is an evident need for accelerated development of new and more effective antifungal drugs, especially for treating systemic infections. This chapter will summarize recent work on existing agents, new agents under development, and on new potential targets for antifungal drug design.

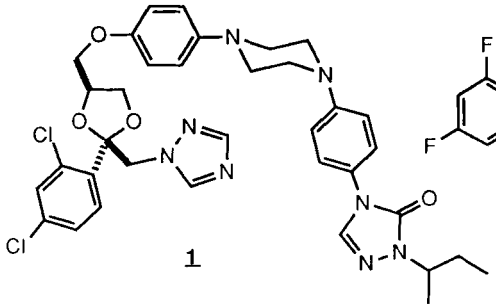
CLASSICAL ANTIFUNGAL AGENTS

Polyene macrolides - Amphotericin B, a fungicidal agent which causes disruption of the fungal membrane, remains the most useful of the systemic antifungal drugs despite its nephrotoxicity. Most of the current work in this field is devoted to the reduction of the toxicity of Amphotericin B by altering its pharmacokinetic properties, using either liposomal preparations or colloidal suspensions of the drug complexed with other constituents of cell membranes (11-14). Efforts to reduce the toxicity of Amphotericin B by chemical modification have not been successful thus far (15). Resistance to the drug has not been considered to be a major problem (16), but a recent survey suggests that resistance among species of *Candida* and *Torulopsis* is becoming significant, and may be due to changes in the fungal population (17).

Azoles - The azoles affect fungal membranes by inhibiting the cytochrome P450-linked monooxygenase component of C14-lanosterol demethylase. Ketoconazole, the leading member of this class is orally active, and has been widely used for many years. This agent, in common with other members of this class, suffers from several disadvantages; it is fungistatic rather than fungicidal, it inhibits the biosynthesis of testosterone, it has some hepatotoxicity, and it is poorly absorbed into the cerebrospinal fluid.

New and improved azoles such as itraconazole (**1**) and fluconazole (**2**) are marketed in some countries, and many others, including Sch-39304 (**3**) and ICI-195739 (**4**) are in various stages of development. These newer triazoles have a high degree of specificity for the fungal enzyme and have mean elimination half-lives 3-4 times that of ketoconazole (18, 19). Some of the agents are claimed to be active against *Aspergillus fumigatus*, which is resistant to ketoconazole; others are active against *Cryptococcus*, which is a major cause of meningitis. Due to the efficient penetration of *C. albicans* by compounds such as ICI-195739, and to the selectivity of these agents for the fungal enzyme, they are

effective at doses lower than those used with the earlier azoles. Recent cloning of the gene encoding lanosterol 14-α-demethylase in *S. cerevisiae* and *C. albicans* will permit detailed comparison of the fungal genes with the P450 gene family of higher eukaryotes. This should assist rational design of azole antifungals by exploitation of the unique structural features of specific fungal enzymes (20-23).



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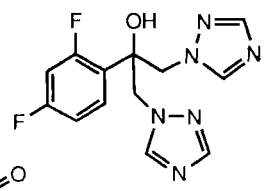

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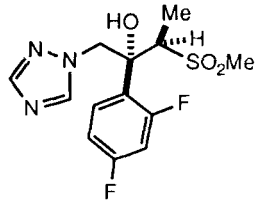

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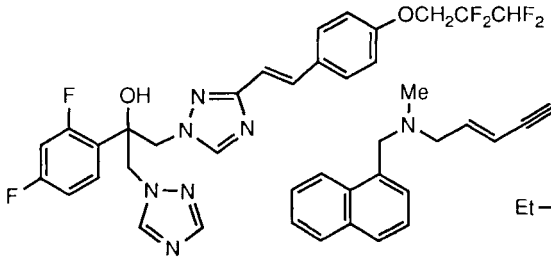
The concentration of antifungal drug research on one specific enzyme in the ergosterol biosynthetic pathway has caused concern, since resistance to some of the azoles has been documented (24-27). Fortunately, other enzymes in the ergosterol biosynthetic pathway have recently been identified as antifungal targets; squalene epoxidase is inhibited by the allylamine class of antifungals, and Δ^{14}-reductase and Δ^{8}-Δ^{7}-isomerase are inhibited by the morpholine class (28,29). Terbinafine (5) and amorolfine (6) are representatives of these classes.



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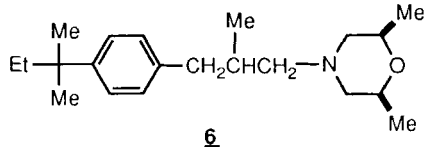

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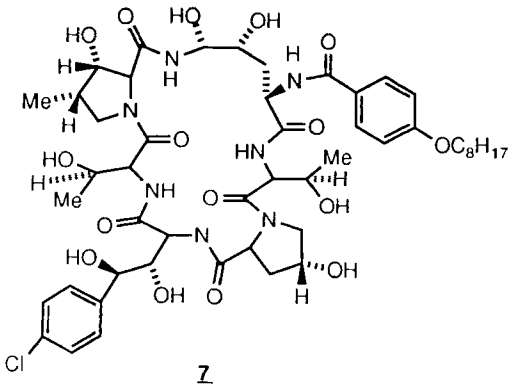
5-Fluorocytosine - 5-Fluorocytosine is metabolized via the salvage pathway. It is taken up by cytosine permease and metabolized to 5-fluorouridine triphosphate, which is a precursor in the biosynthesis of RNA, and to 5-fluorodeoxyuridylate, which is an inhibitor of thymidylate synthase (30,31). The synthesis of these two metabolites does not seem to be linked; both products can severely affect cell growth. The major liabilities of 5-fluorocytosine are bone marrow suppression and development of resistance (32-34). The mutations which confer resistance are recessive, and it is unclear why homozygosity occurs so frequently in a diploid asexual organism such as *C. albicans*. It is possible that mitotic recombination or chromosomal instability commonly occur in this organism. Recent observations of the high frequency of chromosomal instability in *C. albicans* may provide some explanation of this phenomenon (35-38).

Cell Wall Synthesis Inhibitors - Agents currently under development include a semi-synthetic polypeptide, cilofungin (7), which inhibits the enzyme ß-1,3-D-glucan synthase (39), and nikkomycin Z (8), which inhibits chitin synthase (40,41). Cilofungin was derived from echinocandin. It has a narrow spectrum of activity, directed primarily against *C. albicans* and *C. tropicalis*. Nikkomycin Z is not effective against *Candida* species, but has activity against other species of lesser clinical importance.

Efforts to develop other inhibitors of chitin synthase and glucan synthase (polyoxin D, papulacandin B) have not yielded agents effective against medically important fungi (42-44). A new lipopeptide similar to echinocandin B (L-671,329) which has a spectrum of activity similar to cilofungin has been reported (45,46).



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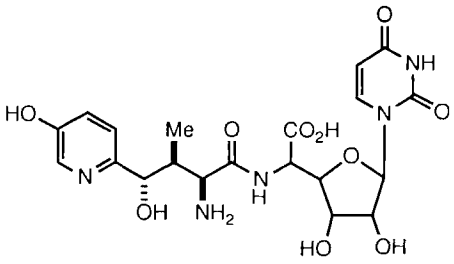

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IDENTIFICATION AND EVALUATION OF NEW TARGETS

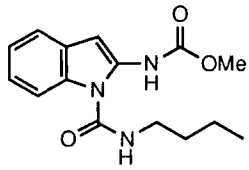
The technique of gene disruption (or gene transplacement) which was developed for non-pathogenic yeasts and filamentous fungi (47-51) is being extended to pathogenic fungi. Specific gene sequences are isolated and manipulated ex vivo, and then returned to the organism, replacing the normal allele by the modified allele (50-53). When successful, this method establishes whether the isolated gene sequence is essential for the life of the organism, and whether it is associated with virulence. Using gene disruption methodology, the two genes encoding chitin synthase in *S. cerevisiae* were identified, one of which was essential for cell division (54). Gene disruptions have been performed in *C. albicans* with the genes encoding orotidine-5'-phosphate decarboxylase and 3-isopropylmalate dehydrogenase (50,51). In the next phase of this work, similar experiments will be performed with genes which encode functions which can be considered as targets for antifungal therapy.

The difficulties which have been experienced in identifying specific fungal targets are due in part to the functional conservation of protein sequence which encompasses all eukaryotic organisms. This has been demonstrated in studies in which *S. cerevisiae* was used as a model of a complex eukaryotic cell (55-61). Nevertheless, it has been found that even in relatively conserved systems, the difference between fungal and mammalian sequences is sufficient, in some instances, to permit selective attack on essential fungal functions. This is illustrated below, in discussion of four areas of interest: tubulins, secreted proteinases, protein synthesis and cell surface components.

Tubulins - Some of the cytoskeletal proteins have been of interest for many years as pharmacological targets. Among these are the microtubules (MT) (62,63) which play a major role in cell division, secretion, motility and cell morphology. The MT are complex structures consisting of two major proteins, the α and β tubulins, and MT associated proteins that stabilize the MT (64). Additional endogenous factors such as GTP, Mg^{+2} and Ca^{+2} modulate the assembly/disassembly process of MT. The microtubules in the cell are in a dynamic equilibrium undergoing addition and subtraction of tubulin. The equilibrium is precisely regulated and any imbalance in the synthesis or polymerization of the α and β tubulins is harmful to the cell (65,66). The α and β tubulins from both lower eukaryotes and higher eukaryotes including man have been isolated and sequenced. Both proteins appear to be highly conserved and functionally interchangeable even across different species (67-69).

A number of inhibitors of tubulin assembly or disassembly have been identified, with the majority of the most potent compounds being derived from natural products (70-72). Some are currently in use as antineoplastic agents and others are undergoing clinical investigation primarily for cancer chemotherapy. Irreversible arrest of cell division by inhibition of microtubule assembly/disassembly is a lethal event to the affected cell.

In addition to the natural tubulin binders, synthetic agents such as the benzimidazole carbamates have been developed and used as anthelminthic agents since the 1960s. The fungicide benomyl (**9**), of which the active component is methyl benzimidazole-2-yl carbamate (**10**), has been in use against fungal plant pathogens for more than 20 years. This tubulin binding agent displays high affinity for fungal tubulin and low affinity for plant and mammalian tubulin (73). Nocodazole (**11**), an experimental antineoplastic agent that is an analog of **10**, displays a high affinity to both fungal and mammalian β tubulin. Resistance to the benzimidazole carbamates is still not widespread and relatively few reports of resistance have appeared. Thus, a range of tubulin binding agents are currently in use as selective drugs.



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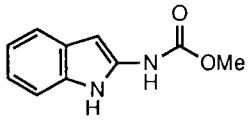

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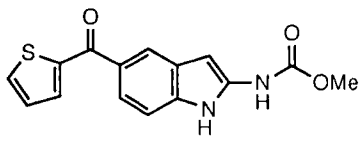

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Three pharmacologically relevant binding sites on the tubulins have been defined by the use of competitive binding experiments, molecular genetic analysis and binding studies with labeled photoaffinity probes (74,75). These sites are referred to as the colchicine, vinblastine and taxol sites according to the inhibitor that interacts with each site. It is apparent that in spite of the high degree of conservation, (a) not all tubulins possess all three binding sites; for example, the vinblastine site has been shown clearly only in mammals and echinoderms; (b) the colchicine binding site displays species differentiation; and (c) modified benzimidazole carbamates show different binding affinities to tubulins from different organisms (76). In retrospect, these findings are not surprising since the tubulin binding agents from natural sources display a high degree of species specificity that may serve as a natural defense mechanism for these organisms (71).

It appears that all fungal tubulins are sensitive to **9** and **10**. Until most recently it was assumed that some inherent property of *C. albicans* β tubulin makes it resistant to benzimidazole carbamates. Recent studies have shown that the nucleic acid sequence of the *C. albicans* β tubulin is very similar to *S. cerevisiae* which is sensitive to **9** and **10** (77). Furthermore, expression of *C. albicans* β tubulin in cells of *S. cerevisiae* in which their single gene of β tubulin had been inactivated does not confer resistance to these cells (78). These results indicate that the resistance to compounds **9** and **10** is not due to an inherent property of the β tubulin of *C. albicans,* but results from some other resistance mechanism found in *C. albicans.* The gene that confers resistance has been isolated and characterized. It confers resistance to other agents in addition to the tubulin binding agents, but its sequence is not related to the multiple drug resistance gene of higher organisms (79,80). Therefore, although the structure-activity relationships of the benzimidazole carbamates are known to the point at which rational design of fungal specific agents can be performed, additional modification is required to bypass the resistance mechanism. For detection of new leads from either natural sources or synthetic derivatives, genetic systems are available for tests with intact cells based on simple phenotypic detection of chromosome nondisjunction. This is a typical characteristic of tubulin binding agents that disrupt the mitotic apparatus (81). Other mechanistically based tests can be developed which are

based on the rescue of overexpression of dominant lethal functions such as overexpression of the β tubulin (66,82).

Secreted Proteinases - Fungal secreted proteinases, principally those of pathogenic *Candida* species, constitute a group of enzymes that have recently received attention as potential virulence factors (83). The attention has arisen due to the fact that a positive correlation can be demonstrated between the secretion of proteinases and the virulence of specific species. Among the *Candida* species, *C. albicans* and *C. tropicalis* are the most important pathogens. Most isolates of both species secrete a proteinase; isolates of the weaker pathogens such as *C. parapsilosis* either do not secrete the proteinase or display weak activity (84). The relationship between virulence and secretion of proteinase gained additional support by studies of proteinase deficient natural variants and mutants of *C. albicans* (85,86). The role of such secreted proteinases is assumed to be involved with adherence to tissues, tissue penetration, invasiveness and evasion of the immune system. Recently, a linkage between phenotypic switching in *C. albicans* and proteinase expression was reported (87).

The proteinase of *Candida sp* which is implicated in virulence is an inducible secreted acid proteinase of ca. 44,000 MW and a pH optimum of 3.2. It is inhibited by pepstatin and is classified as an aspartyl proteinase, although it is not inhibited by diazoacetylnorleucine methyl ester and epoxy(p-nitrophenoxy)propane. Bovine serum albumin, bovine hemoglobin and a range of immunoglobulins can be proteolytically digested by the secreted proteinase and are potential substrates; however, the sequence specificity of the enzyme is not known. The enzyme is secreted in vivo in substantial amounts; it has been proposed as a diagnostic indicator. Recent studies on the initial stages of infection using immunological methodologies to follow the expression of the proteinase provided additional support that the proteinase is secreted during the early infection phase (88,89). Furthermore, purified *Candida* proteinase was shown to disrupt intercellular junctions in an in vitro assay with chorioallantoic membrane (86).

The nucleotide sequence of a *C. albicans* aspartyl proteinase gene was shown to be 72% homologous to proteinase A (PrA) which is the vacuolar proteinase of *S. cerevisiae* (90). The *S. cerevisiae* enzyme is not secreted and acts as a posttranslational activator of a number of hydrolases. The sequence specificity of the latter is also not known. The isolation of the gene encoding the secreted proteinase of *C. albicans* should allow the evaluation of its role as a virulence factor by gene disruption. The *S. cerevisiae* gene has been disrupted but growth of the cell was unaffected. It is assumed that functional redundancy can compensate for the loss of PrA. Recent studies with new synthetic proteinase inhibitors that are effective inhibitors of the HIV aspartyl proteinase (91) have shown inhibition of the inducible proteinase activity of *C. albicans* and a decrease in adherence to endothelial cells (92). None of the inhibitors was fungicidal to *C. albicans*.

Additional secreted aspartyl proteinases from *C. albicans* have been reported. Also, other secreted proteinases from fungal pathogens are known. Some of these proteinases are serine proteinases with a basic pH optimum and have the capacity to digest keratin and elastin (93). Thus, this is an area of interest although it is not clear that a proteinase inhibitor alone can be an effective drug. Currently, there are no nontoxic inhibitors that can be tested in animal models to establish the concept of proteinase inhibitors as antifungal drugs.

Protein Synthesis - In search for targets unique to fungi, an elongation factor, EF3, has recently been identified in *S. cerevisiae* (94). In vitro studies with isolated components of the translation system have shown that EF3 is required uniquely by the yeast ribosomes. This factor was also found in a number of other fungi, including pathogenic fungi, though not in higher eukaryotes. EF3 participates in the elongation cycle by stimulating the function of EF1-α in binding aminoacyl-tRNA to the ribosome (95). EF3 was purified and some of its enzymatic activities have been characterized. With current detailed knowledge of protein synthesis and the in vitro systems available from lower and higher eukaryotes,

the intervention in translation directed against a factor such as EF3 appears to be an attractive target (96).

Surface Components - The difficulties encountered in affecting intracellular targets in *C. albicans* have prompted reconsideration of strategies aimed at surface components and membrane-associated enzymatic reactions. These include transglycosylation reactions and cross-linking reactions between chitin and glucans or between chitin and mannoproteins as a part of cell wall synthesis (97). Additionally, a membrane-associated enzyme such as H^+-ATPase that functions as an electrogenic proton pump which regulates intracellular pH and membrane potential represents a possible target (98). However, the enzymatics of the cell wall reactions are not known and the essentiality and extent of redundancy of membrane ATPases in the fungal pathogens have not been established.

Very recently a surface component which may constitute a new potential target was suggested by studies on the action of a fungal secreted polypeptide that is toxic to *C. albicans* (99). Polypeptides secreted by fungi and lethal to fungi have been known for some time as "killer" proteins (100). Studies on the polypeptide secreted by *Pichia anomala* have shown that antiidiotype antibodies derived against the antitoxin antibodies are lethal to *C albicans*. Furthermore, immunization of mice with the antitoxin antibodies protects the mice from infection (101,102). These studies suggest the presence of an essential surface component, possibly a receptor, that upon inactivation causes cell death. Characterization of such a receptor may provide a new target for drug development.

Conclusions - While progress is continuing in the study of existing agents and the development of new ones, developments in genetic methodology and advances in knowledge of the biochemistry of fungi offer the prospect of identifying new antifungal targets. Gene disruption is proving to be a useful technique for the identification of genes which are either essential for the life of the organism or are responsible for virulence. Unique structural characteristics of fungal tubulins, secreted proteinases, elongation factors and membrane-bound receptors are being investigated in the search for novel antifungal targets.

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Chapter 16. Recent Advances In Anti-retroviral Chemotherapy for AIDS

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Introduction - The urgent need for effective anti-retroviral therapy for AIDS has increased significantly during the 9 years since the first documented cases of the disease. The number of cases of AIDS reported in the United States since June, 1981 surpassed 110,000 in November, 1989, and more than half of these cases were recorded after 1987 (1,2). In the first six months of 1989, 11% of the reported US cases were in women; prior to 1985, this proportion was only 7%. Outside the US, more than 70,000 additional cases of AIDS have been reported to the World Health Organization by 152 nations. In the US alone, the total number of HIV-infected people is estimated between 1 and 1.5 million.

Previous reviews have outlined the retroviral life cycle and identified several targets for therapeutic intervention (3-6). Inhibitors of the HIV reverse transcriptase, especially dideoxynucleoside analogs, have continued to play a predominant role in the clinic, and their pharmacology and SAR have been reviewed (7,8). Inhibition of the HIV protease appears to be a major new thrust of basic research.

Reverse Transcriptase Inhibitors: Clinical Trials - Efficacy in two clinical trials led to the approval of 3'-azido-3'-deoxythymidine (AZT) for prophylactic use among HIV-infected individuals (9). Early symptomatic and asymptomatic patients who received AZT at 1200 and 500 mg/d respectively progressed to AIDS or advanced ARC with less than half the frequency of placebo recipients (10). These differences were statistically significant in patients whose CD4 cell levels were less than 500/μl at entry. The incidence of severe, drug-related toxicity was low in both studies.

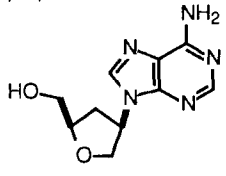
In patients with AIDS and advanced ARC, the hematologic toxicities of AZT continue to be dose limiting, and frequently require dose reductions, transfusions, or cessation of AZT therapy (11). After an initial period of clinical improvement, the CD4 cell counts and HIV p24 antigen levels of most AIDS patients treated with AZT gradually return to baseline values, and, although life is prolonged and improved in quality, mortality rates increase significantly after 6-12 months of therapy (11-15). It is not known if diminished drug efficacy in patients with advanced disease is due to an underlying, irreversible immunologic deficit, an overwhelming viral burden, drug toxicity, loss of a cell function that mediates drug efficacy, or the emergence of more virulent or drug resistant strains of HIV (16). In one study, virus isolates from most AIDS or ARC patients who had received AZT for at least 6 months exhibited decreased *in vitro* sensitivity to the drug (17). These AZT resistant strains have been shown to share 3 to 4 specific mutations in the reverse transcriptase gene (18). Although some strains exhibited cross-resistance to 3'-azido-2',3'-dideoxyuridine (AZDU, CS-87), no or only partial cross resistance was observed to other dideoxynucleoside analogs, e.g., DDC, D4T, DDI, and BCH-189 (17,19).

The limited benefits of therapy with AZT have encouraged the intensive investigation of nucleoside analogs. In clinical trials with 2', 3'-dideoxycytidine (DDC), painful peripheral neuropathy was induced in AIDS and advanced ARC patients by oral doses as low as 0.005 mg/kg every 4 hours (20). Although the median reduction in p24 levels was 64% at this dose, the significant increases in CD4 cell count and skin test reactivity experienced with AZT were not observed and the trial was of insufficient duration to demonstrate an effect on the course of the disease. The low incidence of hematologic toxicity, however, suggested that alternating regimens of DDC and AZT might attenuate the toxicity of a continuous course of a single agent. In an initial study, weekly alternation of the two agents led to substantial improvements in CD4 cell counts, p24 levels, and weight gain with less toxicity than either drug alone (21). In cell culture, a 3-day-alternating regimen of AZT and DDC exhibited a prolonged time to viral breakthrough (22).

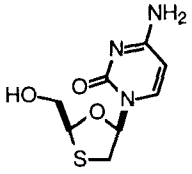
In a phase I clinical trial, AIDS or ARC patients who received 2,' 3'-dideoxyinosine (DDI, 7) intra-

venously for 2 weeks and then continued with oral administration showed significant immunologic improvements after 6 weeks (23). Five of the six patients who had detectable serum HIV p24 antigen at entry became p24 antigen negative by the sixth week of therapy and remained so for at least 28 weeks. Although the average plasma half-life of DDI was only 35 minutes, these changes occurred even with twice a day dosing, presumably as a consequence of the >12 hour intracellular half-life of the active metabolite, 2',3'-dideoxyadenosine-5'-triphosphate (24). Macrocytic bone marrow suppression, the dose-limiting toxicity of AZT, was not observed at oral doses of 6.4 to 19.2 mg/kg/day for up to 42 weeks. This finding may reflect DDI's relatively high in vitro therapeutic index and lack of toxicity for human marrow progenitor cells (25). However, dysethesia of the feet and pancreatitis have been observed at higher doses.

Reverse Transcriptase Inhibitors: Preclinical Findings - Because of its acid lability, DDI must be administered on an empty stomach after ingestion of antacids. Transposition of the ribose ring oxygen and 3'-methylene generated the acid stable analog Iso-DDA, (1), which exhibited activity (ED_{50} = 5-15 μM ; CD_{50} >200 μM) comparable to DDI in the ATH8 cell viability assay (26). Iso-DDG was also active in this assay (ED_{50} = 10-50 μM; CD_{50} > 500 μM), but Iso-DDI was inactive (ED_{50} >100 μM).



1



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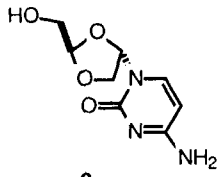
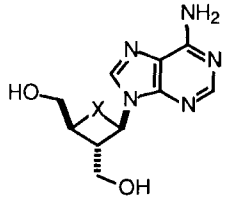
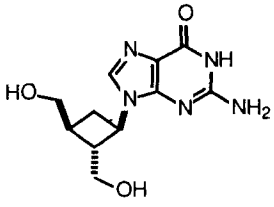


3



X=O: 4
X=CH_2: 5



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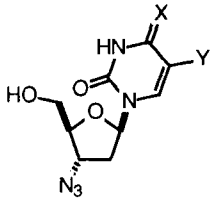


X= O, Y=Et: 7 X=O, Y=H: 8
X=NH, Y=H: 9 X=NH, Y=Me: 10

The decision to explore the clinical utility of 2',3'-dideoxy-2',3'-didehydrothymidine (D4T) was supported by extensive in vitro and in vivo studies. Although the anti-HIV activity of D4T is very close to that of AZT in a variety of cell lines (e.g., in CEM cells: AZT ED_{50} = 0.10 μM , CD_{50} = 29 μM; D4T ED_{50} = 0.15 μM, CD_{50} = 90 μM (27)), D4T, unlike AZT, is metabolized intracellularly to its 5'- triphosphate without grossly suppressing the levels of thymidine 5'-triphosphate required for normal DNA synthesis (27-30). This suggested that the hematologic toxicities associated with AZT might not be induced by D4T. In fact, the in vitro CD_{50} of D4T against human CFU-GM (colony forming units granulocyte-monocyte) was 100 μM, a value 100 times greater than that observed for AZT (27). Against BFU-E (burst forming units-erythrocyte), the CD_{50} of D4T was 10 μM. In mice, oral administration of 250-1000 mg/kg/d of D4T for 30 days induced significantly less anemia and erthrocytopenia than the same doses of AZT (31) or 3'-deoxy-3'-fluorothymidine (DFT (32)). Unlike AZT, D4T was not glucoronidated in the monkey (31), and preliminary reports indicate a half-life >5 hours in humans (33). Reductions in p24 levels were said to occur at doses of 2-4 mg/kg/d in the absence of hematopoietic toxicity. However, peripheral neuropathy was noted at doses higher than 4 mg/kg/d (32).

Racemic pyrimidine 2',3'-dideoxynucleoside analogs in which the 3'-methylene is substituted with sulfur or oxygen were reported to possess more selective in vitro anti-HIV activity than AZT (34, 35). In HIV-1 infected CEM cells, both BCH-189 (2) and AZT increased cell viability to 50% at 0.3-0.4 μM, but unlike AZT, BCH-189 was not toxic to these cells at 100 μM. The most active compound in the dioxolane series, BCH-203 (3), was shown to have the unnatural trans-configuration. Although about half as potent as AZT in CEM cells, BCH-203 was less inhibitory to human CFU-GM than either AZT or BCH-189.

(±)-Carbocyclic-2',3'-didehydro-2',3'-dideoxyguanosine ((±)-carbovir) was selected as the most potent and selective in vitro inhibitor of HIV-1 from a group of 33 carbocyclic nucleoside analogs (36). The ED_{50} for inhibition of HIV-1 induced cytopathic effects in ATH8, MT-2, and CEM cells ranged from 0.15 to to 0.19 μg/ml; concentrations greater than 35 μg/ml were required to cause 50% inhibition of cell growth. (±)-Carbovir-triphosphate and AZT-triphosphate exhibited similar inhibitory potencies (IC_{50}'s=0.03 μM) against the HIV-1 reverse transcriptase, but (±)-carbovir-triphosphate was considerably more selective with respect to the cellular DNA polymerases β and γ (36). (±)-Carbovir and AZT synergistically inhibited HIV production in H9 cells with an optimal combination ratio of 1:0.032 /(±)-carbovir:AZT (37).

Two carbocyclic analogs of oxetanocin (**4**), (±)-cyclobut-A (**5**) and (±)-cyclobut-G (**6**), were reported to provide 50% protection against the infectivity and cytopathogenic effects of HIV-1 in ATH8 cells at a concentration of 1 μM; >100 μM of either agent was required to induce 50% cytotoxicity in uninfected ATH8 cells (38). By comparison,10 μM of oxetanocin-A was required to effect 50% protection in this assay, and, in an MT4-cell assay system, oxetanocin-A was reported to be 6 times less potent than DDA (39). Both (±)-cyclobut-A and (±)-cyclobut-G completely suppressed HIV-1 p24 gag protein expression in monocytes/ macrophages at a concentration of 0.5 μM (38). Notably, both compounds also exhibited selective in vitro activity against HSV-1, HSV-2, HCMV, VZV, and EBV at concentrations from 0.05 to 6 μg/ml (40). In addition to their direct contributions to morbidity and mortality in AIDS patients, these herpesviruses may play a more insidious role in the pathogenesis of AIDS by enhancing the replication and cytopathicity of HIV.

Parenteral administration of another broad spectrum antiviral agent, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), slowed the progression of murine immunodeficiency disease when treatment was initiated on the day of infection, and increased the survival time of mice superinfected with a neurovirulent strain of HSV-1 (41). In cats, continuous intravenous infusion of 12.5 mg/kg/d of PMEA beginning 1 day prior to inoculation with feline leukemia virus prevented or delayed the onset of infection. However, moderate anemia was evident after the 3 week treatment period, and doses of 25 or 100 mg/kg/d were toxic (42).

Previous studies identified 3'-deoxy-3'-fluorothymidine (DFT) as one of the most potent inhibitors of HIV replication (ED_{50} = 0.001 μM in MT4 cells), but the compound was also rather toxic (CD_{50} =0.197 μM (8)). Extensive SAR studies led to a DFT analogue, 2',3'-dideoxy-3'-fluoro-5-chlorouridine which was considerably less toxic than DFT but still effective against HIV in MT-4 cells (ED_{50} = 0.38 μM; CD_{50}= 535 μM (43)).

Four AZT analogs, CS-85 (**7**), CS-87 (**8**), CS-91 (**9**), and CS-92 (**10**), emerged from systematic SAR studies as potent (ED_{50} =0.056-1.19 μM) and selective (CD_{50} >100 μM) inhibitors of HIV in peripheral blood mononuclear cells (44). Previously, CS-85, CS-91, and CS-92 had been found to be inactive in ATH8 cells. CS-87 was more than 10-fold less toxic than AZT to human CFU-GM and BFU-E and has been selected for phase I trials (33).

The in vitro antiviral efficacy of the dideoxynucleosides is a complex function of the formation and degradation of the mono-, di-, and triphosphate metabolites, the perturbation of normal nucleoside metabolism, the selective inhibition of the reverse transcriptase , and the efficiency of chain termination (45). Consequently, the discovery of nucleoside analogs with useful anti-HIV activity has been primarily an empirical process. In a limited series of compounds, in vitro activity has been correlated with the presence of an unusual C3'-exo conformation in the solid state (46). AZT however, appears to adopt C2'-endo and C3'-endo conformations in solution (47). The relative in vivo hematologic toxicity of nucleoside analogs appears to be in general agreement with results from in vitro assays using human bone marrow progenitor cells, and may be related to inhibition of DNA polymerase γ and the consequent loss of mitochondrial DNA (48,49). Contrary to an earlier report, dideoxynucleosides effectively inhibit the replication of HIV in monocytes/macrophages, cells thought to serve as a viral reservoir in vivo (50).

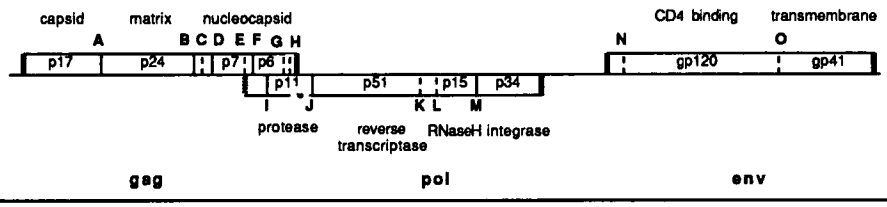
Several compounds have been identified which potentiate the activity or diminish the toxicity of dideoxynucleoside analogs in vitro. At a concentration of 2 μM, dipyridamole, an inhibitor of carrier-mediated nucleoside transport across cell membranes, decreased the ED_{50} for AZT in monocytes/macrophages by a factor >5 without exacerbating its in vitro bone marrow toxicity (51). At in vitro concentrations of 10-100 μM, 2'-deoxycytidine protected human bone marrow progenitor cells against the cytotoxicity of AZT without diminution of anti-retroviral activity in HUT-102 cells

(52). The ability of various cytokines, such as erythropoietin and granulocyte-macrophage colony stimulating factor, to counteract the marrow suppressive effects of AZT is being studied clinically (33).

HIV Protease Inhibitors - Postranslational cleavage of the HIV gag, gag-pol, and env polyproteins is required to generate the structural proteins and enzymes found in mature, infectious virions (53). N and C terminal sequencing of these proteins has identified a remarkably diverse array of specific cleavage sites (54-56). Cleavage at sites I and J (see Table I) within gag-pol produces a 99 amino acid peptide containing the Asp-Thr-Gly triad characteristic of aspartyl proteases (57,58). Proteins encompassing this peptide sequence have been expressed in E. Coli and found to exhibit autoprocessing (57,59). Transfection of SW480 human colon carcinoma cells with HIV-1 proviral DNA in which the putative catalytic Asp residue was mutated to Gln yielded virions containing gag p55 but lacking gag p24, a product of proteolytic processing (60). The inability of these mutant virions to infect MT-4 lymphoid cells established the essential role of the HIV protease in the viral life cycle and suggested that chemical inhibition of the wild-type protease should render the virus noninfectious. However, the proteolytic events critical for infectivity are not fully understood. Transfection of monkey COS-M6 cells with HIV-1 proviral DNA containing a deletion mutation in the protease gene produced virions containing ring-shaped nucleoids similar to immature HIV particles released from lymphocytes (61). These noninfectious mutant virions had reduced reverse transcriptase activity. Similarly, protease/ reverse transcriptase and protease/reverse transcriptase/ endonuclease polyproteins containing Ala in place of the catalytic Asp residue accumulated as enzymatically inert polyproteins when expressed in E. Coli. Trans complementation by a second, wild type copy of the protease led to normal processing and restoration of reverse transcriptase activity (62). Mutation of the N or C terminal cleavage sites of p24 to Ser/Arg blocked proteolytic processing at these sites, and virions containing either of these mutations exhibit altered morphology and fail to induce syncytia formation or viral protein expression in Sup-T1 cells (63).

Peptides modeling 7 of the 15 known sites of proteolytic processing within the HIV-1 polyproteins have been shown to be accurately cleaved by purified synthetic or recombinant HIV-1

Table I. Sites of Proteolytic Processing Within HIV-1 Polyproteins[a,a]



	CLEAVAGE SITE[b]	CLEAVAGE SEQUENCE	SPECIFIC CLEAVAGE BY HIV-1 PROTEASE[c]
A	gag 132/133	ValSerGlnAsnTyr / ProIleValGlnAsn	(+); 55, 56, 67, 72
B	gag 363/364	LysAlaArgValLeu / AlaGluAlaMetSer	(+); 55, 56, 67, 72
C	gag 367/368	LeuAlaGluAlaMet / SerGlnValThrAsn	(-); 55
D	gag 377/378	ThrAlaThrIleMet / MetGlnArgGlyAsn	(+); 55, 56, 67, 72
E	gag 432/433	GluArgGlnAlaAsn / PheLeuGlyLysIle	(?)
F	gag 448/449	ArgProGlyAsnPhe / LeuGlnSerArgPro	(+); 55, 67
G	gag 478/479	SerPheArgSerGly / ValGluThrThrThr	(?)
H	gag 496/497	AspLysGluLeuTyr / ProLeuThrSerLeu	(?)
I	pol 68/69	ValSerPheAsnPhe / ProGlnIleThrLeu	(+); 55, 56, 67, 72
J	pol 167/168	CysThrLeuAsnPhe / ProIleSerProIle	(+); 55, 56, 67, 72
K	pol 593-603	GlnLeuGluLysGlu /(?) ProIleValGlyAla	(-); 67
L	pol 607/608	GlyAlaGluThrPhe / TyrValAspGlyAla	(?)
M	pol 727/728	IleArgLysIleLeu / PheLeuAspGlyIle	(+); 56, 67
N	env 37/38	MetIleCysSerAla / ThrGluLysLeuTrp	(?)
O	env 518/519	GlnArgGluLysArg / AlaValGlyIleGly	(-); 56

[a](a) ▌: site of initiation or termination; | : site cleaved by purified recombinant or synthetic protease; | and ¦ : cleavage site determined by sequence or compositional analysis of purified viral protein; ▮ :site of frameshift; (b) see ref. 116; (c) corresponding synthetic peptide cleaved (+) or not cleaved (-) at expected residue by purified recombinant or synthetic protease; (?): undetermined; reference.

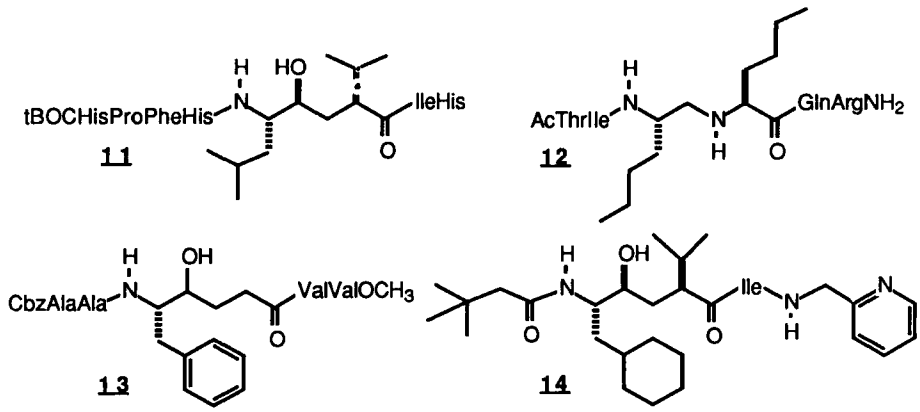
protease. A discrete cleavage site responsible for the generation of the p51/p66 reverse trans-

criptase heterodimer has not yet been defined (64). Trypsin, chymotrypsin, and E. Coli proteases cleave at multiple sites within the pol 593-603 sequence (65,66), but the model peptide LeuGluLysGlu/ProIleVal was not a substrate for the HIV protease (64,67). A nearby cleavage site at pol 607/608 has been proposed from a determination of the total amino acid composition of purified p51, but this has not been confirmed (68). Failure of the HIV protease to cleave a model peptide based on the env 518/519 site is consistent with processing of gp 160 by a cellular tryptase (69,70). Trimming of the envelope protein signal peptide at site N may also be mediated by a cellular enzyme.

The determinants of substrate specificity for the HIV-1 protease are not readily apparent from simple sequence analysis around the scissile bonds. Although cleavages at sites A, I, and J have the canonical (Ser, Thr)-X-Asn-(Phe,Tyr) /Pro-(Ile, Gln) retroviral sequence in common (71), the only obvious similarities of this sequence to other confirmed cleavage sites are a strong preference for hydrophobic residues in the P1 and P1' sites and a trend toward Asn, Gln, or hydrophobic residues in the P2 and P2' sites. Substitution of Leu-Phe, the normal pol 727/728 cleavage site, for Phe-Pro in the pol 68/69 cleavage site, blocked processing at the mutated site (73). This suggests that peptide conformation is an important determinant of substrate specificity, an idea supported by the failure of the protease to process denatured gag proteins (74).The relative efficiencies of cleavage also depend on the length of the model peptide utilized and the pH of the assay. At pH 5.5 and 6.0, peptides corresponding to the pol 68/69 and gag 377/378 cleavage sites exhibited V_{max}/K_m values 3 to 33 times greater than those values at other sites (56, 72). Six to seven residues appear to be required for efficient hydrolysis. At the gag 132/133 locus, SerGlnAsnTyr / ProIleVal is cleaved only 10% more slowly than the longer peptide, HisSerSerGlnValSerGlnAsnTyr / ProIleVal-GlnAsnIle (56). A hexapeptide model of the gag 377/378 site, AcThrIleMet / MetGlnArg-NH2 (K_m= 1.4 mM, V_{max} = 725 nmoles/min/mg), is the shortest substrate to have been reported (75).

The six to seven residue binding site these results implied was remarkably consistent with an earlier structural model which predicted that the HIV protease functions as a C2 symmetrical dimer (76). This conjecture was confirmed by biophysical characterization (77,78) and by X-ray crystallographic analyses of both the recombinant and synthetic protein (79-81). As predicted, each identical subunit contributes a single aspartyl residue to the catalytic site, and inhibition of the enzyme by pepstatin (K_i = 1.1 μM) confirmed the aspartyl protease classification (78). A systematic study of the standard transition state analogs previously used to develop inhibitors of renin found the hydroxyethylene isostere **13** (K_i = 48 nM) to be the most potent structural type (82). Related compounds with hydrophobic substituents in the P1' site, such as the known renin inhibitor **11** (K_i = 5 nM) and U-81749 (**14**, K_i = 70 nM) were also highly effective (83,84). The structure of a complex between HIV-1 protease and the reduced peptide inhibitor **12** (K_i = 780 nM) has been determined at 2.3 angstrom resolution; binding of the inhibitor induced movements as large as 7 angstroms in the protease "flap" region and involved complementary interactions with all six residues of the inhibitor in an extended conformation (59,85). The recent development of a fluorogenic protease inhibition assay should facilitate large scale screening (86).

After initial reports that 100 μM pepstatin exerted measurable anti-HIV activity in H9 cells, more potent inhibitors of the protease have been examined in a variety of cell lines (87,88). Treat-



ment of chronically infected CEM/IIIB cells with 20 μM of the hydroxyethylene isostere **13** reduced the proteolytic processing of the HIV gag and gag-pol proteins to a level where relatively little p17, p24, or p51/p66 could be detected by Western blot analysis of cell extracts (89). Addition of 25 μM of **13** to cultures of Molt 4 cells immediately after they had been exposed to 200 infectious units of HIV prevented the formation of syncytia and reduced the 7 day levels of particle-associated reverse transcriptase activity and p24 antigen in the culture supernatants to <2% of the untreated controls (89). Similarly, addition of 1 uM of **14** to HIV-1 infected peripheral blood lymphocytes reduced the 4 day levels of supernatant HIV RNA by 99% (84). Together, these experiments provide strong evidence that inhibition of the HIV-1 protease can attenuate viral infectivity in cell culture.

Although not mediated by the HIV protease, endoproteolytic cleavage of the envelope protein gp160 to generate gp120 and gp41 is necessary for membrane fusion and viral infectivity (70). Mutagenesis of the tryptic-like cleavage site O to a chymotryptic-like site, GlyGluGluPhe /, led to the production of virions whose lost fusogenic potential could be qualitatively restored by the addition of exogenous chymotrypsin. It is significant that in the assembly of wild-type virions, gp 160 is cleaved intracellularly and then transported to the plasma membrane without mediating fusion with adjacent membranes (90). The implied requirement for a secondary activation step may be related to the ability of trypstatin, a potent inhibitor of trypsin (K_i = 14 nM), to inhibit HIV-1 induced syncytia formation in cell culture at a concentration of 1 μM (91). Interestingly, residues 316-328 of gp 120 and residues 7-19 of trypstatin share 50% homology and a highly conserved β-turn (GlyProGlyArg). Whether virus selective toxicity can be achieved by inhibiting the cellular, trypsin-like protease(s) involved in envelope glycoprotein maturation and function is unknown.

Myristoylation Inhibitors - Mutation of the terminal glycine residue of the gag and gag-pol polyproteins to alanine prevented their N-myristoylation (63). Although proteolytic processing of these unacylated mutant precursors appeared to proceed normally in COS-7 cells, virus assembly was abolished. Octapeptides resembling both the HIV nef and gag N termini were acylated by an N-myristoyl transferase activity found in H9 cells lysates; thus, the relevant transferase is probably a cellular enzyme (92). While total inhibition of the enzyme might be expected to jeopardize cell viability, the marked substrate dependence of myristic acid analog transfer suggested a possible basis for selective toxicity (93). In H9 cells infected with HIV-1 at an moi of 0.01-0.001, 13-oxamyristic acid reduced supernatant reverse transcriptase activity to 20% of control at 10 μM and to <10% of control at 100 μM with no effect on the rates of DNA and protein synthesis or cell growth. In preliminary communications, N-myristoyl-glycinal-diethylacetal and tetradecanal were reported to inhibit the acylation of p17 and replication of HIV in cell culture (92, 94).

Glucosidase Inhibitors - Interaction of the viral envelope protein gp120 with CD4 receptors governs the cellular tropism of HIV. Both gp120 and the transmembrane protein gp41 are heavily glycosylated, and the carbohydrate residues play an important role in viral infectivity and cytopathogenicity (95). Glycosylation of both viral and cellular membrane glycoproteins usually begins cotranslationally with the transfer of $Glc_3Man_9(GlcNAc)_2$ to asparagine residues of the nascent peptide (96). Removal of the three glucose residues in the endoplasmic reticulum by glucosidases I and II followed by further processing in the Golgi complex generates the complex array of oligosaccharide moieties found in the mature glycoprotein. Several azacyclic transition state inhibitors of glucosidase I have been found to alter HIV glycoprotein synthesis and to reduce the production of infectious virus at nontoxic concentrations in vitro (97). One of the most potent of these compounds, N-butyldeoxynojirimycin (BuDNJ), reduced the infectivity of T-45 cell supernatants by $>10^5$ at 0.05 mg/ml after 10 days in culture (98). Cells were qualitatively spared from virus induced cytopathogenic effects by as little as 0.01 mg/ml of BuDNJ, while cell growth was unaffected by the compound at 0.5 mg/ml. Prolonged exposure of HIV-infected T-45 cells to 0.1 mg/ml of BuDNJ gradually increased the time required for cell aliquots to induce cytopathogenic effects in uninfected, drug free cultures. After 55 days, infected cells appear to be virtually eliminated from drug treated cultures. In a preliminary communication, 6-O-butyrylcastanospermine was reported to be 20 times more potent than castanospermine against glucosidase I and to reduce the number of syncytia induced by HIV-1 in JM cells from 100 to 50 at 0.15 μg/ml; host cell toxicity was not evident at concentrations up to 200 μg/ml (99).

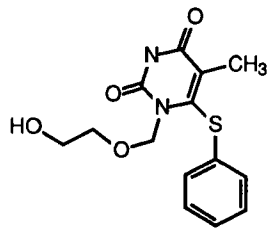
Protein Kinase C Inhibitors - Productive binding of HIV-1 to the CD4 receptor may depend on phosphorylation of the CD4 molecule by protein kinase C (100). Glycrrhizin, a tricarboxylic acid of MW 823, was shown to inhibit protein kinase C with an IC_{50} between 0.075 -0.15 mM and to almost completely suppress HIV-1-induced cytopathogenicity, virus antigen expression, and giant cell formation at 0.6 mM with no cytotoxicity. In a small, uncontrolled, clinical trial, continuous

intravenous adminstration of glycyrrhizin to 3 AIDS patients at 400-1600 mg/kg/d for periods of more than one month appeared to cause significant decreases in serum HIV p24 levels without serious side effects (101). A sustained increase in T4 counts, however, was not achieved.

Inhibitors of viral adsorption - Pentosan polysulfate, a sulfated polysaccharide, was found to protect MT-4 cells from the cytopathic effects of HIV-1 at a concentrations between 0.19 -2500 μg/ml without cytotoxicity (102). Like dextran sulfate and heparin, pentosan polysulfate inhibits the association of virus with target cells, probably by blocking the interaction of gp120 with the CD4 receptor (103). Unfortunately, the clinical investigation of sulfated polysaccharides has been hampered by their probable lack of oral bioavailability in humans and their anticoagulant properties. In a phase I trial, orally administered dextran sulfate caused significant G.I. toxicity but had no effect on CD4 cell counts or levels of p24 antigen (104). The oral absorption of another sulfated polysaccharide, Hoe/Bay 946, was reported to be 4-40% in healthy subjects, and a clinical pilot study is underway in HIV-infected patients (105).

The utility of recombinant soluble CD4 (rsCD4) as a molecular decoy has been reviewed previously (106), and recent advances in this area are discussed elsewhere in this series (59). In general, rsCD4 molecules have been well tolerated in phase I studies, but consistent effects on the levels of CD4 cells or p24 antigen have not been reported during short courses of therapy (33).

Miscellaneous inhibitors - A novel acyclic nucleoside analog, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (**15**), was found to selectively inhibit (ED_{50} = 7.0 μM, CD_{50} = 740 μM) the cytopathic effect of HIV-1 in MT-4 cells (107). Remarkably, **15** was inactive against a variety of other



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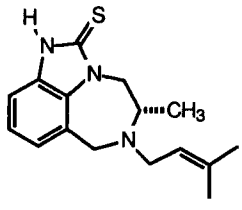

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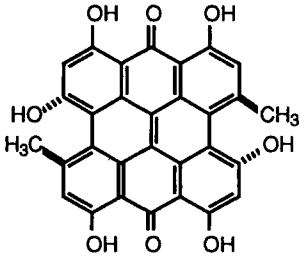

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retroviruses, including HIV-2. Neither **15** nor its triphosphate inhibited the HIV-1 reverse transcriptase, and the compound was not a substrate for thymidine kinase. Derivatives of tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-one and -thione (TIBO) have been reported as the most specific and potent inhibitors of in vitro HIV-1 replication studied so far (108). In MT4 cells, the TIBO derivative R82150 (**16**) exhibited an ED_{50} = 0.028 μM and a CD_{50} >870 μM. The relative anti-HIV-1 activity of a series of TIBO derivatives has been correlated with their IC_{50} for HIV-1 reverse trancriptase. Plasma levels exceeding the anti-HIV-1 ED_{50} of **16** were maintained for 24 h in healthy males following the oral ingestion of a single 200 mg dose and the compound appeared to be well tolerated. A ribosome-inactivating protein, GLQ223, reduced the expression of p24 in acutely infected VB T cells to 27% of control at a concentration of 0.016 μg/ml with less than 20% inhibition of of host cell protein or DNA synthesis (109). Exposure of chronically infected monocytes/macrophages to 0.5 μg/ml of GLQ223 for 3 h appeared to cause the selective death of HIV-infected cells within 2 weeks. Hypericin (**17**) was reported to directly inactivate HIV virions, but the compound was toxic to human T-cells in vitro (110). The antifungal antibiotics, benanomicins A and B, inhibited HIV-1 infection and syncytia formation in MT-4 cells at 10-30 μg/mL without toxicity (111). A variety of antisense, phosphate modified oligonucleotides have been found to inhibit HIV replication in cell culture (112). The virus encoded trans-activator protein (TAT) is essential for viral replication, and cell lines containing reporter genes expressed under the control of the TAT gene product have been developed to screen for inhibitors of TAT function (113, 114).

Conclusion - The efficacy of AZT has proven the fundamental assumption that the clinical course of HIV-infection can be improved by anti-retroviral therapy. An unprecedented number of anti-retroviral agents, identified through targeted approaches or screening, may now be considered for clinical trials. Increasingly, animal models of retroviral infection will be used to discriminate between

these compounds (115). Combinations of drugs, each interfering with a different stage of the viral life cycle, are likely to be more efficacious and less toxic than single agents. Although beyond the scope of this review, the complementary therapeutic potential of cytokines and immunomodulators, e.g., interferon-α and diethyldithiocarbamate (Imuthiol), should be recognized.

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Section IV - Metabolic Diseases and Endocrine Function

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Chapter 17. Agents for the Treatment of Peptic Ulcer Disease

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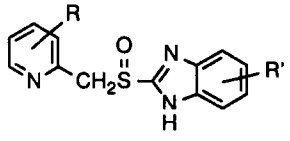
Introduction - The etiology of peptic ulcer disease is far from clear. It is believed to result from an imbalance between the "defensive factors" (1) (such as bicarbonate secretion, mucus secretion, blood flow and the epithelial cell layer) and the aggressive forces (such as acid secretion, pepsin and bile). However, what underlies a breakdown in the defense mechanisms is not understood. The concept of "cytoprotection" (2) and the involvement of prostaglandins in this process has received widespread attention. However, cytoprotection may be a misnomer since, even in the presence of exogenously administered prostaglandins, mucosal damaging agents, such as ethanol, cause destruction of the surface epithelium (3). Nevertheless, deeper cells are preserved and the surface epithelium rapidly reconstitutes under the protection of a mucoid cap (4). In clinical studies in peptic ulcer disease, synthetic prostaglandins have shown efficacies comparable to H_2-antagonists (5); side effects such as diarrhea and uterotonic activity have been seen (5). Their use in mucosal injury caused by NSAID's may prove to be a more logical therapeutic indication. Recently the role of trophic peptides such as epidermal growth factor (EGF) (6) in the maintenance of mucosal integrity has received attention and could possibly lead to novel drug therapy (7).

Much of the therapy of peptic ulcer disease deals with the aggressive agents and in particular acid. Antisecretory drugs such as the H_2-antagonists and, more recently, the H^+/K^+-ATPase inhibitors are the principal agents in use. Recent studies have focussed on the degree and duration of acid suppression required for optimal therapy of acid related diseases (8). A single dose of an H_2-antagonist at night has been found to have approximately equal efficacy in duodenal ulcer (DU) as qid dosing, emphasizing the importance of the control of nocturnal secretion. However the situation is less clear for gastric ulcer (GU) and gastro-esophageal reflux disease (GERD): the greater efficacy of omeprazole, which suppresses acid secretion over a 24 hour period suggests that in these diseases (as well as in DU) greater acid inhibition equates with improved efficacy. This conclusion is supported by a meta-analysis relating acid inhibition to rapidity of healing (9). The optimal acid inhibition profile for each disease has yet to be established; the importance of this is highlighted by potential toxicity problems associated with omeprazole (10). In rat carcinogenicity studies omeprazole produced gastric carcinoid tumours which are believed to be caused by the sustained hypergastrinemia associated with prolonged anacidity. However, a debate over a direct genotoxic effect for omeprazole has recently opened (11,12). Although no comparable toxicity has been demonstrated in man to date, gastrin can act as a tumour growth factor (13). Safety concerns are reflected in the fact that the use of omeprazole in peptic ulcer disease is usually restricted to short term therapy in patients failing to respond to H_2-antagonist therapy.

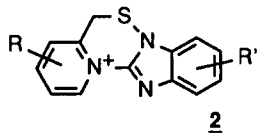
Despite their efficacy in the short term healing of peptic ulcer disease, antisecretory agents are not permanent cures: relapse occurs in 80-90% of cases in the first one to two years after cessation of therapy. The reason for this is unclear, but could be associated with the persistence of the organism Campylobacter pylori (now called Helicobacter pylori (14)). This organism is found in a high proportion of patients with antral gastritis and it has been suggested that it has a role in peptic ulceration (15). Therapy with bismuth salts, which are bactericidal to H. pylori, is associated with lower relapse rates than those healed with H_2-antagonists (16). While there is little doubt that the organism is a major cause of chronic gastritis, a role in other gastroduodenal diseases is as yet

unproven (17). The use of antibiotic therapy either alone or in combination with bismuth for peptic ulcer disease requires further controlled clinical trials before conclusions can be drawn. However, preliminary studies are encouraging (18).

H^+/K^+-ATPase Inhibitors - A notable recent development has been the FDA approval of omeprazole **1** (19,20). However, because of continued concern over gastric carcinoids observed in long term rat toxicology studies, approval has been limited to its use in severe erosive esophagitis diagnosed by endoscopy, short term treatment of symptomatic gastro-esophageal reflux disease poorly responsive to conventional treatment, and long term treatment of Zollinger-Ellison syndrome. Approval for general use in peptic ulcer disease was not given; however, its use for refractory DU and GU has been approved in a number of European countries. Clinical trials continue to demonstrate the superior efficacy of omeprazole over H_2-receptor antagonists. Much of the available data has appeared in a recent symposium proceedings (21).



1 R = 3-CH_3, 4-OCH_3, 5-CH_3; R' = 5-OCH_3
8 R = 3-CH_3, 4-OCH_2CF_3; R' = H
9 R = 3-CH_3, 4-$O(CH_2)_3OCH_3$; R' = H
10 R = 3-OCH_3, 4-OCH_3; R' = 5-OCF_2H
11 R = 3-Cl, 4-morpholino; R' = 5-OCH_3
12 R = 3-CH_3, 4-OCH_3, 5-CH_3; R' = 4,5-$CO_2CH_2CH_2$



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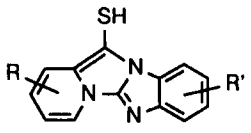

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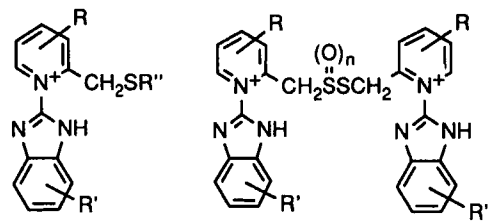


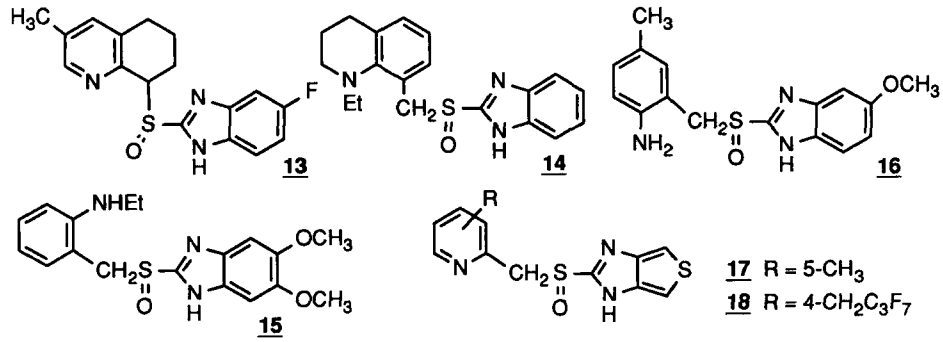
3 R'' = OH
4 R'' = SEnz

5 n = 1
6 n = 0

Structure activity relationships of omeprazole analogues have been reviewed (22). Further details of the mechanism of action and the underlying chemistry of the irreversible inhibition of the gastric H^+/K^+-ATPase by omeprazole and related compounds have now appeared (23-30). Acid activation gives rise to the cyclic sulfenamide **2** via the sulfenic acid **3**. Compound **2** is believed to be the primary biologically active species, binding to a critical thiol group on the enzyme as the disulfide **4**. Further reactions, particularly at higher concentrations, include the formation of dimers such as the thiolsulfinate **5** and disulfide **6**. An alternative pathway from **3** presumably via a thioaldehyde, leads to a series of monomeric and dimeric compounds derived from the tetracyclic thiol **7**.

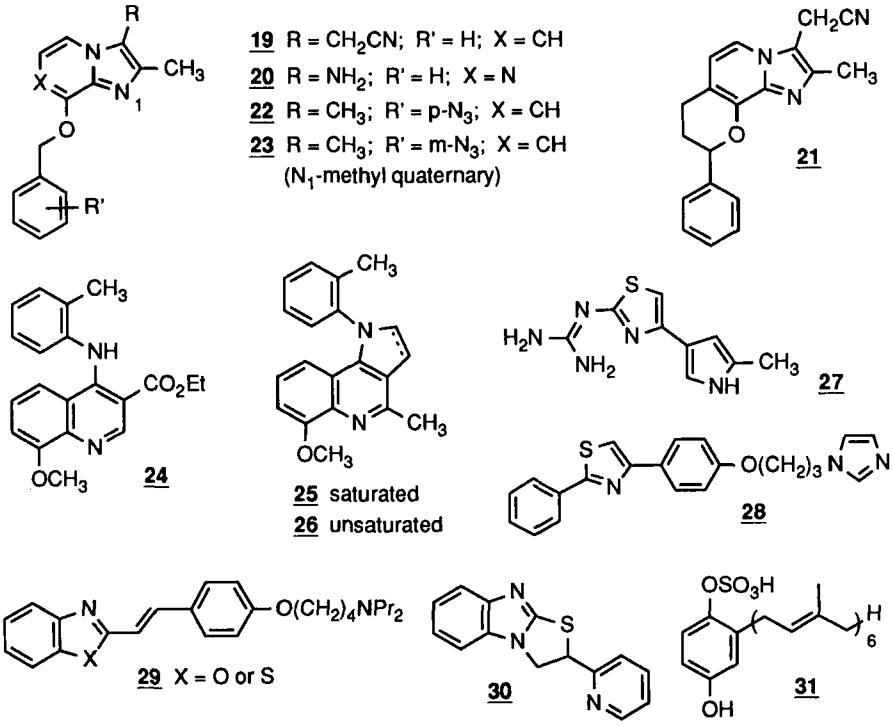
Lansoprazole (**8**, AG-1749) is currently in Phase III trials in Japan (31,32). The detailed biochemistry (33) and pharmacology (34) has been described. In humans, a similar antisecretory potency to omeprazole and a capacity to abolish aspirin induced mucosal damage has been reported (35,36). More rapid DU healing rates than ranitidine have also been reported (37). Other compounds currently undergoing Phase I studies include **9** (E-3810) (38) and **10** (BY 1023/SK&F 96022) (39), which has a good stability profile (40). Stability considerations were key in selecting **11** (SK&F 95601) for further development from a series of 4-aminopyridyl derivatives (41). The factors influencing activity and stability in this series have been discussed (41).

The omeprazole analog **12** was one of the most potent from a series recently described (42). Compound **13** (OPC 22321) was selected from a series of conformationally restrained tetrahydroquinoline derivatives as a potent antisecretory/antiulcer agent (43). Compound **14** was the most potent from a related series based on aniline rather than pyridine (44). Other examples of this type recently described include **15** (S 3337) (45) and **16** (46). As with the analogous pyridines, activity has also been shown to correlate with pK_a (46); the compounds presumably undergo similar reactions to those of omeprazole at low pH. The benzimidazole can be replaced by thienoimidazole as in compounds **17** and **18** (S 1924 and HOE 731) (47,48). HOE 731 is said to



have a different pharmacodynamic profile at higher doses compared to omeprazole, suggesting hypergastrinemia may be less pronounced for this compound.

The concern over prolonged inhibition of acid secretion and sustained hypergastrinemia, referred to earlier, has stimulated an interest in shorter acting, reversible inhibitors of the gastric H^+/K^+-ATPase. SCH 28080 (**19**) was evaluated clinically in the early 80's as an antisecretory agent of unknown mechanism of action, but dropped due to liver toxicity (49). Attempts to eliminate the hepatotoxicity by modifying the metabolism of these compounds (50,51) have led to the selection of SCH 32651 (**20**). Most recently, in a conformational study using rigid analogs, notably **21**, it was concluded that an extended conformation of the benzyloxy group was important for activity (52). Compounds of this type have now been shown to be reversible, K^+-competive inhibitors of the gastric H^+/K^+-ATPase (53,54); the term "K^+-site inhibitor" has been adopted. Being a weak base (pK_a **19** = 5.5) like omeprazole, these compounds can accumulate in the acidic compartments of the parietal cell. However, in this case the compounds bind non-covalently in the protonated form to the luminal face of the enzyme (54,55). Azido derivatives, e.g. **22** (56) and **23** (57), have been



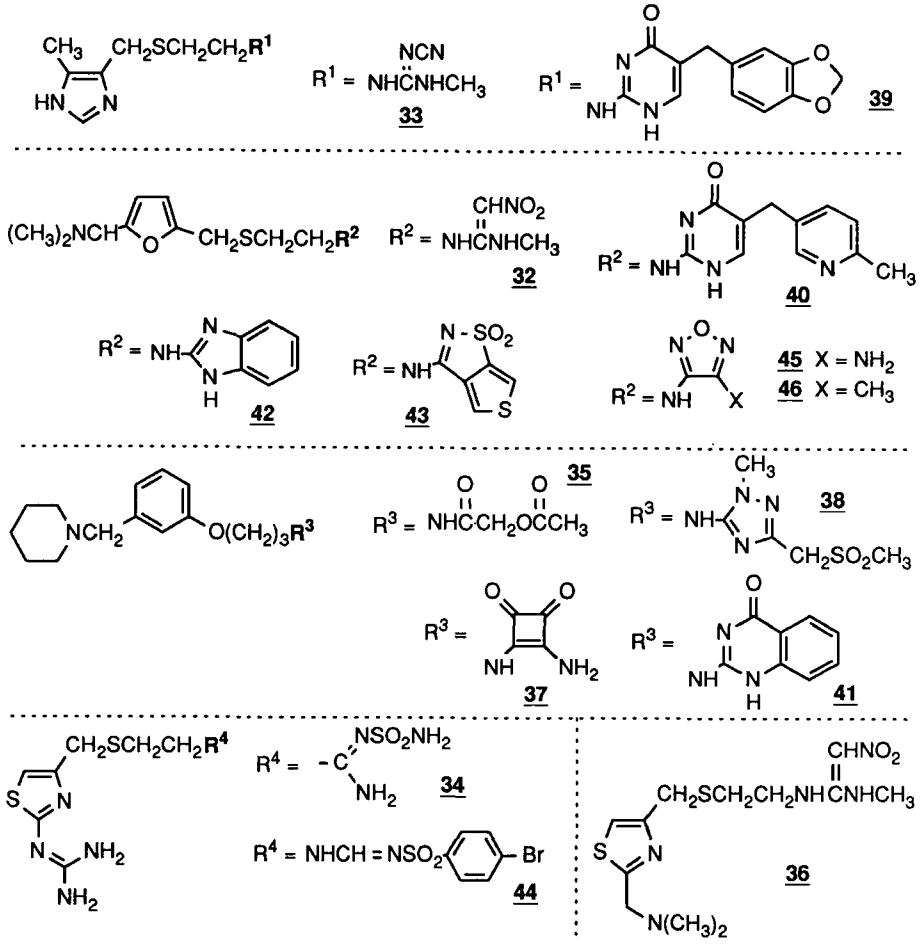
used as photoaffinity probes in an attempt to identify the binding site for these inhibitors on the amino acid sequence for the H^+/K^+-ATPase.

The quinoline derivative **24** has now also been shown to be a reversible K^+-competitive inhibitor of the gastric H^+/K^+-ATPase (58). Conformationally restrained analogs, e.g. **25** and **26**, showed activity approaching that of **24** against the isolated enzyme; however, in vivo activity in the rat after iv administration was significantly less (58). The guanidinothiazole derivative **27** represents a further example of a "K^+-site inhibitor" (59). Using this as a starting point, SAR studies, despite leading to compounds substantially more potent against the isolated enzyme, gave only a three-fold increase in in vivo potency (60). From a series of aryloxyalkylamines, compounds **28** and **29** were identified as the most interesting, with activities in the pylorus-ligated rat model comparable to ranitidine (61). The antisecretory activity appears to be a consequence of H^+/K^+-ATPase inhibition, but no information as to whether these were "K^+-site inhibitors" was given. Similarly, the antisecretory activity of 2-cyano-3-(ethylthio-3-methylthio)-2-propenoic acid methyl ester (sic) (AY-28080) and WY-26769 (**30**) may also be a consequence of H^+/K^+-ATPase inhibition (62,63). The latter compound is said to inhibit the enzyme non-K^+-competitively (63). A number of gastric H^+/K^+-ATPase inhibitors have been identified from marine organisms, e.g. the polyacetylene siphonodiol from Siphonochalina Truncata (64) and the hexaprenylhydroquinone sulphate **31** isolated from a sponge of the Dysidea genus (65). Other miscellaneous H^+/K^+-ATPase inhibitors reported include polyamines such as spermine (66), the macrolide antibiotic copiamycin A (67) and the polypeptide bee venom toxin melittin (68). The latter compound has been shown to bind to a site distinct from that of SCH 28080 (68).

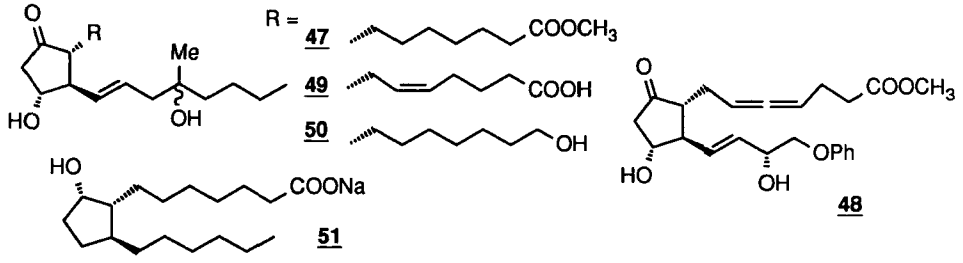
Histamine H_2-Receptor Antagonists - Currently, five H_2-antagonists are marketed worldwide with ranitidine (**32**) outselling cimetidine (**33**) as the world's number one drug (69). The medicinal chemistry has been reviewed (70), and two symposia proceedings review recent developments with particular emphasis on famotidine (**34**) (71) and roxatidine acetate (**35**) (72). The general pharmacology of nizatidine (**36**) has been reported in detail (73). The optimum dosing regime for DU continues to be debated with a recent healing study on famotidine (74) and intragastric acidity measurements with SK&F 94482 (BMY 25368, **37**) (75,76) and sufotidine (**38**) (77) questioning the need for evening dosing with these agents. In an attempt to achieve the healing rate of omeprazole for DU, repeat dosing of ranitidine (300 mg qds) has been studied. Although a significant improvement at 2 weeks was observed over the standard dose, this was not significant at 4 weeks (78). Despite conflicting results, a recent critical analysis of available clinical data concluded that H_2-antagonists were an effective treatment for GERD; mainly in moderate to severe cases (79).

The medicinal chemistry leading to famotidine has been described (80). Similarly, the medicinal chemistry leading to oxmetidine (**39**) and the long acting H_2-antagonist lupitidine (**40**) has been reported (81-83). In a related series of quinazolones, **41** (NO-794) was found to be some 10 times more potent than ranitidine in the pylorus-ligated rat, but of comparable potency on the guinea pig right atrium (84). Other bicyclic systems reported include the 2-aminobenzimidazole **42** (of similar potency to ranitidine in the lumen perfused rat (85)), and Wy-45727 (**43**) (86). The sulfonylamidine **44** inhibited histamine stimulated acid secretion in the rat with an ED_{50} similar to ranitidine (87). The diaminofurazan **45** was some 15-20 times more potent than ranitidine on the guinea pig right atrium, but the corresponding monoamino analog **46** was only of similar activity (88,89); potency, however, could be further increased by substitution on the primary amino group (90). A number of recent SAR studies continue to focus on the dipole moment and its orientation in the polar end group (91-93). The effect of substitution in the phenyl ring of lamtidine analogs has been investigated, but more potent compounds were not identified (94).

Prostaglandins - The cytoprotective actions of endogenous and exogenous PGs have been the subject of several reviews (95-100). Though clinical and preclinical work on synthetic analogs of PGE_1 and PGE_2 is continuing, it remains controversial whether these compounds have therapeutic advantages in treating peptic ulcer disease separate from their antisecretory action (96,97). Their role in the prophylaxis of NSAID-induced ulcer may be clearer, and this area has been the subject of several studies (98,101). This class of compounds has been reviewed in comparison with histamine H_2-antagonists (102).



Misoprostol (47) is now on the market in many countries. A dose of 400 μg bid was as effective as ranitidine (150 mg, bid) in healing GU (103). The major clinical side effect is diarrhea; postprandial administration of misoprostol produced less adverse effects on gastrointestinal transit *than preprandial administration* (104). *Administration with food reduced peak plasma* concentrations of the active metabolite, misoprostol acid, without affecting AUC (105). Effect on intragastric pH appeared weak, as 800 μg and 600 μg single doses, but not 200 μg qid, reduced 24-h pH relative to placebo (106). In vitro, misoprostol inhibits acid secretion both by a direct effect on the parietal cell and by inhibiting endogenous histamine release (107). In contrast to natural PGs, it inhibited basal as well as pentagastrin-stimulated acid secretion (108,109). The effect of misoprostol in reducing NSAID-induced mucosal damage has been reviewed (110), and further

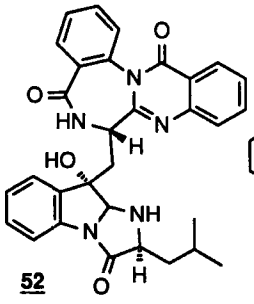


studies continue to be reported. Misoprostol was more effective than placebo in reducing gastroduodenal injury from aspirin (111) or ibuprofen (112), and was more effective than cimetidine in protecting against tolmetin (113). Histological effects of aspirin on dog stomach with misoprostol pretreatment have been reported (114). Misoprostol has been reported to be more effective than ranitidine or sucralfate in treating NSAID-induced ulcers, and its use in all patients over 50 receiving NSAIDs has been advocated (115), though this policy has been criticized (116).

Enprostil (**48**) has been the subject of a review (117), and its metabolism has been reported in detail (118). SAR studies have shown that modifications at C-1 (the ester group) and C-11 generally give active compounds, whereas modifications at other sites generally led to loss of activity (119). In vitro, enprostil inhibited pentagastrin-stimulated acid secretion for longer than misoprostol (109). Unlike misoprostol, enprostil was effective against histamine-stimulated secretion (109), which mirrors the results with the natural analogs PGE_1 and PGE_2. In protecting against gastric blood loss induced by aspirin, a dose of 7 µg bid was as efficacious as 35 µg bid (120), although only the higher dose significantly increased the pH of gastric washings. The rate of gastric emptying in volunteers has variously been reported as enhanced (121) or unchanged (122) after enprostil (70 µg). Both basal and food-stimulated acid output and gastric juice volume were significantly reduced, and serum gastrin was unaffected (121). In rats, enprostil reduced the trophic effect of an H^+/K^+-ATPase inhibitor on the gastric oxyntic mucosa, though without preventing hypergastrinemia (123).

Arbaprostil (**49**), currently awaiting approval in Japan, has been the subject of a symposium report (124). A dose of 10 µg qid, which had little effect on acid secretion, was not efficacious in healing GU (125). Rioprostil (**50**) has been the subject of a symposium (126). After slow delivery from a 24-hour release osmotic pump, rioprostil was 37 times more potent in protecting against aspirin-induced ulcer in dogs than when given as a single oral bolus (127). Rosaprostol (**51**) has been shown to be effective in protecting against NSAID-induced ulcers (128,129).

Gastrin Antagonists - During the last few years, increased interest in the actions of gastrin (130) has coincided with major advances in the design of potent inhibitors. This field has been extensively reviewed (131-133). The breakthrough came with the discovery of asperlicin (**52**), which was used as a starting point for the development of the simpler, more potent and orally active benzodiazepine derivatives MK-329 (**53**, previously called L 364,718) and L 365,260 (**54**). MK-329 is selective for peripheral CCK-A receptors (IC_{50} 0.08 nM) relative to gastrin or brain CCK receptors (134); however, although the affinity for gastrin receptors is 4 orders of magnitude lower, MK-329 was still among the most potent gastrin antagonists known at the time of its discovery. L 365,260 has nanomolar potency against gastrin and CCK-B receptors, and is two orders of magnitude less potent against CCK-A receptors (135-137). In rats, L 365,260 inhibited pentagastrin-stimulated acid secretion, but had no significant effect on basal secretion in rats, nor histamine- or carbechol-stimulated secretion in mice (137). Potency and duration of action were species-dependant, being least in the dog. The clinical relevance of this series of compounds remains to be assessed, but they seem certain to be valuable pharmacological tools.



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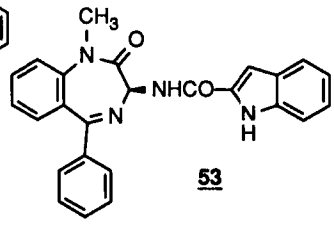

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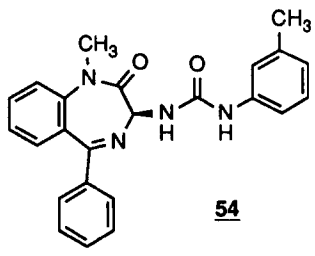

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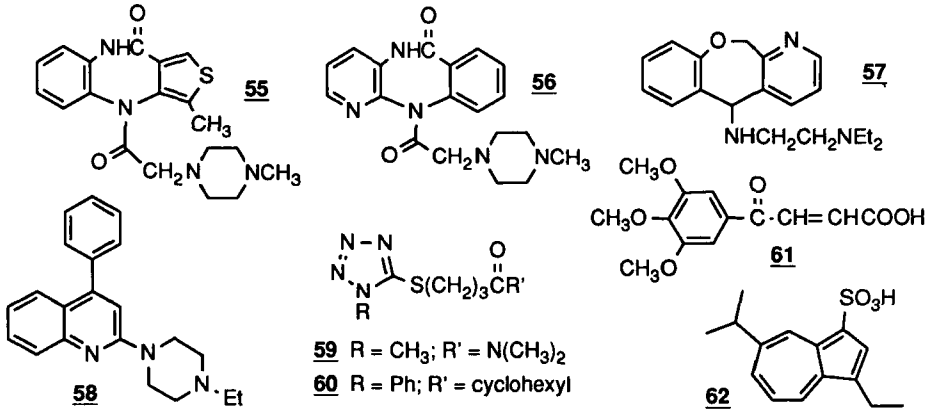
Muscarinic M_1-Antagonists - The clinical significance and current status of muscarinic M_1-receptor antagonists and their usefulness compared with H_2-antagonists has been reviewed (138-140). In a recent double-blind study, telenzepine (**55**) (3 mg nocte) was shown to be as effective as pirenzepine (**56**) (50 mg bid) for the treatment of DU (141). The advantage of combined



pirenzepine/H_2-antagonist therapy for DU continues to be debated, but recent studies suggest a significant improvement over monotherapy (142,143). Antiulcer activity of telenzepine, over and above that due to inhibition of acid secretion, has been demonstrated in rats (144). In a related series of compounds, KW5805 (**57**) was identified as a potent antiulcer agent in a stress-induced ulcer model in rats, comparable to pirenzepine, but with apparently much lower affinity for the M_1-receptor (145).

Other Agents - The most up-to-date clinical information on the mucosal protectant, sucralfate, has been reviewed (146). Comparable efficacy with H_2-antagonists continues to be demonstrated. AD-2646 (**58**) was found to be more potent than cimetidine in the pylorus-ligated rat and stress induced ulcer models (147,148). Compounds **59** and **60** (OPC 12182) were identified from a series of tetrazole derivatives, using an acetic acid induced ulcer model, as being at least 10 times more potent than cimetidine (149,150). RU 38086 (**61**) was selected for clinical evaluation on the basis of its antisecretory and antiulcer potency (151) and KT1-32 (**62**) was identified as a potent anti ulcer agent using the Shay rat (152).

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Chapter 18. Modified Serum Lipoproteins and Atherosclerosis

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Introduction - Myocardial infarction and stroke account for more than 50% of the deaths in the western hemisphere. The underlying disease is atherosclerosis, a complex pathologic process involving the intimal layer of the arteries. It is generally accepted that the fatty streak is the earliest lesion of atherosclerosis. These early lesions contain lipid-laden macrophages as well as some lipid-laden smooth muscle cells (SMC) (1). Apart from phospholipids, free and esterified cholesterol are the main lipid constituents of these lipid-rich lesions. Hypercholesterolemia and hyper-β-lipoproteinemia are considered to be major risk factors in the development of atherosclerosis. Thus, the focus of recent research on the pathophysiology of the disease is to study the formation of lipid-laden cells (so-called foam cells because of their foamy appearance) by incubating various cell types mainly with low density lipoprotein (LDL). However, as it was shown by Goldstein and Brown, synthesis of the LDL receptor and cholesterol biosynthesis are normally finely tuned to prevent an intra-cellular accumulation of cholesterol (2). The aim of this chapter will be to describe the various modifications of lipoproteins leading to their uptake by macrophages and SMC in an unregulated fashion which in turn cause excess lipid deposition in the arterial wall.

CHEMICALLY AND ENZYMATICALLY MODIFIED LIPOPROTEINS

Uptake by Macrophages - Cultured mouse peritoneal macrophages (MPM) take up native LDL to a very small degree and do not accumulate cholesterol esters when exposed to even high concentrations for a long period. Yet LDL modified chemically by acetylation enters the cells by receptor mediated endocytosis; this results in a massive cholesterol accumulation within the macrophages (3). The ε-amino group of the lysine residues of apolipoprotein B (apo B) become blocked by acetylation, the net negative charge being strongly enhanced. Ac-LDL does not bind to the LDL receptor; however, it binds the ac-LDL receptor (or scavenger receptor) on macrophages (3). This receptor is also expressed on monocytes freshly prepared from the blood and increases as much as 20 fold upon cultivation. Other treatment of LDL such as acetoacetylation, malelylation, succinylation, carbamylation and incubation with malondialdehyde (MDA) also modify LDL to a form which is recognized by the ac-LDL receptor (3). The modification of LDL by MDA has been studied extensively since MDA is produced physiologically during phagocytosis by macrophages or by thrombocytes undergoing the release reaction (4). Lipoprotein(a) (Lp(a)), a human serum lipoprotein with a significant atherogenic potential, is converted by modification with MDA to a form readily taken up by monocyte macrophages (5). ince MDA is a final product of lipid peroxidation of arachidonic acid, other break-down products of unsaturated fatty acids were investigated to determine their ability to modify LDL. 4-Hydroxynonenal (HNE), which results from peroxidation of arachidonic acid or linoleic acid, is highly reactive towards LDL (6). LDL treated with HNE shows reduced affinity to the LDL receptor on fibroblasts (7); however, incubation of LDL with HNE above 4 mM consistently ended in an aggregation of the lipoprotein. These aggregates were taken up by both the J774 macrophages and MPM leading to lipid-loading of the cells as observed by oil red staining of the cells (8). The uptake, however, appeared to be facilitated mainly by phagocytosis and not via the LDL or ac-LDL receptor, since competition with excess amounts of LDL or ac-LDL failed to inhibit degradation of labeled aggregated

HNE-treated LDL (8). Another study modifying LDL concomitantly with MDA and HNE demonstrated that the presence of MDA prevented the formation of aggregates of LDL. Using a constant amount of HNE and increasing levels of MDA resulted in an elevated uptake of LDL. Cross competition studies with labeled ac-LDL or labeled LDL, treated simultaneously by MDA and HNE, showed an uptake of MDA-HNE treated LDL by the ac-LDL receptor on macrophages (9). LDL, which was modified by water soluble products derived from autoxidized unsaturated fatty acids, avoiding oxidation of LDL itself, was rapidly degraded by cultured macrophages through the ac-LDL receptor. In an attempt to characterize the modifying compounds to determine which of them might be responsible for the recognition of this modified LDL by the ac-LDL receptor, LDL was incubated with acrolein, crotonaldehyde, pentenal, heptenal and nonenal. Of these, only nonenal in presence of NaCNBH3, could modify LDL in such a way that it stimulated its degradation rate in MPM comparable to oxidized LDL (ox-LDL) (10).

Since workers continually exposed to CS_2 develop hypercholesterolemia, this agent was investigated for its ability to modify LDL (11). Lysine and tryptophan residues were assumed to be affected by CS_2 treatment. CS_2-modified LDL was processed by the LDL receptor in cultured fibroblasts to a lower degree than native LDL. Cholesteryl ester formation in J774 macrophages was markedly stimulated by this modified LDL (11). Cholesteryl ester formation in the monocyte macrophages precedes their conversion to foam cells.

Non-enzymatic glycosylation of LDL is also involved in the reaction with the lysine residues of apo B. This glycosylation leads to an enhanced cholesteryl ester synthesis in human monocyte macrophages. Apart from a slightly reduced uptake via the classic LDL receptor, a low affinity, high capacity pathway seemed to mediate uptake and degradation of glycosylated LDL (12). Glycosylated LDL was also found to be taken up by platelets to a higher degree than control LDL and caused an increased platelet reactivity towards aggregating agents, probably due to an altered structure of the platelet membrane (13). Studies on the metabolism of a LDL-proteoglycan complex by cultured MPM revealed a receptor-mediated pathway in macrophages, but not via the LDL or ac-LDL receptor (14).

Activation of monocyte macrophages by LDL immune complexes caused an enhanced expression of the LDL receptor and stimulated the uptake of native LDL by these cells (15). Digestion of LDL by polymorphonuclear cell elastase degraded apo B, doubled the size of LDL and resulted in an enhanced uptake of the modified lipoprotein by cultured human monocyte macrophages via the LDL receptor (16). Granules of stimulated mast cells were shown to bind and take up LDL, modifying the lipoprotein by proteolysis. This modification resulted in a fusion of LDL to larger particles and stimulated cholesterol ester formation in cultured MPM (17). Furthermore, stimulation of rat peritoneal mast cells enhanced the uptake of LDL by rat peritoneal macrophages in vivo (18). Note however that mast cells and macrophages might coexist in the arterial intima. An enhanced uptake of aggregated LDL (caused by vortexing) by cultured MPM was probably due to phagocytosis and partially mediated via the LDL receptor (19). Modification of LDL by platelet secretory products induced an enhanced uptake in cultured MPM through a receptor mediated process shared at least in part with native LDL (20). A significant stimulation of both the LDL and the ac-LDL receptor activities also was obtained with LDL incubated with secretory products of human moncyte macrophages or human SMC (21).

Uptake by Cultured Aortic Smooth Muscle Cells - Apart from monocyte macrophages, lipid-laden foam cells can derive from arterial SMC (1). Recently it has been shown that desialylation of LDL caused a substantial increase of intracellular cholesteryl esters, free cholesterol and triglycerides in cultured intimal cells of smooth muscle origin. LDL, which was isolated from plasma of patients with coronary heart disease, had a 2 to 5 fold decreased level of sialic acid as compared to LDL from healthy donors; this LDL

induced lipid accumulation in cultured cells (22). Studies with SMC isolated from the intima of human aorta showed that insoluble associates of LDL with certain compounds raised the intracellular amount of total cholesterol significantly. These substances included dextran sulfate, gelatin, particles of aortic elastin or collagenase-resistant aortic matrix, latex beads and goat polyclonal antibodies against LDL. High density lipoprotein (HDL) and very low density lipoprotein (VLDL) in the presence of agents capable of forming insoluble complexes, stimulated intracellular lipid accumulation too (23). Murine resident peritoneal macrophages modified β-VLDL, probably by a secreted neutral serine protease. No indication for oxidative modification of β-VLDL was found. The modified β-VLDL enhanced the accumulation of cholesteryl esters by cultured SMC (24).

OXIDIZED LIPOPROTEINS

Oxidation of LDL by Endothelial Cells - In 1981 it was shown that incubation of LDL with endothelial cells (EC) led to a modified form of LDL which was recognized by the receptor for ac-LDL on macrophages (25). A free-radical oxidation (e.g. lipid peroxidation) was later shown to be involved in the alteration of LDL by EC (26-28). The oxidation of LDL was accompanied by a nonenzymatic cleavage of the peptide bonds of apo B (29); the solubilized fractions of apo B of delipidated ox-LDL were demonstrated to be recognized by the ac-LDL receptor (30). EC lipoxygenase might play an important role in the EC-mediated LDL modification since inhibitors of this enzyme markedly reduced LDL oxidation, whereas superoxide dismutase showed only weak inhibition (31). Prior to this however, an EC line, which generated superoxide anion inefficiently, was shown to be unable to modify LDL oxidatively (32). Since LDL, which was added to the EC in a dialysis bag, did not undergo oxidative modification, a direct cell interaction may be necessary for its modification (31). Enzymatic modification of LDL using purified lipoxygenase plus phospholipase A2 resulted in a modified LDL which was degraded by MPM. This degradation was competitively inhibited by EC-modified LDL but only partially suppressed by ac-LDL (33).

Oxidation of LDL by Smooth Muscle Cells and Monocytes - Since extracellular superoxide was detected in cultured monkey and human SMC, this cell system was tested for its capacity to oxidatively modify LDL (27). The results indicate that in culture arterial SMC generate superoxide and oxidize LDL in the presence of micromolar concentrations of Cu^{++} or Fe^{++} by a L-cystine-dependent process. Uptake and degradation of the modified LDL by human fibroblasts were reduced whereas uptake and degradation by macrophages were enhanced compared to native LDL; this suggests the involvement of the ac-LDL receptor. It was further assumed that the disulfide, L-cystine, might be taken up by the cells in a Ca^{2+}, Mg^{2+} dependent process and secreted in the reduced form, L-cysteine, which in turn takes part in the generation of the superoxide anion (34). The role of thiol compounds in the oxidation of LDL leading to recognition by the ac-LDL receptor has been confirmed independently (35). Rabbit aortic EC, rabbit SMC, monkey arterial SMC and human skin fibroblasts all modify LDL and generate more superoxide anion than an EC line not capable of oxidizing LDL (32). Incubation of LDL with stimulated monocytes led to an enhanced production of superoxide and to an increase in LDL oxidation. Superoxide seemed to be necessary for initiation of LDL oxidation but its progression seemed to be independent of it (36). An optimal oxidation of LDL by human monocytes as well as by two myelomonocytic cell lines was found after exposure of these cells to opsonized zymosan (37). Superoxide anion released by activated monocytes was reported to be important in starting the oxidation of LDL, since addition of superoxide dismutase at the beginning of the incubation inhibited oxidation of LDL in a dose dependent manner. Yet, a superoxide anion-independent free radical propagation process seemed to occur during further LDL oxidation since the latter process was almost completely blocked by butylated hydroxy toluene, a general free radical scavenger, but not by superoxide dismutase (38).

Macrophage Receptors for Ox-LDL - The ac-LDL receptor has been reported to recognize ox-LDL in all the relevant studies cited above. However, a class of receptors was found to exist on cultured MPM, which recognized LDL oxidized by incubation with EC, but not ac-LDL (39). Another report showed that at least three receptors are expressed on cultured MPM. One was specific for ac-LDL, one specific for Cu^{++}-oxidized LDL, and another a common receptor for both ac-LDL and ox-LDL (40).

Structural Properties of Oxidized Lipoproteins - The way by which LDL can be modified by EC can also be mimicked by incubation of the lipoprotein in the presence of micromolar concentrations of $CuCl_2$ (41). Using those conditions it was shown that degradation of arachidonic acid and linoleic acid started after a lag phase. This suggests that the lipophilic antioxidants, like α- and γ- tocopherol, β-carotene and lycopine must be destroyed before degradation of the unsaturated fatty acids occurs (42). Apart from MDA, which was the major product, hexanal, HNE, 4-hydroxyoctenal, 4-hydroxyhexenal, propanal and 2,4-heptadienal were other aldehydic products detected (42,43). However, the pattern of apo B (of delipidated ox-LDL solubilized in SDS) obtained by three-dimensional fluorescence spectroscopy showed that the maximum was comparable with that of apo B of HNE-treated LDL rather than that obtained with apo B of MDA-treated LDL (28). Investigation of the surface of Cu^{++}-oxidized LDL, Lp(a) and VLDL with a polyclonal antiserum raised against HNE-treated LDL, indicated that HNE and, to a minor degree, 4-hydroxyoctenal, formed epitopes on the surface of the oxidized species of these lipoproteins (44). Using monoclonal antibodies against distinctly different epitopes of apo B, it was proved that rapid fragmentation of the protein is accompanied by disappearance of immunoreactive apo B upon Cu^{++}-mediated oxidation of LDL (45).

Cytotoxic and Chemotactic Properties of Oxidized Lipoproteins - Oxidative modifications of LDL may be relevant to the atherogenic process in several other ways. These include cytotoxic effects as well as recruitment and retention of monocyte macrophages. LDL which was oxidized either in the presence of cultured umbilical vein EC, bovine aortic SMC, monocytes or neutrophils was found to become cytotoxic to proliferating fibroblasts. The toxic components were suggested to reside in the lipid moiety of LDL; the toxicity of oxidized LDL was determined to be selective for the S phase of the cell cycle (28). Superoxide anion participates in monocyte-mediated conversion of LDL to a cytotoxin (38). This potent toxin might be responsible for the tissue damage in atherosclerotic lesions. A protective role of intracellular glutathione against Fe^{++}-oxidized LDL was found in cultured EC and it was suggested that the mechanism of the toxicity of ox-LDL was related to the depletion of intracellular content of glutathione (46).

Oxidation of LDL is accompanied by activation of an intrinsic phospholipase A2, further characterized as the platelet-activating factor acetylhydrolase (47). Lysophosphatidylcholine is a potent chemotactic factor for monocytes (48) and may play a role in the attraction of monocytes by ox-LDL and by oxidized β-VLDL leading to invasion of these cells into the subendothelial space (49,50). However, note that 4-hydroxyalkenals, shown to be generated during oxidation of LDL (28,29) were also found to be chemotactic to neutrophils (51).

Effect on Growth Factors, Prostanoids and Thrombocytes - PDGF is the major mitogen for SMC. Production of the PDGF-like protein by cultured vascular EC can be inhibited specifically by the lipid moiety of ox-LDL. The inhibition was dependent on the level of LDL oxidation. Inhibition by ac-LDL was also dependent on oxidation of the lipoprotein (52). Ac-LDL was demonstrated to impair erythroid growth factor release from EC (53). Pretreatment of cultured murine peritoneal macrophages by lipid extracts of ox-LDL resulted in a concentration and time dependent suppression of inflammatory gene expression including tumour necrosis factor (54). Exposure of EC to minimally oxidized LDL resulted in a dramatic induction of mRNA for M-CSF, CM-CSF and G-CSF whereas native and MDA-modified LDL had no effect (55). Thus, this ox-LDL

may induce an EC response favoring the early cellular events in atherosclerosis.

Ox-LDL enhanced prostanoid production and release (mainly PGI2) by cultured human saphenous vein EC (56). Cu^{++}-oxidized LDL was incorporated by macrophages and stimulated prostaglandin E2, leukotriene C4 and 6-keto-PGF1α syntheses as estimated by radioimmunoassay. Prostaglandin E2 and 6-keto-PGF1α were additionally identified by coupled GC/MS. Native LDL, ac-LDL and β-VLDL had no effect, though the latter were more efficiently incorporated than ox-LDL (57). Autoxidized LDL contained large amounts of soluble material which was shown to cross-react with antibodies to prostaglandin E2 but not 6-keto-PGF1α (58).

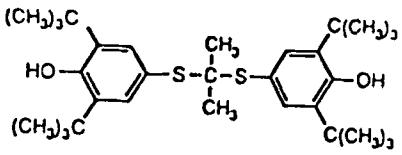
Native and Cu^{++}-oxidized lipoproteins differed significantly in their effects on platelets. Ox-LDL as well as oxidized HDL caused platelet aggregation, probably due to a change in the membrane fluidity of the thrombocytes caused by the oxidized forms of the lipoproteins. The enhanced platelet response could not be attributed to increased production of thromboxane A2 since cyclooxygenase inhibitors like aspirin or indomethacin had little inhibitory effect (59).

Possible Oxidation of Lipoproteins in vivo - As known for many years ceroid, composed of oxidized lipid and protein, occurs in human atherosclerotic plaques. Human ceroid and strongly Cu^{++}-oxidized LDL had many properties in common and it was assumed that human ceroid might be an end product of LDL oxidation in vivo (60). Furthermore, autoantibodies against ox-LDL and ceroid were found in the plasma of chronic periaortitis patients, whereas in young healthy donors no detectable antibodies existed (61,60). A MDA-altered protein was detected in atheroma of Watanabe heritable hyperlipidemic (WHHL) rabbits. For this investigation a monoclonal antibody against MDA-modified LDL was used. However, this antibody did not react with ox-LDL (62). Monoclonal antibodies raised against LDL oxidized in vitro either in a cell free system or by arterial SMC were also able to react with atheromata from WHHL rabbits, but not with aortic tissue from control rabbits (63). Polyclonal antibodies against HNE-lysine and MDA-lysine adducts and a monoclonal antibody against Cu^{++}-oxidized LDL were shown to recognize materials in the atherosclerotic lesions of WHHL rabbits. LDL extracted from these lesions was recognized by the antiserum against MDA-modified LDL. Autoantibodies against MDA-LDL were demonstrated in rabbit and human sera (64). Concomitantly, it was suspected that autoantibodies may bind to ox-LDL in the arterial wall and contribute to foam cell formation by uptake through the Fc-receptor on macrophages (65). Foam cells deriving from macrophages from diet-induced hypercholesterolemic rabbits were isolated from the aortas and found to have a positive immunoreactivity with antisera directed against MDA-modified and HNE-modified LDL (66). LDL isolated from the vascular tissue of WHHL rabbits was chemically analyzed and mild lipid peroxidation was detected to be evident in this LDL (67).

Cigarette smoking was demonstrated to render LDL more susceptible to oxidative modification by incubation with cultured SMC as compared to LDL from nonsmokers. LDL which was isolated from smokers was metabolized by cultured peritoneal macrophages twice as avidly as LDL of nonsmokers (68). This was in agreement with the findings that treatment of LDL with cigarette smoke extract leads to cholesteryl ester accumulation in cultured macrophages (69).

Pharmacological Agents - Probucol (**1**), a lipid lowering drug, can also act as an antioxidant to protect LDL against Cu^{++}-mediated oxidation (70,71). Prevention of the progression of atherosclerosis in WHHL rabbits has also been reported independently by two groups (71,72). Since probucol did not prevent foam cell formation of macrophages of WHHL rabbits or mice directly, it was concluded that the antiatherosclerotic effect of this drug in WHHL rabbits was mainly due to its inhibitory effect on the oxidation of LDL (73). However, no effect of probucol treatment on the formation of atheroma was seen in cholesterol-fed rabbits kept at comparable plasma cholesterol levels (74). Other hindered phenols and analogs were also tested with regard to their hypolipidemic and

hypoglycemic properties combined with the ability to prevent lipid peroxidation. CS-045 (2) was selected for further development as an appropriate agent (75).



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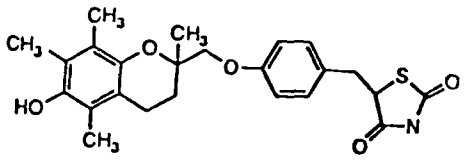

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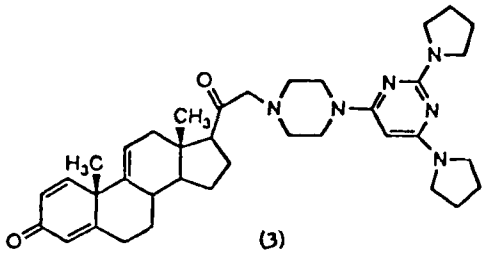

(3)

U74006F (3) was selected for clinical trials from a series of 21-amino steroids, all potent inhibitors of iron-dependent lipid peroxidation, for clinical trials (76). So far however, except probucol, none of these compounds has been tested with regard to its ability to prevent oxidative modification of lipoproteins. 17β-Estradiol inhibited Cu^{++}-mediated oxidation of LDL and the formation of cholesteryl ester in cultured P388 macrophages. The mechanism of the protective effect of this hormone remains to be elucidated (77).

Conclusion - Modification of lipoproteins, especially LDL, seems to be prerequisite to their atherogenicity. Several types of lipoprotein modification were found in vitro to lead to lipid loading of SMC. In recent years attention has focussed on the alteration of the ε-amino groups of the lysine residues on apo B. This modification of LDL may occur in vivo in proportion to the exposure of LDL to oxidative conditions. However, it is too early to assume that such a modification is the only major route by which macrophages are transformed to foam cells in the atherosclerotic process.

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Chapter 19. Biochemistry and Inhibition of Collagenase and Stromelysin

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Introduction - The collagenases and stromelysin are extracellular, calcium dependent, zinc endoproteinases whose substrates include the macromolecular components of the extracellular matrix (1,2). The primary endogenous substrate for the collagenases are the collagens; proteoglycan is thought to be an important substrate for stromelysin. The naming of these enzymes in the literature has not been consistent. For example, stromelysin (3), proteoglycanase (4), transin (3) and MMP-3 (1) have been used to describe the same enzyme. In an attempt to bring order to the nomenclature, the name "matrixin" has been proposed as a way to identify the family of matrix metalloproteinases (MMP) from animal sources (5). However, we will use MMP to refer to the enzyme class in this chapter. It has been suggested that MMPs should display the following properties (5):

1. Zinc is essential for activity.
2. Calcium is required for maximal activity.
3. A proenzyme form, or zymogen, exists.
4. Enzymatic activity is inhibited by a "tissue inhibitor of metalloproteinases" (TIMP).
5. Some macromolecular component of the extracellular matrix is a substrate.

Of the approximately ten human MMPs which have been tentatively identified to date, six have been isolated and have had their enzymatic activity studied: these are fibroblast collagenase (HFC or MMP-1) (6), stromelysin (HFS or MMP-3) (7), neutrophil collagenase (HNC or MMP-8) (8), 72 kDa gelatinase (HFG or MMP-2) (9), 92 kDa/neutrophil gelatinase (HNG or MMP-9) (10), and uterine metalloproteinase (PUMP-1 or MMP-7) (11).

The amino acid sequences of HFC (12), HFS (3, 13), HFG (9), HNG (14), and PUMP-1 (15), have been deduced from the corresponding cDNA. Although a three dimensional structure has not been determined for any member of the MMPs, the preparation of diffraction-quality crystals of porcine synovial collagenase has been reported (16).

This chapter will focus on the biochemistry and inhibition of three of these enzymes: HFC, HNC, and HFS. We will use the more descriptive acronymic nomenclature throughout. Related reviews have appeared that more broadly cover inhibitors of metalloproteinases (17), and more narrowly cover inhibitors of HFC (18).

SUBSTRATE SPECIFICITY

Action on Natural Substrates - Collagens and proteoglycans are the major organic molecules of cartilage and bone. There are over ten distinct collagen types known (19). Types I, II and III collagen are reasonably well characterized and are structurally related, consisting of three-stranded helical soluble monomers which associate to form rod-like fibrils. HFC (20) and HNC (21) are the only known human enzymes which can degrade soluble and fibrillar collagen of types I, II and III in the helical region. Since the major collagen of cartilage is type II collagen, this implicates these enzymes in diseases of

cartilage destruction (19). HFS does not degrade helical collagen, but can degrade proteoglycans, laminin, type IV collagen and fibronectin (7).

The Sequence Specificity of Collagenase - The nomenclature of Schlecter and Berger (22), in which the hydrolyzed bond of the substrate is between the P_1 and P_1' residue, is used to describe peptide substrates. It is generally accepted that HFC and HNC cleave the collagen triple helix at a specific site across all three chains. The sequence cleaved by HFC in many collagens is known (23); **1** and **2** are the sequences of the cleavage sites in the α1 and α2 chains of calf type I collagen, respectively. Collagen has a repetitive sequence, and several sequences occur with high homology to that of the cleavage site. It has been proposed that disruption of the helical structure at the cleavage site of collagen results in an appropriate conformation for interaction with the enzyme, and is thus responsible for the specific sequence required by HFC (23). This hypothesis was strengthened by the observation that HFC hydrolyzed the octapeptide Gly-Pro-Leu-Gly-Ile-Ala-Gly-Pro faster than **1** without detectable hydrolysis of intact collagen at the site of this sequence (23).

The parameter which is maximized in a "good" substrate is the specificity constant, k_{cat}/K_m (24). The specificity constant is equivalent to the second order rate constant for reaction of substrate and enzyme when the concentration of substrate is far below its K_m. The relationship between highly specific peptide substrates (that is, those with a high k_{cat}/K_M) and the K_i of transition-state inhibitors is well documented (25). Substrates **3** and **4**, with greater specificity constants than **2**, are obtained by substitution of **2** with hydrophobic amino acids, specifically, Leu at P_2 (26), and Leu or Trp at P_2' (27). These peptides have comparable specificities for HFC to type I collagen from rat skin (k_{cat}/K_m = 42.7 $\mu M^{-1}h^{-1}$) (23). Substrate **4** was used in the development of a continous fluorogenic assay for kinetic analysis of potential inhibitors of HFC (27).

	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	k_{cat}/K_m ($\mu M^{-1}h^{-1}$)
1	HGly	Pro	Gln	Gly	Ile	Ala	Gln	GlyOH	0.22
2	HGly	Pro	Gln	Gly	Leu	Ala	Gln	GlyOH	0.35
3	Ac	Pro	Leu	Gly	Leu	Leu	Gly	OC_2H_5	17.5
4	DNP	Pro	Leu	Gly	Leu	Trp	Ala	D-Arg-NH_2	5.4

More that fifty octapeptides which differed from the sequence of **1** by a single amino acid change have been synthesized, and their rates of hydrolysis by HFC, HFS, and HNC measured (28). Some substrates, in which a substitution led to enhanced specificity, are shown in the following table. The data are reported as a % of the specificity measured for the"endogenous" octapeptide sequence **1** from collagen.

	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	HFC[a]	HFS[b]	HNC[c]
5	Gly	Pro	Leu	Gly	Ile	Ala	Gly	Gln	150	190	260
6	Gly	Pro	Gln	Ala	Ile	Ala	Gly	Gln	660	300	320
7	Gly	Pro	Gln	Gly	Leu	Ala	Gly	Gln	130	110	180
8	Gly	Pro	Gln	Gly	Ile	Trp	Gly	Gln	840	280	930
9	Gly	Pro	Gln	Gly	Ile	Ala	Ala	Gln	220	280	120
10	Gly	Pro	Gln	Gly	Ile	Ala	Met	Gln	130	810	34

a) % k_{cat}/K_m, 100%=0.22 $\mu M^{-1}h^{-1}$; b) % k_{cat}/K_m, 100%=0.06 $\mu M^{-1}h^{-1}$; c) % k_{cat}/K_m, 100%= 5.65 $\mu M^{-1}h^{-1}$.

These data demonstrate that these MMPs prefer substrates that are generally more hydrophobic, but are still related, to the sequence cleaved in collagen by HFC. The sequence specificity of HFC, HNC and HFS appear to be rather similar. However, note that the substitution of methionine at P_3' selectively leads to a large increase in specificity for HFS.

The Sequence Specificity of Stromelysin - In addition to the peptides sequences **5** to **10**,

modeled after the HFC cleavage site in collagen, the peptide sequences of several other HFS substrates have been reported:

			P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	k_{cat}/K_m [a]
11	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	LeuMetNH$_2$	19
12				Ile	Gln	Ala	Glu	Asn	Gly		(b)
13				Ala	Ile	His	Ile	Gln	Ala		(b)

(a) $\mu M^{-1}h^{-1}$; (b) no data reported

Structure activity relationships for hydrolysis of **11** (Substance P) were investigated in detail (29). Substitutions of p-nitro-Phe or p-Cl-Phe into the P1′ position, or Tyr into the P2′ position were tolerated, and terminal amino acids could be removed up to the P4 or P3' positions (29). Substitution of Gly with sarcosine at P_3' was not tolerated. Compounds **12** and **13** have been identified as HFS hydrolysis sites in cartilage proteoglycan link protein (30).

CONTROL OF MMP ACTIVITY

MMP activity is highly regulated *in vivo* at several levels: 1) transcription (for non-neutrophil MMPs), 2) activation of the proenzyme, 3) inhibition by endogenous protein inhibitors, and 4) chemotaxis and activation of neutrophils (for neutrophil MMPs). Each of these regulation points may be considered as possible approaches to interruption of proteinase activity in addition to direct inhibition of the active enzyme by exogenous species.

Transcriptional regulation - HFC and HFS are transcriptionally regulated by tumor promoters such as phorbol esters, mediators of inflammation such as IL-1, growth factors, and oncogenes (14). The time-dependant upregulation of HFS and HFC by IL-1, and the presence of elevated IL-1 levels in inflammatory diseases such as rheumatoid arthritis, has led to the strategy of attempting to prevent inflammation-related tissue damage via IL-1 antagonism (31). HNC is not transcriptionally active in mature neutrophils (10).

Proenzyme activation - Activation of the latent proenzyme form of HFC (32), HFS (33) and HNC (34) has been accomplished with organomercurials, serine proteinases, oxidants and surfactants. These diverse methods for activation have been rationalized in terms of a single mechanism termed a "cysteine switch" whereby the sulfhydryl group of Cys^{73} is ligated to the active site zinc ion (35). This event prevents coordination of water and thus does not allow activation of the enzyme. As this cysteine is conserved in all MMPs, this hypothesis is consistent with a common mechanism of activation. The cysteine is located in the 81 residue propeptide which is removed during activation of the MMP by enzymatic proteolysis; the initial step for trypsin-like serine proteinases is cleavage of the Arg^{55}-Asn^{56} bond. It is hypothesized that this initial step results in a destabilization of the Cys^{73}-Zn bond which allows autolysis of the propeptide fragment followed by activation (32). There is evidence that the initial activation step of MMPs *in vitro* by mercurials, disulfides and oxidants also occurs by reaction with the Cys^{73} thiol, followed by coordination of water.

The mechanisms of activation *in vivo* are much less certain. An attractive proposal for HNC activation relies on the "oxidative burst" produced during degranulation of neutrophils (2). The hypochlorite produced by the neutrophil is a known activator of HNC *in vitro*. The postulated activation of HFC and HFS *in vivo* is more complicated. Activated HFC is produced when mixed cultures of fibroblasts and keratinocytes are grown in the presence of plasminogen (36). It is suggested that the fibroblasts produce pro-HFS and pro-HFC and the keratinocytes produce urokinase (uPA) which initiates a proteolytic cascade: uPA activates plasminogen to plasmin which acts as a tryptic enzyme in the activation of pro-HFC and pro-HFS. It has been demonstrated *in vitro* that coactivation of HFC and HFS could result in the enhanced activation of HFC (4,36). Thus,

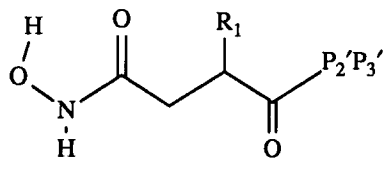
blocking the activation of these MMPs may be a viable medicinal chemistry strategy to prevent tissue damage.

Inactivation by Proteinaceous Inhibitors - α2-Macroglobulins have been reported to be excellent inhibitors of HFC (37) and HFS (38) in vitro, and the sequence of the "bait" region of these inhibitors, which is cleaved by these proteinases, has been determined. The rates of hydrolysis of octapeptide substrates based on these sequences have been measured (23). The specificity constants were found to be approximately the same as those for **1** and **2**. A unifying principle of MMP biochemistry is the specific, high affinity inhibition by the proliferating family of TIMP's (39, 40). However, α2-macroglobulin has been shown to be a more effective inhibitor in competitive experiments, and is present in much higher concentrations in serum or rheumatoid synovial fluid (41).

INHIBITOR DESIGN

In this section we confine ourselves to developments in the field since the review of Johnson (18). Hydroxamates, carboxylates, thiols, and phosphorous-containing (phosphonamidates, phosphonates and phosphinates) peptide-derived inhibitors of HFC have been described. The most potent examples of each class have Leu, Ile or a similar residue at P_1', and Leu, O-methyl Tyr, or Trp at P_2' (18). These data are consistent with the sequences of highly specific substrates **3** and **8**. Substituents at positions which correspond to P_1 and P_2 are frequently beneficial as described below. Most of the available data on MMP inhibition refer to HFC. It is not reported if the inhibitors also have activity versus HFS or HNC. There are no reports that any compound has entered clinical trials for evaluation as a collagenase inhibitor.

Hydroxamate Inhibitors - The peptide hydroxamate **14** was the earliest reported sub-100 nanomolar inhibitor of HFC (42). Actinonin (**15**) was detected in the culture supernatant of an Actinomycete strain (43). Substitutions of isomeric pentyl groups at R_1, and a morpholino substitution at P_3' (see **16** to **19**), failed to increase potency (44, 45). Substitution of the isobutyl group at R_1, **20** to **23**, gave sub-100 nanomolar inhibitors.



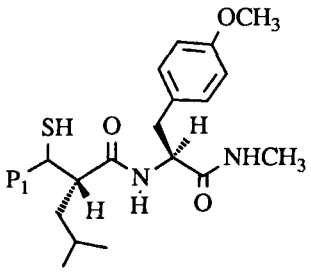
	R_1	P_2'	P_3'	$IC_{50}(\mu M)$
14	Me_2CHCH_2	Tyr(Me)	NHMe	0.020
15	n-pentyl	Val	(a)	0.35
16	n-pentyl	Val	N-morpholino	0.35
17	i-pentyl	Val	N-morpholino	0.35
18	n-pentyl	Ile	N-morpholino	0.35
19	Me_2CHCH_2	Val	NH_2	0.32
20	Me_2CHCH_2	Val	NHBz	0.035
21	Me_2CHCH_2	Val	NHBz(4-CF_3)	0.080
22	Me_2CHCH_2	Val	$NHCH_2CH_2Ph$	0.075

(a)= 2-hydroxymethylpyrrolidino

Thiol Inhibitors - Compound **23** (46) may be considered as the thiol analog of **14** . It is 200 fold less potent than **14** against MMPs. Substitution at the P_1 position (46) with phenyl, yielding **24**, or with methyl, yielding **25**, gives rise to a twenty-fold increase in potency. However, incorporation of a 2-oxopropyl group to give **26** (47), produces a further

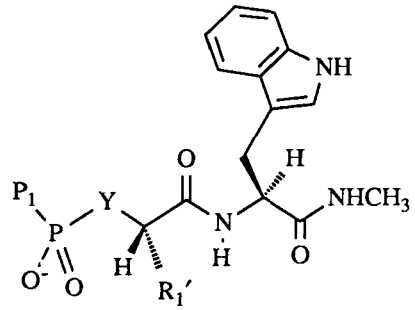
increase in potency, yielding an inhibitor which is equipotent to the hydroxamate **14**.

	P_1	IC_{50} (μM)
23	H	4
24	Ph	0.27
25	Me	0.22
26	CH_3COCH_2	0.014

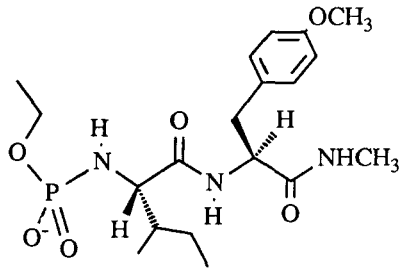


Phosphorous-based Inhibitors - Several phosphorus-containing species have been found to be inhibitors of MMPs, including phosphinates (C-PO_2^--C), phosphonates (C-PO^-_2-O) and phosphonamidates (C-PO_2^--N). Thermolysin (TLN), although not an MMP, is perhaps the best available mechanistic model for HFC. It is a bacterial, zinc endoproteinase whose structure is known and mechanism of action well documented (48). Extremely potent (Ki = 0.07 nM) phosphonamidate inhibitors of TLN have been described (25), and their mode of interaction with the enzyme has been studied crystallographically (49). The potency is attributed in part to a specific hydrogen bond between the phosphonamidate N-H and the carbonyl oxygen of Ala^{113}. Although there are clear differences in inhibitor potency/structure relationships between TLN and the MMPs (for example, phosphoramidon is a potent inhibitor of TLN and is not an inhibitor of HFC (50)), attempts have been made to rationalize HFC inhibitor potency in relation to the data for thermolysin. For HFC, the similarity in K_i's for **28** and **29** have led to the proposal that a similar hydrogen bond is not present in the phosphonamidate-collagenase complex (51). Clearly, the CH_2 of **28** and the oxygen of **27** adjacent to the phosphorus atom cannot form a direct hydrogen bond to a carbonyl oxygen.

Two proposals have been suggested as explanations of the relative potency in a series such as **27** to **29**. One proposal is that the basicity of the phosphorous oxygen influences the potency: the measured pK_as of **27**, **28**, and **29** correlate with inhibitor potency (52). However, this proposal does not explain why **30** (52) is not significantly more potent than **31** (53). The second proposal is based on calculations of inhibitors of



27-31

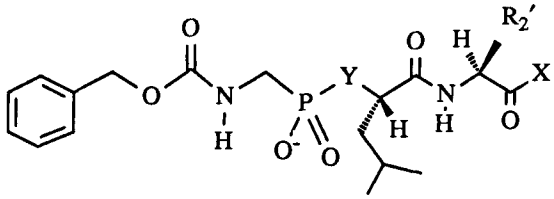


32

	P_1	Y	R_1'	Ki(μM) vs HFC
27	n-hexyl	O	2-methylpropyl	300
28	n-hexyl	CH_2	2-methylpropyl	0.5
29	n-hexyl	NH	2-methylpropyl	0.6
30	n-propyl	NH	2-butyl	2
31	EtO	NH	2-butyl	2
32	-	-	-	31

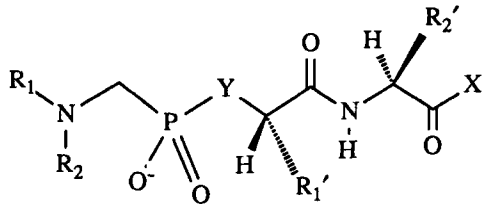
thermolysin 35 to 37 (53). This series is analogous to the series 27 to 29 for HFC (53). These calculations suggest that the oxygen isostere 35 is a weaker inhibitor than 37 due to a repulsive interaction between the inhibitor oxygen and the carbonyl oxygen of Ala^{113}. The calculations also predicted that 36 would be a comparable inhibitor to 37, due to the reduction in solvation free energy of 36 versus 37, which compensates for the hydrogen bond between the NH of 37 and the carbonyl oxygen of Ala^{113}.

Trp, 31, is favored over Tyr(Me), 32, at P_2' (54) and large hydrophobic groups are allowed at P_2 (compare 38 (51) and 41 to 43)(55). Substitution at P_3 (39 and 40) offers no advantage over hydrophobic groups at P_2. Compounds 33 and 34 were evaluated as inhibitors of HNC (56); only weak potency was observed , but an optimal amino acid was not used at P_2' (28).



	Y	R_2'	X	Ki(μM)
33	NH	CH_3	GlyOH	35[a]
34	NH	CH_3	OH	80[a]
35	O	2-methylpropyl	OH	9[b]
36	CH_2	2-methylpropyl	OH	0.011[b]
37	NH	2-methylpropyl	OH	0.009[b]

a) Inhibition measured versus HNC; b) inhibition measured versus thermolysin.



	R_1,R_2	Y	R_1'	R_2'	X	Ki(μM)[e]
38	Phth[a]	NH	2-butyl	2-indolyl	$NHCH_3$	0.05
39	AcProLeu,H	NH	2-butyl	2-indolyl	$NHCH_3$	0.3
40	ZProLeu,H	CH_2	Me_2CHCH_2	Me_2CHCH_2	AlaOH	0.06
41	Napth[b],H	CH_2	Me_2CHCH_2	2-indolyl	$NHCH_3$	0.03
42	Napth[b],H	CH_2	Me_2CHCH_2	2-indolyl	NHBz	0.01
43	Nap[c],H	CH_2	Me_2CHCH_2	(d)		0.11

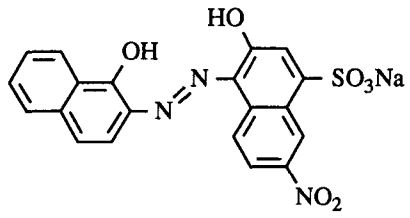
a) Phth is the phthalimide derivative; b) Napth is the 2-napthamic derivative; c) Nap is 1,8-naphthalenedicarboximido; d) N-CR_2'-CO-X is replaced by 3-amino-2-oxo-1-azacyclotridecyl; e) inhibition measured versus HFC.

Gold Inhibitors - Sodium gold thiomalate and sodium gold thiosulfate are noncompetitive inhibitors of HNC (57), and the inhibition of HNC by these agents is reversed by Zn(II). An extrinsic metal binding site on HNC has been proposed to explain these data, and this

proposal is supported by the observation that Cu(II) and Cd(II) are weak inhibitors of HNC (57). While the mechanism of action of Au(I) salts for the treatment of rheumatoid arthritis is not known, it was suggested that the inhibition of neutrophil proteinases could be a factor (58).

Nonpeptide Inhibitors - The only reported competitive, nonpeptide inhibitor of HFC is Eriochrome black T, **44**, which has a K_i of 1.5 υM (59). Since no inhibition of HFG occurred up to 76 μM, it was suggested that **44** is not simply acting as a zinc chelator.

44



Activity of Inhibitors in vivo There have been few reports of the in vivo activity of MMP inhibitors. When given as an intravenous or intraperitoneal injection, **14** reduced the number of lung lesions in mice which were given intravenous injections of melanoma cells (60). Radiological assessment indicated that **43** was effective in preventing joint damage in type II collagen-induced arthritis in the rat (55).

Conclusion Substantial progress has occurred in the cloning, isolation and purification of HFC and HFS in quantities sufficient for mechanistic and structural studies. Peptide-based assays have been developed which are much more accurate and reliable than assays based on the hydrolysis of biological macromolecules. Although potent, peptide-based inhibitors of HFC have been designed, few reports of activity in vivo have appeared. Only when inhibitors with in vivo activity are obtained will the role of the MMPs in disorders such as arthritis and peridontal disease be established.

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Chapter 20. Pharmacological Modulation of Interleukin-1

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Introduction - Interleukin-1 (IL-1) is a member of the class of proinflammatory proteins (cytokines) produced primarily by stimulated mononuclear phagocytes (1-2). It has been shown to play a central role in mediating many immunological and inflammatory responses to infection and tissue injury (3-5). There is accumulating evidence for its role in many chronic inflammatory disorders such as rheumatoid arthritis (6), inflammatory bowel disease (7), psoriasis (8), allergic encephalitis (9), glomerulonephritis (10) and adult respiratory distress syndrome (11). In most cases, an inappropriate production of and/or response to IL-1 has been implicated (12-13). It is therefore thought that the inhibition of IL-1 production and IL-1 function are both important considerations in drug intervention strategies. Advances in gene cloning have provided the recombinant IL-1 and monospecific anti-IL-1 antibody tools necessary to allow better understanding of the synthesis and action of IL-1 at the cellular and molecular levels and to facilitate the search for agents which interfere with IL-1 production or action. Recent reviews on immunomodulators have extensively covered both synthetic and natural products which result in the production or release of cytokines (14-15). For a general review on the interleukins the reader is refered to Volume 23 of this series (16). This chapter summarizes recent findings in the cellular and molecular biology of IL-1 and focuses on agents with therapeutic potential in the treatment of inflammatory disease which are reported to inhibit the synthesis, processing, binding and/or action of IL-1.

STRUCTURE AND FUNCTION OF IL-1

IL-1 exists in two structurally diverse forms (17-19) termed IL-1α and IL-1β, which are encoded by two distinct genes (20), the latter of which is mapped on chromosome 2 (21). Both forms are synthesized as 31 kDa precursor molecules which are subsequently cleaved intracellularly by specific proteolytic enzymes to their respective 17 kDa mature forms (22). The exact secretory pathway for IL-1 is not fully understood since the precursor molecules lack a classical signal cleavage region which facilitates protein transport across plasma membrane. Despite structural diversity, the mature products of both forms have a similar if not identical spectrum of biological activities (5). In addition, both molecules bind to the same cell surface receptor (23). Unless otherwise indicated, IL-1 without a suffix refers to both forms.

IL-1 is also produced by other cell types including synovial fibroblasts, keratinocytes and Langerhans cells of the skin (24), mesangial cells of the kidney (25), glial and microglial cells of the brain (26), endothelial and epithelial cells and lymphocytes (27,28). IL-1 is a highly inducible protein and in monocytes, its synthesis can be triggered by a variety of stimuli such as bacterial lipopolysaccharide (LPS), microorganisms, complement C3 and immune complexes (29). The kinetics of transcription and translation are similar for the two IL-1 forms; however, the half-lives of these product pairs greatly differ (30). There is preliminary evidence for the modulation of the differential expression of the two IL-1 forms. Recent studies suggest the existence of a specific processing enzyme for IL-1β (31-33). A unique intracellular protease cleaves precursor IL-1β to the mature active form in human monocytes at the unusual cleavage site of Asp(116)-Ala(117). This enzymatic activity is distinct from neutrophil elastase in both its peptide cleavage specificity and its reduced sensitivity to elastase inhibitors. While it is not clear which of the two proteolytic enzymes is physiologically relevant, full biological and receptor binding activity requires appropriate proteolysis of the precursor molecule. Since the precursor IL-1β molecule is

biologically inactive, the characterization of this proteolytic enzyme should provide insight into the cleavage process. Transfection experiments demonstrate that proteolysis is an absolute requirement for IL-1 secretion (34). It is also clear that the synthesis of IL-1, unlike many other secretory proteins, does not occur in the rough endoplasmic reticulum compartment (31) or in the microsomes (35).

IL-1 has a broad spectrum of biological activities (29). It regulates lymphocyte development, activation and proliferation, and induces lymphokine expression (36). The pro-inflammatory effects of IL-1 include the production of prostaglandins, neutral proteases and IL-6 by fibroblasts, synovial cells, chondrocytes, endothelial cells, hepatocytes and osteoclasts (37). IL-1 also regulates body temperature and affects the central nervous system (38). In experimental models, IL-1 administered into the joint space elicits many characteristics of antigen-induced arthritis (39).

At least one form of the IL-1 receptor has been purified and its cDNA cloned (40). The 80 kDa receptor displays high affinity binding of IL-1 (Kd of 100 pM) with structural features similar to the other integral membrane receptors of the immunoglobulin superfamily. The x-ray crystal structures of IL-1α (41) and IL-1β (42) respectively have been recently elucidated and should provide insight into the molecular interaction between IL-1 and its receptor. The signal transduction pathway by which IL-1 mediates its effects is not well understood. Several biochemical pathways have been suggested to play a role in mediating IL-1 signaling such as increases in intracellular cAMP (43), activation of phospholipase A_2 (44) and protein kinase A (45). However, previously suggested involvement of protein kinase C, calcium and phosphatidylinositol metabolism in IL-1 signaling has been discounted (46). Electron microscope autoradiographic evidence suggests that internalized IL-1/IL-1-receptor complex accumulate in the nuclei, suggesting a nuclear site for IL-1 receptor signaling (47).

Despite the overwhelming evidence for the role of IL-1 as a mediator in a variety of diseases, its beneficial attributes have also been examined and could be exploited as a therapeutic modality. IL-1, when administered prophylactically can protect against a lethal challenge of radiation (48) and microbial infection (49). IL-1 also has major effects on hematopoiesis (50). It stimulates the stromal cells to produce colony stimulating factors and directly induces proliferation of multi-potential stem cells (51). Furthermore, the ability of IL-1 to lyse certain tumor cells provides rationale for the clinical trial using IL-1 as an anti-tumor agent (52).

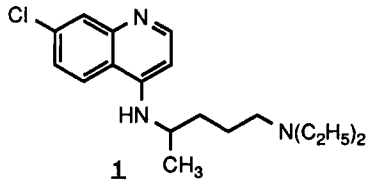
The profound effects of IL-1 on connective tissues, neutrophils and endothelial cells contribute to disease states. For example, IL-1 is found in the joint fluid of arthritic patients at elevated levels (53-55). Activation of osteoclasts and osteoblasts by IL-1 is important for bone remodeling in joint diseases. IL-1 potentiates neutrophil infiltration, degranulation and adhesion to endothelium (56) resulting in hypotension and the capillary leak syndrome (57). Recently, IL-1 has been implicated in the pathogenesis of atherosclerosis (58) and adult respiratory distress syndrome (59).

AGENTS EXHIBITING IL-1 MODULATION

Inhibitors of Synthesis or Release - The disease modifying antirheumatic drugs (DMARDs) chloroquine (**1**), auranofin (**2**), sodium aurothiomalate (**3**) and dexamethasone (**4**), used clinically for the treatment of chronic rheumatoid conditions such as rheumatoid arthritis, have been reported to inhibit IL-1 synthesis (60). While both gold drugs **2** and **3** inhibited mitogen-induced IL-1 release from peripheral blood mononuclear cells (PBMC), only **2** inhibited both spontaneous release and LPS induced release (61). The mechanism of action of **4** was recently shown to be at two levels: In vitro transcription assays with isolated nuclei from glucocorticoid treated cells show inhibition of IL-1 gene transcription, and kinetic studies involving pulse-labeling of mRNAs demonstrate a selective destabilization of the IL-1β mRNA (62).

Tenidap (**5**, CP-66,248) is an antiarthritic drug in clinical phase III trials, and has shown efficacy both in patients with osteo- and rheumatoid arthritis (RA) (63). Clinical improvement in RA

patients was observed by the second week of treatment. After 4 weeks of treatment at 40 to 120 mg daily dose, **5** was judged superior to placebo in all measures tested. This dual inhibitor of 5-lipoxygenase and cyclooxygenase (5-LO/CO) also inhibits the synthesis of both 17 kD IL-1 and 34 kD pro-IL-1 in LPS or zymosan stimulated murine peritoneal macrophages in vitro (64) and is reported to reduce IL-1 activity in the synovial fluid of rheumatoid arthritic patients by 35% in paired measurements of IL-1 from synovial fluid (65). Many analogs of **5** have been patented as antiinflammatory agents and analgesics (66).



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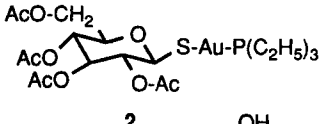

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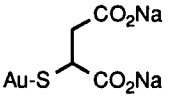

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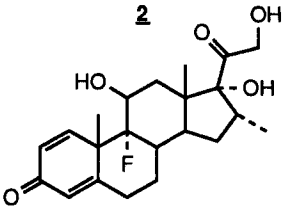
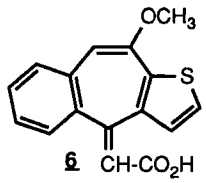


4

The tricyclic ylidene-acetic acid **6** is an antiarthritic, antipyretic and analgesic drug which is reported to block the acute phase reaction in animal models (67). In vitro, **6** blocks the synthesis of prostaglandins from zymosan stimulated murine macrophages and inhibits the release of IL-1α and IL-1β from human monocytes and murine macrophages. Compound **6** and a series of its analogs, particularly the 2-chloro derivative, were recently patented as inhibitors of IL-1 release from human monocytes and mouse macrophages claiming clinical improvement in patients with psoriasis, periodontal disease and Alzheimers disease (68). Results from one clinical study of **6** in arthritic patients reported improvement in clinical measures of disease severity (67).

5



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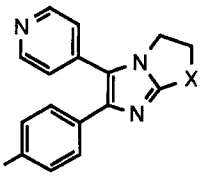
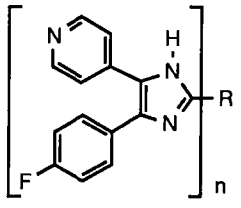


	R	X
7	F	S
8	F	CH_2
9	CH_3O-	$(CH_2)_2$

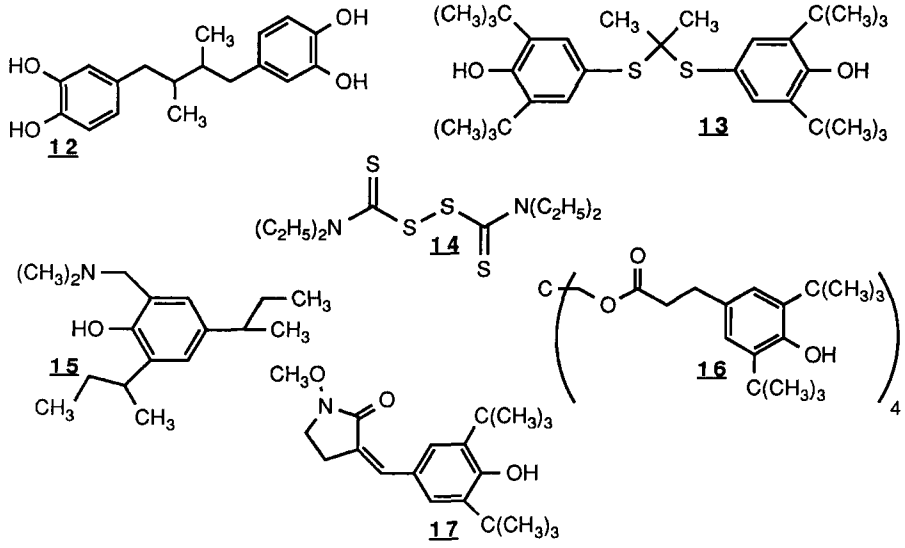


	R	n
10	-SCF_2CF_2H	1
11	-SCH_2COCH_2S-	2

The dual 5-LO/CO inhibitor SK&F 86002 (**7**), has been reported to inhibit the synthesis of IL-1 in human monocytes and human synovial cells in a dose dependent manner (69-71). Examination of analogs showed no correlation between IL-1 inhibition and LTC_4 inhibition in monocytes (71). Study of ibuprofen, phenidone and benoxaprofen in this assay demonstrated that neither selective CO, LO nor dual inhibition was sufficient for downregulation of IL-1 synthesis. The mechanism of action of **7** upon IL-1 production has been demonstrated to be mainly at the level of transcription of IL-1 mRNA in LPS stimulated human monocytes but may also involve a post-transcriptional component (71,72). Both **7** and its sulfur replacement analog **8** significantly

reduced both serum amyloid protein content and disease severity in the murine collagen arthritis model (73). These compounds and additional analogs **9**, **10** and **11** have been recently patented as IL-1 inhibitors (74-76).

The antioxidant and 5-LO inhibitor NDGA (nordihydroguaiaretic acid, **12**) has been observed to inhibit IL-1 production by LPS stimulated monocytes in vitro (69). Probucol (**13**), a hypocholesterolemic drug which possesses antioxidant activity, has been recently reported to inhibit the ex vivo release of IL-1 from LPS stimulated macrophages of mice pretreated orally with 100 mg/kg/day of drug (77,78). In vivo, **13** was observed to inhibit the fall in serum zinc levels induced by iv LPS administration (78). IL-1 induces the production of metallothionein by the liver, which then binds zinc and reduces unbound serum zinc levels (78). The inhibition of this LPS induced zinc lowering effect by 100 mg/kg of **13** dosed for 2 weeks is attributed as direct evidence for inhibition of IL-1 release (78). This inhibition of IL-1 secretion is thought to contribute to the therapeutic effect of **13** in atherosclerosis; levels as low as 1 unit of recombinant IL-1β induce the proliferation of aortic smooth muscle cells (79). Similarly, a group of antioxidants of diverse structure, disulfiram (**14**), **15** and **16** have been recently patented as inhibitors of IL-1 release when dosed orally to CD-1 mice and assayed ex vivo (80). While no indication is given of in vitro inhibition for this group of antioxidants, **13** is specifically reported to have no direct in vitro effect between 10^{-3} and 10^{2} μM, thus the mechanism of IL-1 inhibition in these classes of antioxidants requires further clarification (78).



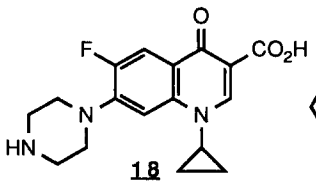
E-5110 (**17**) is a dual 5-LO/CO inhibitor currently in clinical phase II trials; it has recently been reported to inhibit IL-1 synthesis in vitro (81,82). Compound **17** has also been reported to reduce extra- and intracellular IL-1 activity induced by LPS in a dose dependent manner in human monocytes. In this study, hydrocortisone and aurothioglucose were also reported to exhibit potent IL-1 synthesis inhibition. Furthermore, **17** inhibited the IL-1 generation induced by antigen-antibody complexes, zymosan and silica particles (82). Efficacy has also been demonstrated in the adjuvant induced and type II collagen induced arthritic models as well as the carrageenin paw inflammation model at doses comparable to indomethacin (81).

RP 54745, an "amino-dithiol-one" derivative (structure not available), is a preclinical compound which has shown promise in the MRL/lpr mouse spontaneous arthritis assay. When dosed at 1 to 25 mg/kg/day orally for 3 months, it reduces ESR, serum amyloid protein, αDNA antibody and IgG_{2a} levels while increasing lifespan and improving anatomical score (83). RP 54745 blocked the proliferative action of IL-1β on murine thymocytes in vitro and also inhibited the production of IL-1 induced by LPS in mouse peritoneal macrophages in vitro and in vivo. At 3 μM

in vitro in LPS stimulated macrophages, the expression of IL-1α and β mRNA was selectively inhibited while TNFα mRNA was unaffected (84).

Studies on the effect of quinolone antibiotics, particularly ciprofloxacin (**18**), upon IL-1 production in human monocytes have demonstrated a reduction in extracellular IL-1 activity without effecting cell viability or HLA-DR cell surface antigen expression at drug concentrations 5-10 fold higher than normal serum levels (85). Ciprofloxacin delayed the peak production of IL-1α and β by 24 hr and decreased total IL-1β production, but did not change total IL-1α production. No effect was seen on accumulation of IL-1α or β mRNA, but cAMP was increased (86).

Dietary administration of fish oil containing 2.75 g of eicosapentaenoic acid (**19**) and 1.85 g of docosahexaenoic acid per day for 6 weeks to 9 volunteers followed by ex vivo analysis of the endotoxin stimulated PBMC's, demonstrated a significant depression in IL-1β (61%), IL-1α (39%) and tumor necrosis factor (TNF) synthesis (40%) 10 weeks following cessation of the supplementation. These levels returned to normal after an additional 10 weeks. Neutrophil chemotaxis stimulated by leukotriene B_4 was also observed to be significantly depressed (25%) after 6 weeks of dosing, but recovered 10 weeks after cessation of supplementation (87).



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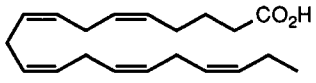

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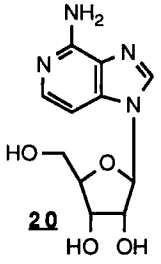


20

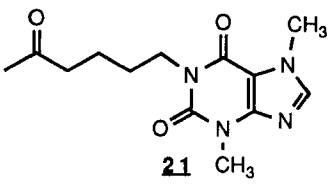
3-Deazaadenosine (**20**), inhibits neutrophil and macrophage chemotaxis, neutrophil adherence to endothelial cells, macrophage phagocytosis and lymphocyte-mediated cytolysis and it is currently in phase I clinical trials as an antiarthritic drug (88,89). Recently **20** has been demonstrated to inhibit IL-1 production by LPS stimulated human PBMCs acting at the level of RNA synthesis. Compound **20** also has been observed to block the effects of added IL-1α on EL-4 cells and on induction of PGE_2 release by human fibroblasts (89).

Inhibitors of IL-1 Mediated Effects - Pentoxifylline (**21**) is a phosphodiesterase (PDE) inhibitor which also increases red blood cell deformability leading to decreased blood viscocity, and increased capillary blood flow. It is used clinically in chronic occlusive arterial disease of the limbs with intermittent claudication. At or near therapeutically achievable levels, **21** has been demonstrated in vitro to block the effects of cytokines, particularly IL-1 and TNF, on neutrophils (90). These cytokine effects include increased PMN adherence to nylon and the priming of neutrophils resulting in increased superoxide production, increased lysozyme release and decreased chemotaxis stimulated by N-formyl-met-leu-phe (FMLP). While **21** is reported to raise cAMP, inhibit FMLP stimulated increase in intracellular calcium and increase binding of FMLP to neutrophils, the mechanism by which it counters the action of cytokines on neutrophils has not been established (90). However, it has been observed that these effects are very similar to the action of adenosine on PMNs (91). Denbufylline (**22**), a closely related xanthine, has been recently patented as a functional inhibitor of the action of cytokines and exhibits a similar profile to **21** on PMNs (92).

Romazarit (**23**, Ro-31-3948), developed from a series of antiinflammatory oxazole and isoxazole propionic acids, is in clinical phase II trials as a DMARD (93). Compound **23** blocks the IL-1 induced activation of human fibroblasts in vitro, antagonizes the rise in serum amyloid P caused by IL-1 injection into mice and was effective in the type II collagen arthritis model in reducing inflammation, bone remodeling and reversing changes in plasma acute phase proteins (94,95). No effect was seen upon PGE_2 synthesis in mononuclear cells, rat renal or sheep

seminal vesicle cyclooxygenase. Significant efficacy was also shown in the spontaneous autoimmune MRL/lpr mouse model (96).

Elevated collagenase and proteoglycanase levels produced by diseased, IL-1 treated, and IL-1 treated diseased human cartilage were reduced by tiaprofenic acid (24, at 4 μM) and dexamethasone (at 1 μM) (97). Similarly, tricyclic compounds 25 and 26 have been recently claimed to inhibit the IL-1 induced release of neutral protease from chondrocytes (98,99). Antiinflammatory activity was demonstrated in the carrageenan paw edema assay (25, 38% inhibition at 50 mg/kg po.; 26, showed a flat dose response curve with 59-64% inhibition at 30-100 mg/kg po.). In a recent in vitro study of a wide variety of representative antiinflammatory and antiarthritic drugs, potent inhibition of IL-1α induced cartilage proteoglycan resorption was observed only with analogs of 1 and 2 (100).



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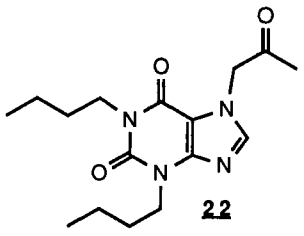

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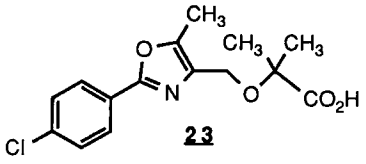

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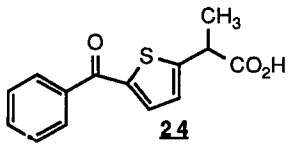

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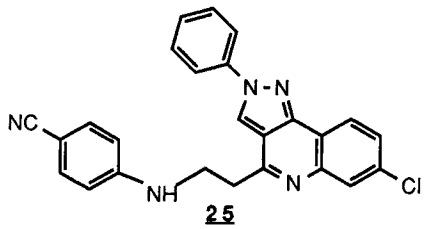

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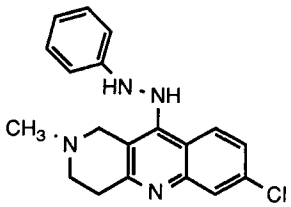

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Receptor Antagonism - The purification and cloning of the IL-1 receptor has provided the necessary reagents to identify the critical structural requirements for the interaction of IL-1 and its receptor (101). The use of a recombinant soluble IL-1 receptor has been shown to ameliorate IL-1 mediated effects in vitro (102). Inhibition of IL-1 action has been accomplished by an anti-IL-1 receptor antibody demonstrating the validity of the receptor antagonist approach (103). The classical method by which a receptor antagonist is discovered involves establishing the pharmacophore of the ligand. Despite the availability of the crystal structure of human IL-1β (42) and epitope mapping studies (104), the nature of the exact pharmacophore remains unclear. It is generally agreed that the critical receptor binding site of IL-1 entails 3 non-contiguous regions which upon proper folding yield the tertiary structure constituting the binding site. Receptor based

screening for small molecule antagonists has been intensely pursued, but no definitive receptor antagonist has been reported to date.

A number of natural macromolecular IL-1 inhibitors have been described (105,106). However, the structures of these inhibitors are not known and they are poorly characterized. Some, such as uromodulin and the "urinary derived molecule", have been refuted as true inhibitors due to experimental artifacts. The recent report of a natural macromolecular IL-1 receptor antagonist (107) provides a strong case for the existence of counter regulatory molecules, suggesting that the imbalance between IL-1 and its natural inhibitors may predispose many disease states. The 22 kDa receptor antagonist molecule is structurally distinct from IL-1 although it is also produced by activated monocytes in a fashion similar to that of IL-1 (108). The molecule competes specifically with the IL-1 receptor with a similar binding affinity to IL-1 but is devoid of intrinsic agonist activity (109). This natural IL-1 receptor antagonist should be a valuable tool as well as a potential therapeutic drug.

Conclusions - Identification of agents which modulate IL-1 synthesis and action is beginning to provide new avenues for therapeutic intervention into disease states in which aberrant IL-1 production or action is implicated. Recent developments in the cellular and molecular biology of IL-1 and the status of agents which inhibit IL-1 production or function have been reviewed to provide an assessment of current knowledge in this rapidly growing area. Further progress in establishing the molecular targets of IL-1 synthesis inhibitors, the nature of the IL-1-receptor interaction and the post-receptor targets for IL-1 action should provide a greater insight into the rational design of inhibitors with improved selectivity for the treatment of chronic inflammatory disease.

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Chapter 21. Macrocyclic Immunomodulators

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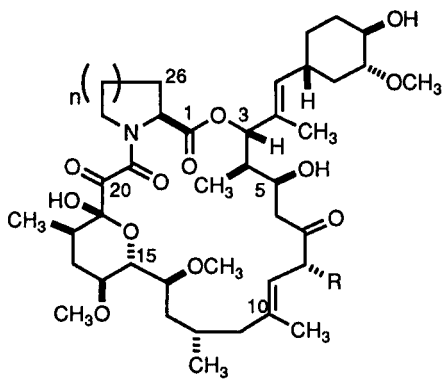
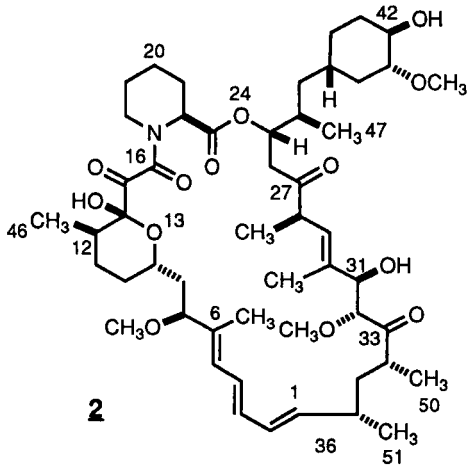
Introduction - The prototype for an emerging class of novel macrocyclic immunomodulatory agents is cyclosporin A(CsA). It is a clinically important drug and is the subject of numerous review articles including isolation, synthesis, and SAR (1), fungal metabolites with immunosuppressive activities (2), pharmacology and therapeutic monitoring (3), nephrotoxicity (4), and mode of immunosuppression (5). Therefore, CsA will not be discussed in this chapter except with respect to mechanism of action. What follows is a summary of the chemistry, in vitro and in vivo immunopharmacology, and mechanism of action of the newer macrocyclic immunomodulators: FK-506 (**1a**), rapamycin (**2**), and the didemnins (**3**).



1a R = $CH_2CH{=}CH_2$, n = 2
1b R = CH_2CH_3, n = 2
1c R = CH_3, n = 2
1d R = $CH_2CH{=}CH_2$, n = 1



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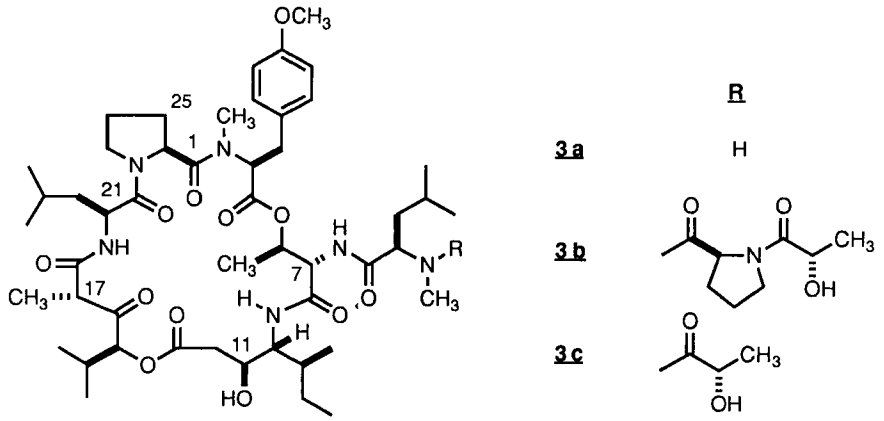
Chemistry - FK-506 was isolated from a strain of S. tsukubaensis from the Tsukuba district in Japan in 1987 (6). It is a 23-membered macrocyclic lactone containing the unusual hemiketal masked α,β-diketopipecolate moiety. In 1975, rapamycin (RPM) was isolated from a strain of S. hygroscopicus from Easter Island (7,8). It is a 31-membered macrocyclic lactone containing the same unusual substructure. The didemnins (D) are a class of cyclic depsipeptides, first isolated from samples of a Caribbean compound tunicate, Tridemnum solidum, in 1978 (9,10). Three major didemnins (A-C) [Didemnin A (DA) (**3a**), Didemnin B (DB) (**3b**), and Didemnin C (DC) (**3c**)] and many minor components were characterized. The basic structure incorporates several unique features including Me_2Tyr, Hip (hydroxyisovalerylpropionic acid), and isostatine residues.



The single crystal x-ray structures of FK-506 (6), RPM (11), and DB (12) have been solved. Interestingly, in both FK-506 and RPM, the amide carbonyl and the α-ketone are orthogonal with hydrogen bonding occurring between the hemiketal alcohol and the α-ketone. The major differences between RPM and FK-506 crystal structures lie in the 180° rotation of the amide carbonyl and the pseudorotation of the C-O bond of the lactone. This is, in part, due to the difference in absolute stereochemistry at the juncture of the cyclohexyl side chain with the macrolide. DB possesses a β-II turn around the Leu-Pro-Me_2Tyr residues of the macrocycle. In contrast to acyclic polypeptides where a β-II turn is stabilized by hydrogen bonding, the Leu-Pro-Me_2Tyr adopts a β-II conformation solely through a "scaffolding" effect .

The NMR assignments of FK-506 (6), RPM (13) and the didemnins (14,15) suggest that in $CDCl_3$ all three macrocycles exist as mixtures of conformations (FK-506, 3:1; RPM , 4:1; DA and DB, 8:1:1). DA and DB have been studied quite extensively via two-dimensional NMR techniques including 500 MHz DQF-COSY, NOESY, TOCSY, ROESY, and proton-detected heteronuclear long-range correlation experiments using semiselective C-atom pulses to determine the solution phase conformation (16-18). Molecular mechanics (using MOMO) and restrained molecular dynamics (using GROMOS) were performed using the x-ray coordinates as the initial geometry in conjunction with distance dependent NOESY data for constrained minimization. The x-ray structure of DB closely parallels the solution conformation with no conformational differences between DA and DB, suggesting that their differences in activity are not conformationally related (16).

Total syntheses of both FK-506 (19) and didemnins A-C (20,21) are reported employing in the key macrocyclization step the Mukaiyama reagent under high dilution (22) and an activated pentafluorophenyl ester in a two phase system (23), respectively. Although a total synthesis of RPM has yet to appear, several FK-506 fragment publications refer to work which may be applicable to RPM. For example, the cyclohexyl (24-26) and tricarbonyl systems (27-31), structural features common to both FK-506 and RPM, were the targets of recent efforts. Finally, fragmentation reactions of FK-506 involving LiOH result in either a benzylic acid rearrangement (32) or macrolactone hydrolysis (33), indicating that subtle differences in protecting groups or reaction conditions greatly influence the reaction products.

Structure-Activity Relationships - Several natural products related to FK-506 were isolated. FR-900520 (**1b**) and FR-900523 (**1c**), produced by a strain of S. hygroscopicus yakushimaensis, have ethyl and methyl groups, respectively, replacing the allyl side chain of FK-506 at C-8 (34). Another analog, FR-900525 (**1d**), produced by S. tsukubaensis, differs from FK-506 in replacement of the pipecolic acid with a proline (35). They are all active in the MLR (IC_{50}s: FK-506, 0.32 nM; FR900520, 0.55 nM; FR900523,1.6 nM; FR900525, 1.9 nM), displaying small differences in immunosuppression. RPM is tenfold more effective as an immunosuppressive and anti-candida agent than 32-demethoxyRPM, another natural product found in S. hygroscopicus (36). Over 30 different derivatives of the didemnins have been isolated or derivatized and present a wide distribution of activities. Since DB is much more active than DA, the N-terminal prolyl lactate is important for enhanced activity. Modification through addition of an O-benzyl group to the prolyl lactate in DB leads to an increase in activity. The most potent modification is the mono-iodo derivative, made by chloramine T iodination of the Me_2Tyr residue (IC_{50} 8.1 pM). These modifications may reflect that an increase in lipophilicity is important for cellular uptake (94,37).

In Vitro Effects on T cells and B cells - FK-506, RPM, and DB inhibit splenocyte proliferation in vitro using the lectins (ConA and PHA) and monoclonal antibodies to CD3 or Ly-6C (with PMA) (38-40). With ConA stimulus, DB is the most potent (IC_{50} 0.18 nM), whereas, FK-506 has IC_{50}s 50 to 100 fold lower than CsA, and RPM has an IC_{50} intermediate between CsA and FK-506. FK-506 reacts rather broadly against T-helper and cytotoxic T cell (CTL) cell populations (41). FK-506 (IC_{50} 0.3 nM) and CsA (IC_{50} 21 nM) inhibit the production of CTLs (42). FK-506 (39) inhibits an early Ca^{2+} dependent step in activation while RPM (39) and DB (43) do not . FK-506 and CsA effects are additive at suboptimal concentrations of both drugs but FK-506 does not enhance CsA's effects at optimal concentrations of CsA (44). However, RPM is synergistic with CsA, lowering the doses of both drugs needed for immunosuppression (45). A T cell subset analysis is published for FK-506 (46). DB, like CsA, inhibits prolactin binding to T cells (47).

FK-506 inhibits in vitro proliferation of murine B lymphocytes induced by goat anti-mouse IgM although it does not affect the induction of proliferation of bacterial lipopolysaccharide (LPS) (48). Although DB inhibits splenocyte proliferation using LPS (IC_{50} < 0.09 nM), proliferation in murine B cell hybridomas (IC_{50} of < 1 nM) using serum as the stimulus (49), and ongoing DNA synthesis in murine bone marrow cells during a 4 h exposure time (IC_{50} 162 nM) (47), it increases antibody production on a per cell basis (49).

Mechanism of Action - Cellular Effects. FK-506, like CsA, inhibits expression and accumulation of mRNAs for GM-CSF, IL-2, IL-3, IL-4, IFN-γ, TNF-α, and c-myb, but not B-cell stimulating factor (50-52). Unlike FK-506, RPM does not suppress the expression and production of IL-2 or IL-2R. In fact, the levels of production of E class activation genes are elevated twofold. RPM exerts its immunosuppressive effects through impairment of the response of T cells to lymphokines (39). RPM and FK-506 are mutually antagonistic (53), suggesting the involvement of a common receptor. FK-506 and DB inhibit the Ca^{2+}/calmodulin dependent phosphorylation of eEF-2 in vitro and biological

effects of the phorbol ester TPA on mouse skin in vivo (54,55). DB has inhibitory effects on protein synthesis, DNA, and RNA synthesis (43, 56).

In Vivo Immunopharmacology - FK-506 has decreased the symptoms in collagen induced arthritis (57-59), uveoretinitis (60), and the MRL/lpr mouse model of SLE (61). RPM prevents the development of humoral IgE-like antibody and is effective in animal models of autoimmune diseases, including experimental allergic encephalomyelitis (62), arthritis [adjuvant (62), collagen induced (63)], and the MRL/lpr mouse model of SLE (63). RPM is also effective in the popliteal lymph node host vs. graft assay, an in vivo MLR (64), and the mBSA delayed type hypersensitivity assay (63). DB stimulates murine haemagglutinating antibody responses and induces leukocytosis (65).

FK-506 is efficacious in prolongation of allograft [heart (66-68), liver(69-71), kidney (72-76), limb (77,78)] and xenograft [heart (79-81), skin (82)] transplantations. It is currently in the clinic for use in organ transplantation (83). RPM (84,85) and DB (86,87) are also effective in prolonging allograft transplantations. RPM, unlike FK-506, is effective in preventing the expression of transplant coronary disease, a major cause of rejection in heart transplantations (88).

Other Antiproliferative Effects - FK-506 is active against filamentous fungi (89) while RPM is active against Candida species (especially C. albicans) and filamentous fungi (90,91). RPM (92,93) and, especially, the didemnins (94), exhibit antiproliferative activity against certain tumors. DB has undergone Phase II clinical trials for colorectal cancer (95). DA and DB are effective antiviral agents against herpes simplex, Rift Valley fever, rhino- and parainfluenza, and dengue viruses, but not against rabies (94).

Toxicity - FK-506 and RPM display species specific vasculitis in the dog. With FK-506, the vasculitis is systemic (75), while with RPM the vasculitis is limited to the GI tract (85). Nephrotoxicity, a major side effect of CsA, has not been reported with FK-506, RPM, or DB. Interestingly, the nephrotoxicity of CsA is potentiated by FK-506, making combination therapy unlikely (96). DB, currently in clinical trials, has shown reversible hepatotoxicity (97) and some elevation of transaminase activity (98).

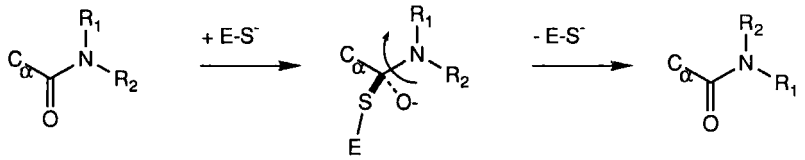
Potential Therapeutic Uses - Both approved and experimental uses of CsA in man are indicative of the therapeutic potential this class of macrocyclic immunomodulators may have as a whole. However, as with CsA, such potential is tempered by the effects of immunosuppression itself and by a limited understanding of the immune system. Reviews of CsA in dermatologic disorders (99), organ transplantation [kidney (100), liver (101), bone marrow (102), heart (103)], autoimmune diseases [renal (104), neurological (105)], rheumatoid arthritis (106), diabetes mellitus (107), and cancer (108) were published.

Mechanism of Action - Immunophilins and Prolyl Isomerases - The search for a possible cellular receptor(s) for CsA led to the isolation and characterization of an erythrocyte CsA binding protein, cyclophilin (CyP), in 1984 (109,110). CyP is a low molecular weight (MW 17,737), basic protein (pI 9.6) concentrated in the cytosol and nucleus. CyPs have been isolated from calf thymus (109), human blood (111), human spleen (112), N. crassa , and S. cerevesiae (113,114), and

are detected at various concentrations in all eukaryotic tissues with the largest concentrations occuring in PBLs of patients with T cell lymphocytic leukemias, lymphomas, and systemic mastocytosis colon adenocarcinomas. In the mouse, highest concentrations are found in the kidney and brain, which may correlate with the oligurin and tubulointerstitial nephritis as well as with CNS side effects found in CsA treatment (112). The amino acid sequences of the isomeric CyPs are highly conserved (60-96%). CyP has been sequenced (111), cloned (115,116) and expressed (117). A high degree of correlation exists between CyP binding and immunosuppression (118-121). The K_d for CsA binding to CyP is 200 nM while the IC_{50} for inhibition of IL-2 secretion is nearly the same (109), suggesting a role for CyP in immunoregulation. CyP has in vivo anti-inflammatory and immunosuppressive effects and may act by inducing the production of endogenous inhibitors of CyP (122). A 40 % homology exists between CyP and eukaryotic elongation factor 2 (eEF-2), prompting the suggestion that CyP may be a nucleotide binding protein and possibly play a role in protein biosynthesis (123). A homolog of CyP (40 % homology) has been detected in the Drosophila melanogaster ninaA mutant. This mutant, although able to express opsin, has a tenfold reduction in the levels of rhodopsin. Encoding for this homolog, with its possible role in visual transduction, suggests the involvement of the CyP family in diverse signal transduction processes (124,125).

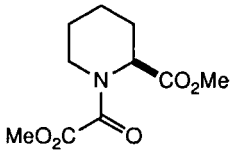
Recently, two research groups independently identified CyP as being identical to peptidyl prolyl cis,trans-isomerase (PPIase), an enzyme which catalyzes the slow protein folding around prolyl amide bonds (126,127). PPIase catalyzes the folding of RNAase T1, cytochrome c (128), murine Ig light chain, the S fragment of RNAase A, porcine RNAase A (129), and type III collagen (130). However, it was not found to catalyze the folding of other proteins thought to depend on a prolyl isomerization for folding. This may be due to inaccessability of the pertinent residues to PPIase or sequence specificity requirements of PPIase (128). PPIase possesses a sulfhydryl group at or near the active site as shown by enzymic inactivation by thiophilic reagents (109,126). The presence of this sulfhydryl group and the detection of a β deuterium isotope effect have led to a postulated mechanism for isomerization as shown in Scheme 1 (131).

Scheme 1 - Proposed Mechanism for PPIase

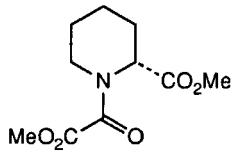


The existence of CyP prompted the search for a similar cellular receptor for FK-506. Initially, it was thought incorrectly that the FK-506 binding protein (FKBP) and CyP were very similar, and preliminary experimental data supported this (132). FKBP was independently isolated by two groups from a Jurkat T-cell line (133,134) and bovine thymus (135) in 1989. Like CyP, FKBP is a small protein (MW 11,747) with prolyl isomerase activity, although it catalyzes the isomerization of the substrate Succ-Ala-Ala-Pro-Phe-p-nitroanilide (136) at 25-fold higher concentrations than CyP. CsA does not bind to FKBP and FK-506 does not bind to CyP (134). FKBP has been cloned and sequenced (135).

Interestingly, CyP and FKBP are members of separate classes of prolyl isomerases, displaying no obvious sequence homology (135). FKBP is highly conserved and ubiquitous - found in T cells, fibroblasts, Spodoptera frugiperda, and the PC-12 rat cell line (137). The K_d for FK-506 binding is 0.8 nM while the IC_{50} for inhibition of IL-2 secretion is 1.1 nM, suggesting the relevance of FKBP in immunoregulation (19). A high correlation exists between FKBP binding and immunosuppressive activity as measured in the MLR (19). The L-pipecolate (**4**) has a K_i of 500 nM to FKBP with no immunosuppressive activity while its enantiomer (**5**) does not bind to FKBP (19) which may show the importance of the α-keto amide in binding, but more must be needed for immunosuppression.

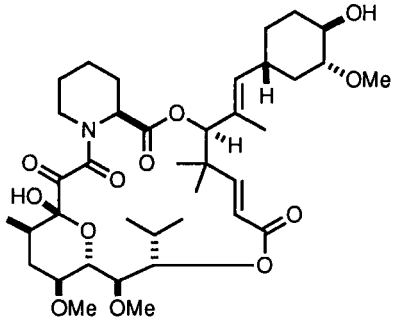


4



5

Schreiber synthesized a macrocycle, 506BD (**6**), which has a different bridge between the pipecolic acid and the pyran, the conformation of the compound being similar to the conformation of FK-506. Compound **6** afforded 95 % inhibition of FKBP at 70 nM (FK-506, 75 %). No immunosuppressive data were given (19).



6

Although a specific RPM or DB binding protein has not been reported, differences in mechanism of action between CsA, FK-506, DB, and RPM make the existence of such a protein plausible. RPM binds weakly to CyP (K_i 8.2 μM) and elutes FKBP off of an FK-506 affinity resin (135).

Summary - FK-506, RPM, and DB may be found to be safer and more efficacious agents for transplantation rejection and autoimmune disease than CsA. The emergence of these new chemical classes may give us a greater understanding of the factors which control the immune system. FK-506 and CsA both act by inhibiting the production of growth promoting lymphokines, although FK-506 is effective at much lower doses. RPM, although structurally similar to FK-506, acts by inhibiting the response of T cells to lymphokines. The mechanism of action of DB remains unknown, but seems to be more antiproliferative than either FK-506 or RPM. The importance and relevance of

immunophilins needs to be delineated, but they may have a role in immunological signal transduction. In addition, or alternatively, the immunophilins may be involved in the renal or vascular toxicity observed with this emerging class of novel macrocyclic immunomodulatory agents.

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Chapter 22. New Approaches to Diabetes

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Introduction - A substantial increase in the understanding of the pathology and physiology of diabetes mellitus has occurred over the past decade. As a result, new strategies for intervention have appeared in a disease where few therapy options currently exist: inhibitors of carbohydrate metabolism have recently been reviewed (1) as have biguanide inhibitors of gluconeogenesis (2); sulfonylurea insulin secretogogues will not be discussed here, but have been extensively reviewed elsewhere (3-5); inhibitors of fatty acid oxidation have also been summarized (6). This chapter will discuss several promising new approaches that may represent potential therapeutic breakthroughs in the treatment of hyperglycemia in diabetes mellitus, with emphasis on insulins, insulin release, insulin action and counter-regulatory hormones.

INSULINS

Insulin - Therapy of insulin dependent diabetes mellitus (IDDM or Type I diabetes) attempts to replace insulin in a manner that mimics the physiologic pattern of release seen in the non-diabetic (7,8). In addition, insulin is used in the treatment of non-insulin dependent diabetes mellitus (NIDDM or Type II diabetes) when oral therapy alone fails (9). While management of the acute symptoms of IDDM is generally achievable, the long-term complications of diabetes, including retinopathy, nephropathy, and neuropathy are not currently well-treated. However, there are clinical data suggesting that strict metabolic control in IDDM can improve long-term prognosis (10-12). Confirmation in longer term, large prospective studies of the effect of primary intervention with intensive insulin therapy has been shown to be feasible (13,14). Risk of symptomatic and severe hypoglycemic episodes associated with intensive insulin therapy is greater, however, than that seen with conventional treatment. New therapies are targetted towards improving control without chronic tolerance difficulties such as increased hypoglycemic risk.

Human insulin - Bacterial expression of human insulin paved the way for its approval as the first recombinant biological for use in humans (15). Use has grown over the past decade and, while no marked clinical differences have been described over porcine insulin, recommendations have been made for starting newly diagnosed diabetics on human insulin (16); however, the clinical advantage for human over animal insulins has been questioned (17). A controversy has arisen over apparent increased hypoglycemic unawareness and hypoglycemic episodes in patients switched from beef to human insulin, and this remains a point of very active debate (18-23).

Modified insulins - Protein engineering has been used to modify pharmacological and pharmacokinetic properties of insulin to provide improved

metabolic control, and reduce risk of inappropriate insulin action leading to hypoglycemic episodes. Basal-bolus insulin replacement uses injections of rapid, short-acting formulations to cover post-prandial glucose surges and longer-acting preparations to control fasting blood glucose. In the latter, insulin is predominately hexameric and following injection must dissociate to enter the capillary circulation (24,25). Mutations in the domain responsible for hexamer stabilization (B9Ser->B9Asp, B27Thr->B27Glu) yields a monomeric analog (26). Following subcutaneous administration this analog shows very rapid absorbtion and prompt onset of action, suitable for administration at the start of a meal to respond to a subsequent glucose excursion and avoiding continuing activity for many hours after the meal (27). Alteration of the charge state of the insulin molecule (B30Thr-CO_2H -> B30Thr-$CONH_2$, B27Thr->B27Arg) increases the isoelectric point (6.8 vs. 5.4 for insulin) and acid solubility over native insulin (28). This derivative crystallizes under physiological conditions, affording prolonged action after injection of a mildly acidic solution. Further modification prevents side chain deamidation (A21Asn -> A21Gly) generating an acid stable derivative with very slow, but reproducible absorption kinetics in man following subcutaneous injection (first order absorption kinetics (T1/2 ~35hr) with low intra- and interpatient variability) (29). This analog may provide once daily injection for sustained control of fasting blood glucose without the unpredictability of absorption demonstrated with current formulations.

Modification of receptor affinity can alter insulin pharmacokinetics by changing receptor mediated clearance. Studies with two derivatives (INA1: B10His->B10Glu, 300% in vitro potency of insulin, and INA2: B9Ser->B9Asp and B27Thr->B27Glu, 30% in vitro potency of insulin) show in vivo half life is inversely related to binding. In vivo activities, as measured by blood glucose fall during prolonged infusions, were virtually identical, but INA2 had a more prolonged effect than insulin, and thus lower potency analogs may provide another class of long-acting insulins (30).

Proinsulin - Proinsulin, the physiologic precursor of active insulin, has a less pronounced, but longer duration of action than human insulin. Apparent selectivity for hepatic versus peripheral action of proinsulin (relative to insulin) has been cited as the basis for its potential use in NIDDM, where fasting hyperglycemia is attributed to high hepatic glucose output (31). This property, however, has been linked to differences in clearance rather than intrinsic selectivity (32). In one clinical study, an increased frequency of acute myocardial infarct in patients receiving proinsulin versus controls raised safety concerns and human trials have been suspended (7).

Incretins - The gut-derived humoral stimuli of insulin secretion have been termed incretins (33). The incretin effect describes the difference in insulin secretion resulting from oral glucose challenge as compared to a similar challenge given intravenously. Several gut derived peptidic hormones, derived from processing of proglucagon prohormones, have recently been discovered which have characteristic properties of incretins. The truncated glucagon-like peptide-I derivatives, GLP-I (7-36) amide and GLP-I (7-37) show potent insulinotropic activities in the rat pancreas and in isolated islet cells (34-36). Infusion of GLP-I (7-36) amide in normal human subjects produced a glucose dependent insulin secretion (37). This peptide shows a synergistic

stimulatory effect with gastric inhibitory peptide (GIP) on insulin release from the rat pancreas (38). Exploitation of the glucose-dependent insulin secretatory effects of these hormones may provide advantages in the treatment of NIDDM.

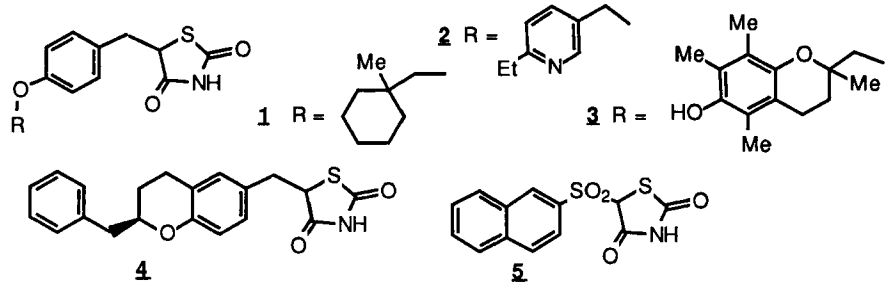
Islet-amyloid Polypeptide (IAPP) or Amylin - Islet amyloid polypeptide (IAPP) or amylin, a 37 amino acid pancreatic peptide, has recently been characterized in amyloid deposits in pancreatic tissue from NIDDM patients (39,40). In addition to association with this common morphologic abnormality of NIDDM, the peptide also decreases both pancreatic insulin release in response to elevated glucose and the action of insulin on peripheral tissue (41,42). It has been postulated that both impaired pancreatic function and peripheral insulin resistance in NIDDM may be related to increased amylin production (43,44). A modified amylin subpeptide, Ser^2,Ser^7 -amylin(1-16) shows weak activity in blocking amylin-induced insulin resistance in isolated rat muscle cells, however no effects on amylin inhibition of pancreatic insulin release were described (45). More potent derivatives may prove useful in the treatment of NIDDM.

Insulin Action - NIDDM is characterized by abnormal insulin secretion from the pancreas, increased basal glucose output by the liver, and insulin resistance in peripheral tissue (46,47). Peripheral insulin resistance significantly contributes to the hyperglycemic state of NIDDM, as demonstrated by euglycemic insulin clamp studies which show a marked reduction in insulin stimulation of glucose disposal (48-50). Current data favor a post-receptor defect in response to insulin. A reduction of glycemia will also alleviate the resistance to insulin action in peripheral tissues (46,51).

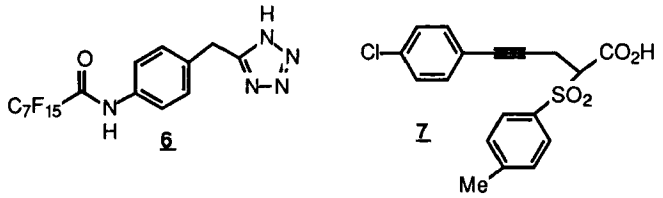
Several agents have recently been identified which enhance insulin action in vivo and in vitro, without stimulating insulin secretion or causing frank hypoglycemia. The prototypical agent in this class, ciglitazone (ADD-3878, **1**) has been shown to lower plasma glucose in genetic animal models of Type II diabetes (ob/ob, db/db and KK-Ay mice, and Zucker fa/fa rat) but has no effect in normal animals (52-56). Insulin sensitivity is improved by **1** in isolated soleus muscle and adipose tissue (57-59). Insulin action in both oxidative and glycolytic skeletal muscle is improved in the high fat fed (HFF) rat model of insulin resistance (60). In addition, **1** lowers plasma triglycerides and free fatty acids and weakly inhibits hepatic gluconeogenesis (61). The molecular mechanism of action of **1** and ciglitazone-like agents has not been elucidated, but does not appear to involve significant effects on insulin binding (58,62-64). A membrane-associated binding protein for sulfonylureas in rat adipocytes has been identified, and **1** can displace the sulfonylurea glyburide from this site (65). Ciglitazone-like effects may be be mediated by a post-receptor stimulation of glucose transporter expression (66). In early clinical studies in NIDDM, **1** lowered fasting blood glucose and triglyceride levels, and improved oral glucose tolerance (67,68). An expanded trial showed moderate decreases in blood glucose but had no significant effects on triglycerides, cholesterol, or body weight. Development of **1** was subsequently stopped (69).

A number of ciglitazone-like thiazolidinediones have been described. Pioglitazone (ADD-4833, **2**) is more potent than ciglitazone and is currently in Phase II trials (70,71). CS-045 (**3**), shows effects on insulin receptor number in obese rats and apparent mechanistic distinctions, particularly its ability to

reduce blood glucose after a single dose in diabetic KK mice (72,73). CP-72467, (4), is more potent than 1 in vivo and like insulin, stimulates glucose transport and glucose transporter expression in vitro (74-79). AY-31637 (5) is equipotent to ciglitazone (80). More potent thiazolidinedione analogs have been disclosed (81-85).



A series of tetrazoles represented by 6, appear to be another example of non-thiazolidinedione ciglitazone-like agents (86). BM-130907, (7), shows insulin sensitizing effects and it normalizes post-receptor defects in the ob/ob mouse in a manner similar to that of ciglitazone (87,88). Compound 7 enhances glucose uptake in isolated rat adipocytes, apparently through stimulation of glucose transporter translocation, while 1 fails to stimulate hexose transport in these cells.



COUNTER-REGULATORY HORMONES

Hypoglycemia or stress can trigger the secretion of several hormones, including glucagon, the catecholamines such as epinephrine, growth hormone and cortisol. These act to counter insulin's action in stimulating peripheral glucose disposal and inhibiting hepatic glucose output (89,90). Modulation of these counterregulatory hormones can impact glycemic control and/or insulin action in diabetic patients.

Glucagon Receptor Antagonists - Insulin and glucagon maintain euglycemia by balancing glucose output by the liver and peripheral disposal (91). Hyperglucagonemia is a common feature in IDDM and NIDDM patients, despite hyperglycemia (92,93). In animals with chemically induced IDDM, 70% of the hypoglycemic action of insulin can be attributed to a reduction of hyperglucagonemia, suggesting glucagon as a target for hypoglycemic therapy (94). Analogs of glucagon inhibit glucagon binding to hepatocyte receptors, and reduce hyperglycemia in animal models of IDDM (95,96). Peptidic antagonists prevent glucagon activation of adenylate cyclase, although

activation of a second pathway resulting in release of inositol phosphates can still occur (97-99). Of these, des-His^1-Glu^9-glucagon amide enhances pancreatic insulin release, blocks effects of exogenous glucagon in rabbits, and reduces the hyperglycemia produced by endogenous glucagon in an animal model of IDDM (100).

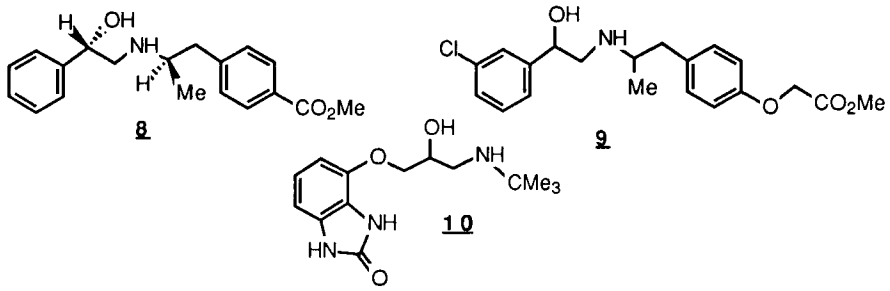
Antagonists of Glucagon and Growth Hormone Secretion - Growth hormone has insulin agonist and antagonistic effects; increased levels may contribute to fasting hyperglycemia and impaired glucose tolerance in diabetics (101-104). The tetradecapeptide hormone somatostatin inhibits glucagon and insulin secretion from the pancreas and growth hormone secretion from the pituitary (105,106). Analogs have been designed to selectively inhibit glucagon and growth hormone secretion without affecting insulin secretion (107,108). The octapeptide analog octreotide, or SMS 201-995, enhances reduction of postprandial hyperglycemia by insulin in IDDM patients by lowering both plasma glucagon and growth hormone levels, and reduces the metabolic changes induced by interruption of continuous subcutaneous insulin infusion in IDDM patients (109-111). A long-acting somatostatin analog, L363,586, has also been shown to reduce fasting and postprandial hyperglycemia after nasal delivery (112). It also reduces the early morning hyperglycemia in IDDM patients by inhibiting growth hormone secretion (113). A potent octapeptide analog of somatostatin-14 (RC-160, D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2) inhibits glucagon secretion and reduces plasma glucose levels after subcutaneous injection in streptozotocin diabetic (IDDM) rats (114,115). The glucagon release resulting from insulin induced hypoglycemia was also attenuated. A slow release delivery system has been developed, which may allow clinical use of this agent (116).

Growth Hormone Fragments - A human growth hormone sub-peptide (hGH_{20K}) has been discovered that lacks the early insulin-like activity of the major form (hGH_{22K}) but retains growth-promoting activity (117). Insulin-like properties of the native hormone may reside in the deleted peptide fragment, hGH_{32-46}, which doubles the insulin response to hyperglycemia and enhances glucose disposal in the conscious dog (118). Both hGH_{32-46} and a closely related peptide hGH_{31-44} have direct insulin-like actions in vitro (119,120). hGH_{32-38}, a smaller fragment of hGH_{32-46}, was shown to stimulate glucose disposal in vivo without significant elevations of plasma insulin (121).

Catecholamines - Acute rises in epinephrine stimulate hepatic glycogenolysis and gluconeogenesis, and inhibit peripheral glucose disposal (122,123). Epinephrine also stimulates lipolysis, resulting in elevations in free fatty acid concentrations which can independently reduce glucose utilization and disposal (124). Epinephrine can elevate plasma glucose and antagonize the actions of insulin (125,126). Paradoxically, prolonged rises in epinephrine can have the opposite effect. Insulin sensitivity is increased with respect to stimulation of glucose transport and glycolysis in muscle from animals exposed to elevations in plasma epinephrine for 120 hours (127).

ß-Agonists - The $ß_2$ agonist terbutaline increases insulin-stimulated glucose metabolism in normal humans (128). However, $ß_2$ agonists have side effects including muscle tremor and blood potassium changes. Other agonists

selective for the atypical or β_3 receptor in brown adipose tissue favorably affect insulin action. BRL26830, (**8**), increases thermogenesis and reduces fat while sparing lean body mass in obese animals and man (129,130). Indeed, muscle growth has been observed in diabetic animals treated with **8** (131). The rodent anti-obesity effects are presumed to be mediated by β_3 adrenoceptors in brown adipose tissue, which are limited in man though thermogenesis appears to occur in muscle of rats treated with **8** (132). Recently atypical (β_3?) adrenoceptors have been discovered in brown adipose tissue and also in liver, muscle and white adipose tissue (133). Thus, selective β_3 agonists may stimulate weight loss without the side effects associated with the stimulation of β_1 (heart) and β_2 (muscle, trachea, uterus) receptors observed with less selective β agonists (134). Although weight loss can improve glycemic control in NIDDM patients, **8** has been shown to improve glycemic control in the absence of changes in body weight (135,136). Similar observations have now been made with BRL35135, (**9**), a more selective β_3 agonist (137). CGP-12177 (**10**) binds to β-receptors in brown adipose tissue and stimulates respiration but is a β-receptor antagonist in other tissues. It also stimulates thermogenesis in vivo without exhibiting sympathomimetic effects in non-brown adipose tissue (138-140).



CONCLUSION

Several new mechanisms for the therapy of diabetes show considerable promise for expansion of the clinical options for treatment of hyperglycemia. As new pharmacological classes of anti-diabetic agents emerge, optimal treatment of NIDDM may require combination therapies. These may be adjusted to suit individual patient needs, particularly if responsiveness to therapies is as heterogeneous as one might expect in so large a clinical population. Insulin replacement therapy of IDDM may be optimized by the development of more physiological insulin replacements, and correction of other secondary hormonal changes.

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SECTION V. Topics In Biology

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Chapter 23. Role of Corticotropin-Releasing Factor in Neuropsychiatric Disorders and Neurodegenerative Diseases

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Introduction - Corticotropin-releasing factor (CRF) was the first hypothalamic hypophysiotrophic factor to be identified. In the 1950's, Guillemin and Rosenberg (1) and Saffran and Schally (2), separately demonstrated that hypothalamic extracts contain a factor that stimulates pituitary secretion of adrenocorticotropic hormone (ACTH). The chemical identity of CRF remained elusive until 1981, when Vale and co-workers at the Salk Institute isolated, sequenced and synthesized a 41-amino-acid CRF from ovine hypothalami (3) (Figure 1). Two years later, the same group identified rat CRF, also a 41-amino-acid peptide but differing from ovine CRF by 7 amino acid residues (4) (Figure 1). In the same year, the human CRF gene was isolated and sequenced (5). Interestingly, human CRF and rat CRF are identical, although their precursor molecules are different. CRF has been localized in the paraventricular nucleus of the hypothalamus in neuronal cell bodies (perikarya), which give rise to axons that terminate in the external layer of the median eminence encircling the pituitary stalk (6). CRF is the major physiologic regulator of the basal and stress-induced release of ACTH, β-endorphin and other pro-opiomelanocortin (POMC)-derived peptides from the pituitary (7,8). Membrane receptors with high affinity for CRF have been identified in the anterior pituitary gland (9). The distribution of these receptors resembles the distribution of ACTH-

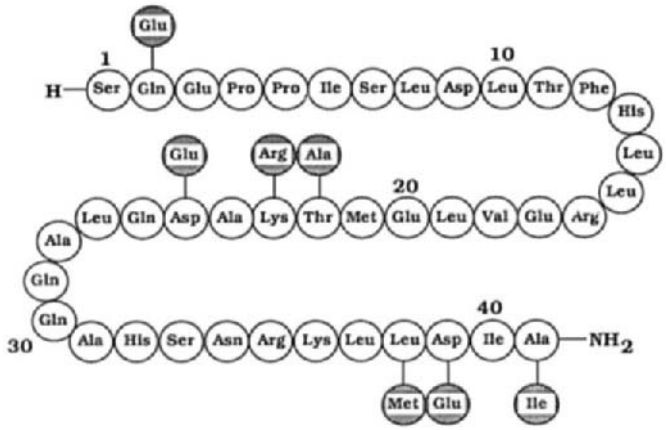

Figure 1. Amino acid sequences of ovine and rat/human corticotropin-releasing factor (CRF). The amino acid sequence of ovine CRF is denoted by the open circles. Rat CRF and human CRF are identical and differ from ovine CRF by seven amino acid residues which are denoted by the shaded circles. Both peptides are amidated at the carboxy terminus and the amidation is essential for biological activity.

containing cells (i.e. corticotrophs). CRF binds to these receptors on ACTH-containing cells initiating the release of ACTH. The process appears to involve both cAMP-mediated and calcium-mediated mechanisms (7,8). ACTH, in turn, stimulates the release of the glucocorticoid cortisol from the adrenal cortex and a negative feedback loop exists whereby circulating levels of glucocorticoid can suppress the release of both CRF and ACTH.

In addition to its endocrine role at the pituitary, there is substantial evidence to suggest that CRF functions outside the pituitary, producing a wide spectrum of electrophysiologic (10,11), autonomic (12,13), and behavioral (14-16) effects characteristic of a central nervous system (CNS) neurotransmitter. Like other hypothalamic neurohormones, CRF is not only localized in the hypothalamus but is ubiquitous in the CNS. Major groups of CRF-producing perikarya and terminal axons have been found in areas of brain that are important in regulating autonomic function, such as the amygdala, the stria terminalis, the lateral hypothalamus, the central gray, the parabrachial nucleus and the dorsal vagal complex (6) (Figure 2). CRF has also been detected immunocytochemically in the cerebral cortex, several periventricular regions, the locus ceruleus, the nucleus accumbens, and the spinal cord (6). Cell membrane receptors with high affinity for CRF have been identified in discrete areas of brain that correlate with immunohistochemical distribution of CRF (17,18). A neurotransmitter role for CRF is further supported by several findings at the cellular level, including its ability to promote the formation of cAMP (19), its calcium-dependent release from brain slices (20), and its ability to selectively alter local cerebral glucose utilization (21).

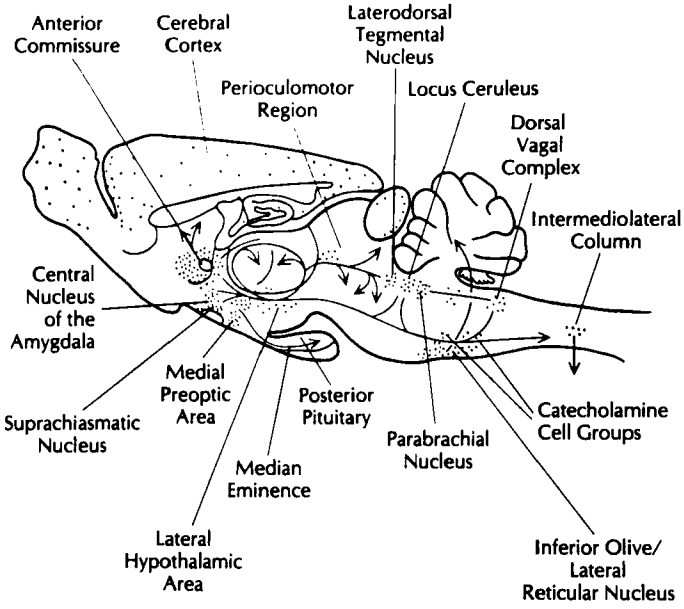

Figure 2. Major groups of CRF-producing neuronal perikarya (dots) and their fiber systems (arrows) are shown in a saggital view of the rat brain. CRF cell bodies and axons are concentrated in the labeled subcortical brain areas, which regulate pituitary and autonomic functions; they are also found in the cerebral cortex, a focal area for investigation of neuropsychiatric disorders and neurodegenerative dementia. (Adapted from P. Sawchenko and L. Swanson, (6); from De Souza, (61); reprinted by permission from Hospital Practice).

CRF plays a crucial role in integrating the body's overall response to stress. The CRF-ACTH-cortisol axis is central to the endocrine response to stress. In addition, intracerebroventricular administration of CRF provokes several stress-like responses including activation of the sympathetic nervous system (12), and inhibition of the parasympathetic nervous system (13) with consequential increases in plasma concentrations of epinephrine, norepinephrine and glucose; increases in heart rate and mean arterial blood pressure; inhibition of gastrointestinal functions including inhibition of gastric acid secretion. The behavioral profile following central administration of CRF is also characteristic of a compound which increases arousal and emotional reactivity to the environment.

These effects of CRF include general arousal, as exhibited by increased locomotion, sniffing, grooming, and rearing in familiar surroundings and increased agitation in unfamiliar surroundings (14,15). In contrast, sexual receptivity (22) and feeding (16) are decreased. Overall, CRF appears to be one of the pivotal CNS neurotransmitters that activates and coordinates the endocrine, behavioral and autonomic responses to stress.

Recent clinical data suggest that CRF may be implicated in endocrine, psychiatric, and neurologic illnesses. This article provides an overview of preclinical and clinical evidence in support of a role for CRF in the etiology and pathophysiology of neuropsychiatric disorders such as depression, anxiety-related disorders and feeding disorders. In addition, the article will describe neurotransmitter defects in neurologic illnesses such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD), progressive supranuclear palsy (PSP) and amyotrophic lateral sclerosis (ALS) as they relate to dysfunction of CRF neurons in the CNS. A possible interaction between CRF and acetylcholine, as it relates to AD, also is addressed. Finally, some strategies directed at developing CRF-related drugs for the treatment of various neuropsychiatric and neurologic illnesses will be briefly described.

NEUROPSYCHIATRIC DISORDERS

Affective Disorders - Many patients with major depression are hypercortisolemic and exhibit an abnormal dexamethasone suppression test (23,24). Given the primary role of CRF in stimulating pituitary-adrenocortical secretion, the hypothesis has been put forth that hypersecretion/hyperactivity of CRF in brain might underlie the hypercortisolemia and symptomatology seen in major depression. The concentration of CRF is significantly increased in the cerebrospinal fluid (CSF) of drug-free depressed individuals (25-27), and a significant positive correlation is observed between CRF concentrations in the CSF and the degree of post-dexamethasone suppression of plasma cortisol (28). Furthermore, the observation of a decrease in CRF binding sites in the frontal cerebral cortex of suicide victims compared to controls is consistent with the hypothesis that CRF is hypersecreted in major depression (29). The increased CSF concentrations of CRF seen in depressed individuals are decreased following treatment with electroconvulsive therapy (30). In addition, a blunted ACTH response to intravenously administered ovine or human CRF is observed in depressed patients when compared to normal controls (31-33). The blunted ACTH response to exogenous CRF seen in depressed patients may be due to the intact negative feedback of cortisol on the corticotrophs, due to a compensatory decrease in CRF receptors subsequent to chronic hypersecretion of the peptide and/or desensitization of the pituitary corticotrophs to respond to CRF.

Preclinical studies provide additional support for the hypothesis that hypersecretion of CRF may be involved in the symptoms seen in human depression. In the rat, intracerebroventricular administration of low doses of CRF produces several behavioral effects including changes in activity (increased locomotion, sniffing, grooming and rearing in a familiar surrounding), increased "emotionality" and assumption of a freeze posture in a foreign environment, decreased feeding and sexual behavior and increased conflict behavior (14-16,22). CRF administered intrathecally in unrestrained monkeys induces a hunched-over posture similar to that observed in primates undergoing separation reactions and their associated "behavioral despair" phase (15,34). The CRF induced behavioral effects in rodents and nonhuman primates described above bear a striking resemblance to the symptoms of human depression and suggest that CRF hypersecretion may be responsible for some of the symptomatology seen in the disorder.

In view of the preclinical and clinical data suggesting a role for CRF in depression, the hypothesis has been put forth that antidepressants may produce their "therapeutic" effects, in part, by decreasing CRF secretion. An increase in CRF binding sites (presumably to compensate for chronic suppression of CRF secretion) is observed in some brain regions such as the brain stem in rats treated chronically with tricyclic antidepressants such as imipramine (35). Although the relevance of the up-regulation in CRF receptors in the brain stem following antidepressant treatment is presently unknown, an important role for this peptide has been postulated in the locus ceruleus of the brain stem. The locus ceruleus receives a rich CRF innervation (6), contains a moderate density of CRF receptors (17), and is markedly activated following iontophoretic or intracerebroventricular administration of CRF (11,21). Furthermore, the concentrations of CRF are selectively increased in the locus ceruleus following application of acute or chronic stress (36). A link between CRF and the locus ceruleus in major depression is further suggested by clinical data that CSF levels of CRF correlate positively with indices of locus ceruleus-noradrenergic activation, such as CSF concentrations of norepinephrine and its metabolite MHPG (37). Given the major involvement of the brain noradrenergic system, in particular in the locus ceruleus in depression (38-

40), and the effects of CRF to activate noradrenergic neurons in this brain region (11,21), it is tempting to speculate that antidepressants may function by suppressing CRF secretion in the locus ceruleus, resulting in the observed increase in brain stem CRF binding sites.

Anxiety-Related Disorders - A number of studies suggest that anxiety-related disorders (such as panic disorder and generalized anxiety disorder) and depression are independent syndromes which share both clinical and biological characteristics. The role that has been proposed for CRF in major depressive disorders along with preclinical data in rats demonstrating effects of CRF administration to produce several behavioral changes characteristic of anxiogenic compounds (14) have led to the suggestion that CRF may also be involved in anxiety-related disorders. A role for CRF in panic disorder has been suggested by observations of blunted ACTH responses to intravenously administered CRF in panic disorder patients when compared to control subjects (41). The blunted ACTH response to CRF in panic disorder patients most likely reflects a process occurring at or above the hypothalamus, resulting in excess secretion of endogenous CRF (41).

Preclinical studies demonstrating interactions between benzodiazepine anxiolytics and CRF provide further evidence for the involvement of CRF in these disorders. Chlordiazepoxide attenuates the "anxiogenic" effects of CRF in both the conflict test (42,43) and in the acoustic startle test (44) in rats. The effects of chlordiazepoxide on these behavioral effects of CRF suggest that these substances may interact in brain. More recent data using the operant conflict test in rats have demonstrated that the benzodiazepine receptor antagonist (Ro15-1788) which was without behavioral activity when given alone, reversed the effects of CRF in a dose-dependent manner whereas the benzodiazepine inverse agonist (FG7142) enhanced the actions of CRF (43). Preliminary studies examining the effects of a CRF receptor antagonist (α-helical ovine CRF_{9-41}) in a variety of behavioral paradigms suggest that the CRF antagonist produces "anxiolytic-like" effects, qualitatively similar to benzodiazepines (14).

Neurochemical, endocrine and receptor binding data also demonstrate interactions between CRF and benzodiazepines. Acute administration of the triazolobenzodiazepines, alprazolam and adinazolam results in increased hypothalamic concentrations of CRF while decreasing the concentrations of CRF in other brain regions, including locus ceruleus, amygdala, pyriform cortex and cingulate cortex (45). Of particular interest is the finding that the two triazolobenzodiazepines exert effects on CRF concentrations in the locus ceruleus and hypothalamus that are opposite to CRF changes seen after stress (45). Chronic administration of diazepam, alprazolam, or adinazolam in rats results in significant decreases in CRF receptors in the frontal cerebral cortex and hippocampus and there is a trend for CRF receptors to be decreased in other brain areas and increased in the anterior pituitary (35). The latter data demonstrating increases in CRF receptor concentrations support the hypothesis for effects of the benzodiazepines to inhibit CRF release which in turn modulate the receptors. Further support for this hypothesis is provided by potent in vitro effects of benzodiazepines (46) and especially the triazolobenzodiazepines (47) to inhibit hypothalamic CRF release. Conversely, the reduced concentrations of CRF in the other brain regions described above following in vivo administration of the benzodiazepines (45) may relate to increased release of CRF, which would be expected to decrease receptors in regions like the frontal cortex and hippocampus (35). Although the mechanisms and sites of action through which the benzodiazepines produce their therapeutic effects in the treatment of panic/anxiety disorders remains to be elucidated, it seems reasonable to speculate that their actions may involve suppression of CRF hypersecretion that may occur in these disorders.

Feeding Disorders - Anorexia nervosa is an eating disorder characterized by tremendous weight loss in the pursuit of thinness. There is similar pathophysiology in anorexia nervosa and in depression including the manifestation of hypercortisolism, hypothalamic hypogonadism and anorexia (48,49). Furthermore, the incidence of depression in anorexia nervosa patients is high (50). Like depressed patients, anorexics show a markedly attenuated ACTH response to intravenously administered CRF (48,49) and significantly higher basal CSF levels of CRF than controls (49). When the underweight anorexic subjects are studied after their body weight had been restored to normal, their basal hypercortisolism, increased levels of CRF in the CSF, and diminished ACTH responses to exogenous CRF all return to normal at varying periods during the recovery phase (48,49). CRF can potently inhibit food consumption in rats which further suggests that the hypersecretion of CRF may be responsible for the weight loss observed in anorexics (16). In addition, the observation that central administration of CRF diminishes a variety of reproductive functions (22,51) lends relevance to the clinical observations of hypogonadism in anorexics.

Obesity is associated with hyperphagia, decreased sympathetic and increased parasympathetic activities (effects that are opposite to those seen following central administration of CRF) (52). Furthermore, the development of obesity in a variety of rodent models is prevented by adrenalectomy (an experimental manipulation associated with increased synthesis and release of hypothalamic CRF) (52). These observations have led to the hypothesis that CRF may be involved in the development of obesity syndromes. Central administration of CRF at a dose that was without affect in lean rats stopped the excessive weight gain of obese animals (53). These effects of CRF appeared unrelated to changes in food intake, since both CRF- and saline-injected rats were pair-fed (53). Rather, they appeared to be due to stimulation of the sympathetic nervous system resulting in increased thermogenesis (53-55) and inhibition of the parasympathetic nervous system (53). In addition, recent data suggest that antiobesity drugs such as fenfluramine are potent stimulators of hypothalamic CRF release (56) which is consistent with the hypothesis that these drugs may produce their weight-reducing effects through increased CRF release. The relevance of these preclinical studies to the involvement of CRF in the development of obesity in humans remains to be demonstrated.

NEURODEGENERATIVE DISEASES

Alzheimer's Disease - Alzheimer's disease (AD) is a progressive degenerative disease of the nervous system characterized neuropathologically by the presence of senile plaques and neurofibrillary tangles in amygdala, hippocampus and neocortex. Several studies have provided evidence in support of alterations in CRF in AD. Radioimmunoassay and immunohistochemical techniques have been used to examine changes in CRF content and morphology, respectively, and receptor binding techniques have been used to determine the consequences of the pre-synaptic changes in the neuropeptide. There are decreases in CRF (57-62) and reciprocal increases in CRF receptors (58,60,61,63) in cerebral cortical areas that are affected in AD such as temporal, parietal and occipital cortex (Figure 3). The reductions in CRF and the increase in CRF receptors are all greater than 50% of the corresponding control values. Neither CRF content nor CRF receptors are changed significantly in other cerebral cortical areas such as the cingulate cortex (60) (Figure 3). Evidence from chemical cross-linking studies indicate that the increased CRF receptor population in cerebral cortex in AD comprises bona fide receptor binding subunits with no apparent change in the molecular structure (63). The up-regulation of cerebral cortical CRF receptors in AD under conditions in which the endogenous peptide is reduced suggests that CRF-receptive cells may be preserved in the cortex in AD. The reduction in cortical CRF content may be due to selective degeneration of CRF neurons intrinsic to the cerebral cortex or it could be due to dysfunction of CRF neurons innervating the cortex from other brain areas. Additional evidence for a role for CRF in AD is provided by observations of decreases in CRF in other brain areas including the caudate (57) and decreased concentrations of CRF in the CSF (64,65). Furthermore, a significant correlation is evident between CSF CRF and the global neuropsychological impairment ratings suggesting that greater cognitive impairment is associated with lower CSF concentrations of CRF (66).

Immunocytochemical observations complement the radioimmunoassay studies demonstrating morphological alterations in CRF neurons in AD. In AD, swollen, tortuous CRF-immunostained axons, termed fiber abnormalities, are clearly distinguishable from the surrounding normal neurons and are also seen in conjunction with amyloid deposits associated with senile plaques (67). Furthermore, the total number of CRF-immunostained axons is reduced in the amygdala of Alzheimer's patients; in effect, the innervation seen in normal controls is lost in diseased persons (67). Interestingly, the expression of CRF antigen in neurons is not globally reduced in Alzheimer's patients. CRF immunostaining of perikarya and axons located in the hypothalamic paraventricular nucleus is much more intense in AD than in controls (67). Increased immunostaining of the paraventricular neurons in AD, if truly representative of increased content of CRF, could be related to increased amounts of CRF mRNA in these cells or increased translation of available mRNA. The increased expression and/or release of CRF from the paraventricular nucleus of the hypothalamus would provide a reasonable explanation for the hypercortisolemia often seen in Alzheimer's patients (68).

Parkinson's Disease - Parkinson's disease (PD) shares certain clinical and pathological features with AD. A substantial number of PD patients (15-40%) eventually develop dementia. Pathological alterations in brains of patients with AD and PD can be quite similar (i.e. senile plaques and neurofibrillary tangles) but only PD patients have extensive Lewy Body inclusions and neuronal loss (69). The neurochemical pathology in the cerebral cortex in PD has not been as extensively studied as in AD.

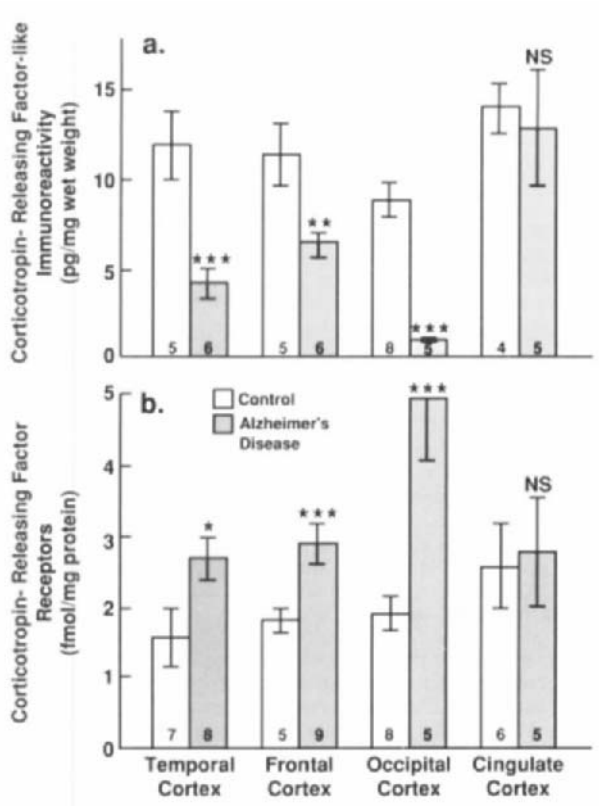

Figure 3. CRF-like immunoreactivity (a) and CRF receptor binding (b) in discrete regions of the cerebral cortex of Alzheimer's patients and controls. All values are means ± S.E.M. The number of subjects in each group is given at the bottom of each histogram. Significant differences from control group at $p < 0.05$, $p < 0.025$ and $p < 0.005$ are denoted by *, ** and ***, respectively. NS, nonsignificant. (From De Souza et al., (60); reprinted by permission from Nature).

In cases of PD with dementia that also show pathological features of AD, CRF content is decreased and shows a pattern similar to those cases exhibiting the pathology of AD alone (58,59,61). Specimens from patients with PD who did not have the histopathology characteristic of AD also demonstrate reductions of CRF content, although the reductions are less marked than in cases of combined AD/PD (58,59,61). The reductions in CRF content in PD may result from the same mechanisms as those in AD. Normal CRF levels have been reported in the hypothalamus in PD (70), suggesting that the loss of CRF in the cerebral cortex is not generalized.

Progressive Supranuclear Palsy - Progressive supranuclear palsy (PSP) is a rare neurodegenerative disorder that shares certain clinical and pathological features with AD. In PSP, neurofibrillary tangles occur, but the ultrastructure of the filaments of neurofibrillary tangles and their distribution differ from those occurring in AD. Due to the rarity of the disorder, only limited data are available from three patients with the disease. In these patients, CRF is decreased to approximately 50% of the control values in the frontal, temporal and occipital lobes (58,59,61). There is no discernable relationship, however, between the clinical symptoms and CRF content in the three lobes. As in AD and PD, the reductions in cortical CRF seen in PSP could be due to either degeneration of intrinsic cortical neurons or to the loss of brain stem neurons projecting to the cerebral cortex. In PSP, loss of neurons also occurs in the pedunculontine nucleus pars compacta of the brain stem (71). Dysfunction in these neurons is probably associated with decreases in cholinergic function in globus pallidus and subthalamic nucleus (72,73). In addition, since a subpopulation of brain stem cholinergic neurons containing CRF project to the cerebral cortex (74), disease of these cells may also contribute to the decrease in cortical CRF in PSP.

Huntington's Disease - The similarity of the changes in CRF found in the context of the three diseases associated with Alzheimer-type pathology raises the possibility that cerebral cortical reduction in CRF is nothing more than a nonspecific sequela of the disease process. To address this issue, CRF radioimmunoassay studies were repeated on brain tissue of patients with Huntington's disease (HD). Cerebral cortical pathology in HD is minimal. Instead, the disorder is characterized neuropathologically by profound neuronal cell loss in the caudate nucleus and putamen (striatum of the basal ganglia) (75). Dysfunction and death of neurons in the striatum are associated with marked changes in a variety of classic and peptidergic neurotransmitters (76).

In HD, the CRF content in the frontal, parietal, temporal, occipital and cingulate cortices and in the globus pallidus is not significantly different from that seen in neurologically normal controls (76). However, the CRF content in the caudate nucleus and putamen of the basal ganglia is significantly reduced; it is less than 40% of the CRF concentrations seen in the caudate nucleus and putamen of controls (76). Thus, HD is the first dementia in which cerebral cortical levels of CRF are relatively unaffected. The localization of the CRF changes to only affected brain regions in the four neurodegenerative disorders described suggest that CRF has an important role in the pathology of these dementias.

Amyotrophic Lateral Sclerosis - Amyotrophic lateral sclerosis (ALS) is a generalized brain and spinal cord disorder associated with progressive degeneration of spinal cord motor neurons (77). CRF was measured in the CSF from five patients with ALS and ten patients with discopathy, which served as a control group (78). The CRF level in the CSF of patients with ALS was approximately 50% of the value seen in controls providing preliminary evidence that CRF may be related to the pathophysiology of ALS.

Interactions of CRF and Acetylcholine - At present, the cerebral cortical cholinergic deficiency seems to be the most severe and consistent neurochemical deficit associated with AD (79). Similar changes occur in PD (80), whereas in PSP, mild cholinergic neuronal loss in the basal forebrain is associated with very low reductions in cerebral cortical choline acetyl transferase (ChAT) activity (71-73). There is some preliminary evidence that there may be a link between alterations in CRF and cholinergic deficits in the three diseases. Reductions in CRF correlate with decreases in ChAT activity in each of the cortical areas examined (58-61). In Alzheimer's, there are significant positive correlations between ChAT activity and reduced CRF in the frontal, temporal, and occipital lobes (58,60). Similarly, significant negative correlations exist between decreased ChAT activity and increased number of CRF receptors in the three cortices (58,60). There also is a highly significant correlation between CRF content and ChAT activity in Parkinson's (59), whereas ChAT activity seemed only slightly changed in PSP.

The correlations between changes in CRF and ChAT activity are complemented by anatomic and behavioral studies in rats, which suggest that CRF and cholinergic systems interact. Colocalization of CRF and acetylcholinesterase is detected in some brain stem nuclei projecting to the frontal cortex (74). Some cholinergic neurons projecting from the amygdala and basal forebrain also contain CRF (74). In studies assessing the effects of CRF on carbachol (a muscarinic cholinergic receptor agonist)-induced behaviors, CRF alone has no visible effect, but inhibits carbachol-induced behaviors, suggesting that CRF may exert a neuromodulatory function on cholinergic systems. Additional evidence for an interaction between acetylcholine and CRF comes from a pharmacological study demonstrating that chronic treatment of rats with atropine (a muscarinic cholinergic receptor antagonist) results in a selective increase in CRF receptors in the frontoparietal lobes (similar to that noted in AD), with no significant alterations in other brain areas including olfactory bulb, cerebellum, striatum or hippocampus (81). These data suggest that the reported reciprocal changes in pre- and post-synaptic markers of CRF in cerebral cortex of patients with AD and possibly PD and PSP may be, in part, a consequence of deficits in the cholinergic projections to the cerebral cortex in these diseases. Additional studies are necessary to determine the functional significance of the interaction between CRF and cholinergic systems.

DRUG DEVELOPMENT STRATEGIES

In view of the evidence described above suggesting that hypersecretion of CRF may underlie some of the symptomatology seen in affective disorders, anxiety-related disorders and in anorexia nervosa, it stands to reason that CRF antagonists may be useful in the treatment of these disorders. Thus, a CRF antagonist may be a useful antidepressant, anxiolytic or "anti-stress" drug. Since the drug would have to act in brain in addition to the pituitary (to counteract the hypercortisolemia), it is essential that the compound cross the blood-brain barrier. While major advances have been made

in the design of metabolically stable peptide analogs, the problem of achieving access to the brain following systemic administration and oral bioavailability with such compounds remains unsolved. The development of small molecules that cross the blood-brain barrier and that have efficacy following oral administration is indeed a challenge.

Recently, potent orally effective nonpeptidal antagonists have been developed for the peptide hormone cholecystokinin (CCK) (82,83). The CCK antagonist was isolated from fermentation broths based on identification of its binding properties to CCK receptors using radioligand receptor assays (82). The structure of the compound was then determined by mass spectrometry, nuclear magnetic resonance and X-ray crystallography (82). Based on the original structure, a variety of chemically related structures were synthesized and demonstrated to have increased potency and specificity at the CCK receptor and to be effective following oral administration (83). The development of CRF antagonists could utilize a similar approach in which fermentation broths and/or synthetic compounds in existing libraries could be screened in a high output receptor binding assay to identify potential leads. These leads could then be screened in a second messenger assay, such as stimulation of adenylate cyclase activity (in the case of CRF) to determine whether they were agonists or antagonists at the receptor. Subsequently, structural modifications could be made to increase their potency and oral bioavailability. The compounds could then be screened in a variety of behavioral and biological assays to further determine their effectiveness.

In view of the noted deficits in brain CRF and up-regulation of CRF receptors in AD, it would appear that the CRF system may offer some hope for potential drug therapy for Alzheimer-type dementia. Here the situation may be analogous to Parkinson's disease, in which a major deficit is loss of dopaminergic axons projecting from the substantia nigra to the caudate nucleus and putamen. Nevertheless, the increase in dopaminergic receptors permits replacement therapy. While CRF agonist-related therapy can be considered for Alzheimer-type dementia, it should be noted that since CRF produces potent anxiogenic "stress-like" effects in rodents, the potential side effects of a CRF agonist must also be considered. Similarly, while a CRF agonist may prove useful in weight reduction in obese individuals, the side effects present a major problem in drug development. Partial CRF agonists may reduce the side-effect profile and represent potentially useful compounds for the treatment of dementias or as weight-reducing agents.

SUMMARY AND CONCLUSIONS

This chapter provides an overview of preclinical and clinical evidence in support of a role for CRF in neuropsychiatric disorders and neurodegenerative diseases. Overall, the data suggest that CRF may be hypersecreted in brain in depression, anxiety-related disorders and anorexia nervosa. In contrast, deficits in brain CRF are apparent in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, progressive supranuclear palsy and amyotrophic lateral sclerosis as they relate to dysfunction of CRF neurons in brain areas affected in the particular disorder. The data suggest that CRF antagonists may represent novel antidepressant/anxiolytic drugs and may be useful in the treatment of neuropsychiatric disorders manifesting hypersecretion of CRF. Finally, some strategies directed at developing CRF-related antagonists for the treatment of the various neuropsychiatric disorders have been briefly described.

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Chapter 24. Ligand Gated and Voltage-Gated Ion Channels

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Introduction - Ion channels are a major class of effectors that control cellular excitability (1) and are regulated by chemical, electrical, visual, volume and other sensory events. Most ion channels are classified as ligand-gated or voltage-gated channels respectively according to the nature of the primary regulating signal (2-6), however these distinctions are not unique. The cell maintains an asymmetric ion distribution with Na^+ and Ca^{2+} being maintained at low and K^+ and Cl^- at high intracellular levels relative to the exterior. This asymmetry maintains the membrane potential and the subsequent generation of ionic currents and it permits the ions themselves, notably Ca^{2+}, to serve as cellular messengers. Molecular biology provides powerful techniques with which to characterize channels, including molecular cloning and expression and site-directed mutagenesis with which to produce "designer channels" (7-12). A resultant major challenge is the generation of structural models that encompass the basic functional properties of channels (13-15).

Channels may be single polypeptides or may be composed of homo- or heteromeric associations of subunits. Channels must contain those structural elements that permit them to regulate ionic currents. These include "gates" that open or close in response to stimulus and sensors that respond to this stimulus and that are linked to the permeation and gating processes (Figure 1). The sensor responds to chemical, electrical or other input and may be an integral component of the channel or may be an extrinsic protein linked to the channel by a messenger(s) (Figure 1). A single channel type may be regulated by multiple receptors through second messengers and a single messenger from a single receptor may regulate multiple channels (16). Channels may also regulate channels. Thus channels that hyperpolarize reduce the activity of depolarizing channels: an ionic or biochemical intermediate may be involved as in the ability of Ca^{2+} to activate Ca^{2+}-dependent K^+ channels (17).

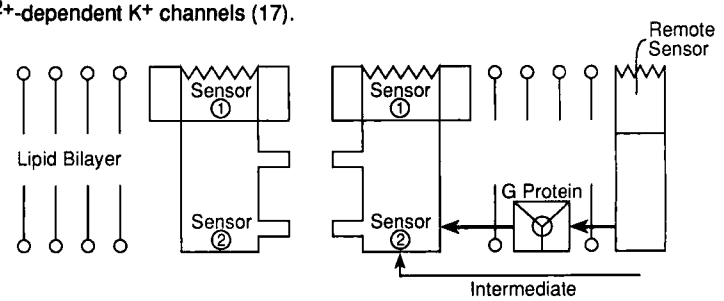

Figure 1. Schematic representation of essential components of an ion channel depicting the presence of both intrinsic and remote sensors and modes of sensor channel communication.

Ion channels may be classified according to several criteria including electrophysiologic properties of permeant ion type, conductance and activation/inactivation kinetics. Ligand sensitivity to agents of physiologic, natural or synthetic origin, provides a further classification usually employed in concert with electro-physiologic criteria. Thus, voltage-sensitive Ca^{2+} channels may be classified into three major types according to the criteria listed in Table 1 (18). Ion channels may therefore be considered as pharmacologic receptors (2,3); accordingly it is expected that:

1. Channels exist as families of proteins with considerable homology within each family (7-9).
2. They possess specific and discrete drug binding sites with defined structure-activity relationships and stereoselectivity (2,3).
3. Both activators and antagonist drugs should exist. These may mimic actions of putative endogenous ligands for the channel (2,3).

4. Ion channels may be associated with one or more guanine nucleotide binding proteins (19,20).
5. Ion channels can be regulated by homologous and heterologous influences (inter alia, 21,22).
6. Ion channel function and expression should be altered during growth and development and in disease states (inter alia, 21,22).

VOLTAGE-GATED ION CHANNELS.

Na^+, K^+ and Ca^{2+} channels function in frequently cooperative fashion to generate cellular excitability, where rapid depolarization mediated through the Na^+ channel precedes Ca^{2+} channel activation and Ca^{2+} influx with repolarization mediated through the final opening of K^+ channels (1).

Channel characterization - The Na^+ channel consists of a major α- subunit (260 kDa) and smaller β_1 (36 kDa) and β_2 (33 kDa) subunits variously expressed according to tissue and species (23,25, 26). The β_2 and α-subunits are linked through disulphide bonds. Mammalian brain contains all three subunits and skeletal muscle the α- and β_1 subunits whereas eel electroplax contains only the α-subunit (23,25,26). All three subunits are hydrophobic and glycosylated, but the α-subunit possesses the major functional properties of the Na^+ channel.

Ca^{2+} channels of several different types exist. Most information is available for the 1,4-dihydropyridine-sensitive L-type channel (Table 1), primarily because of the very high binding site density in skeletal muscle (27,28). Five subunits have been characterized with substantially different physical and biochemical properties, but the non-glycosylated α_1 subunit expresses the pharmacologic and functional properties of the channel and is thus the major component (29-32).

Table 1. Properties of Plasmalemmal Voltage-Gated Ca^{2+} Channels

Property	Channel Class		
	L	T	N
Activation range,mV	-10	-70	-30
Inactivation range,mV	-60 to -10	-100 to -60	-120 to -30
Inactivation rate	Slow	Rapid	Moderate
Conductance	25pS	8pS	13pS
Permeation	$Ba^{2+}>Ca^{2+}$	$Ba^{2+}=Ca^{2+}$	$Ba^{2+}>Ca^{2+}$
Cd^{2+} Sensitivity	Sensitive	Insensitive	Sensitive
1,4-DHP sensitivity	Sensitive	Insensitive	Insensitive
ω-Conotoxin sensitivity	Sensitive	Insensitive	Sensitive

Channel architecture - Schematic arrangements of the subunits of the Na^+ and Ca^{2+} channels are depicted in Figure 2. Corresponding biochemical studies for the K^+ channel are not yet available, but molecular biology studies (23) have established the homologies between these several channels. Primary structures are now available for the α-subunit of the Na^+ channel from eel electroplax (33), rat brain (34) and Drosophila (35), the α_1 (30,36,37), α_2 (38) and β-subunits (38) of the Ca^{2+} channel and K^+ channel proteins from Drosophila (39,40) and rat brain (41-44). Considerable homology exists between the α and α_1 subunits of the Na^+ and Ca^{2+} channels and with the K^+ channel proteins (12,23,45,46). The α_1 subunit of skeletal muscle Ca^{2+} channel consisting of 2005 residues is 29% identical to the rat brain Na^+ channel II subunit or 65% homologous by conservative substitution. Three, I, II and III, sodium channel subtypes have been isolated from rat brain (34) and a homologous tetrodotoxin-resistant channel has been isolated from rat heart and denervated skeletal muscle (47). Ca^{2+} channel subtypes (α_1 subunits) also exist in skeletal muscle, heart (30), brain and smooth muscle where some 66-75% homology exists (48).

The A-type K^+ channel proteins are approximately one quarter the size of the Na^+ and Ca^{2+} channel proteins. For example the protein from Drosophila and a rat brain channel have 616 and 495 residues respectively. Nonetheless, the K^+ channels are significantly homologous to the Na^+ and Ca^{2+} channels each of which is organized into four homologous stretches, with each of these domains being similar to the single domain of the K^+ channel (Figure 3). These homologies indicate that the voltage-gated channels form one "super- channel family" and that K^+ channels may represent the ancestral species. K^+ channels may constitute a remarkably diverse group because they are derived from an extended gene family (49,50) and because the channel is likely an oligomeric assembly of small subunits. Certainly, K^+ channel classification by pharmacologic and electrophysiologic criteria is indicative of a large number of channel subtypes. (1,51-53).

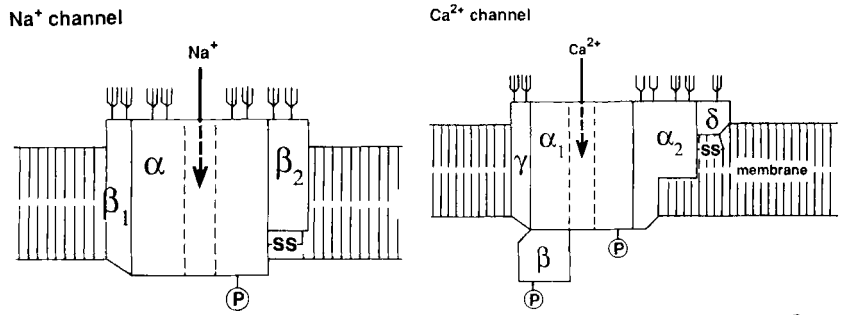

Figure 2. Proposed subunit organizations of the voltage-gated Na^+ and Ca^{2+} channels. Reproduced with permission from Catterall, Science, 242: 50, 1989.

Channel diversity may arise because gene expression differs between tissues (54), because of differences in posttranslational modification (55,56) and because hormonal influences regulate gene expression (57,58). Thus, in skeletal muscle the 212 kDa α_1-subunit (2005 residues) which represents the fully expressed gene may serve as the Ca^{2+} channel, while the 175 kDa form (1873 residues), derived by posttranslational cleavage, may be the voltage sensor linked to the release of Ca^{2+} from the sarcoplasmic reticulum through the ryanodine sensitive release channel (59).

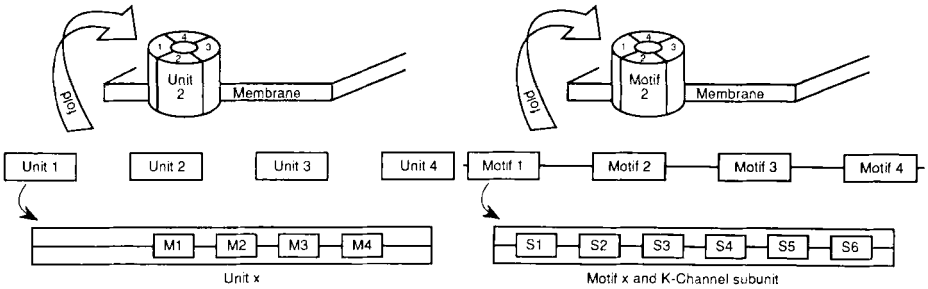

Figure 3. Comparison of organizational features of ligand- and voltage-gated ion channels. Ligand gated channels (left) are composed of assemblies of individual subunits and voltage-gated channels (right) are composed, save for the K^+ channel, of repeating motifs within a single peptide. Reproduced with permission from Stevens, Nature, 328: 198, 1987.

Structure and function - The homologies between the voltage-gated channels extend, most significantly, to their proposed membrane organization. Hydropathy profiles of the channel sequences indicate an identical pattern of hydrophobic and hydrophilic segments which have been interpreted as membrane spanning α-helices and cytoplasmic or extracellular connecting loops. Each of these repeating domains contains six hydrophobic α-helical segments S1- S6: S2 and S3 are negatively charged and S4 exhibits an ordered pattern of five or six positively charged lysine or arginine residues each of which is separated by two intervening hydrophobic units. Since the Ca^{2+} and Na^+ channels each contain four of these repeating domains, and the K^+ channel only one, the functional K^+ channel is likely an oligomeric assembly.

The repeat units of the channel are proposed to be oriented in a pseudosymmetric fashion around a central pore lined by the four S2 segments with the S4 segments, as voltage sensors, situated within the helical cluster (9,14,15,23,60). Models of voltage sensor function in which the S4 segments, stabilized by ionic bridges from positive charges to negative residues on adjacent helical segments, move in an outward sliding motion upon depolarization to transfer one positive charge across the membrane per segment provide a basis both for the conformational changes that must underly the activation process and for the observed (+ 4 to +6 units) gating charge movements (23,33,61). Site-directed mutagenesis studies on the Na^+ channel α-subunit show that all four repeats are necessary for functional channel expression, confirm the importance of the positive charges in the S4 segment and demonstrate that the cytoplasmic domain between repeats 3 and 4 is necessary for channel inactivation (62,63). A modest and conservative substitution in the S4 strand of domain II of the rat Na^+ channel sequence at position 860 (Phe Leu) produces a 20-25mV

shift in the activation potential to more negative values. This likely represents the critical difference between type II and type IIA Na^+ channels (64).

Toxins have played a major role in the characterization of Na^+ channels and five distinct receptor sites have been identified (4). α-Scorpion toxin V from *Leirus quinquestriatus* (LqTx) labels the channel in the first set of transmembrane helices at a site formed from residues 335 to 378 located in an extracellular loop between helices S5 and S6 (65,66). Charybdotoxin interactions with A-type K^+ channels indicate the role of Glu422 as an external residue creating an electrostatic field that attracts the positively charged toxin (67).

Specific drugs have been used to characterize the functional organization of channels. The availability of specific and potent drugs that act at specific channels includes tetrodotoxin, saxitoxin and local anesthetics for Na^+ channels, quaternary ammonium ions for K^+ channels and 1,4-dihydropyridines for Ca^{2+} channels (1-3,23). The Na^+ channel is sensitive to five discrete classes of drugs, the guanidinium toxins that block permeation, the veratridine alkaloids that produce prolonged activation, the α-scorpion toxins which inhibit inactivation, the β-scorpion toxins which shift activation and the brevetoxins which prolong activation. These drugs act at five discrete, but linked, sites (23). A similar pattern of multiple ligand sensitivity is observed with the L class of voltage-dependent Ca^{2+} channel (24).

LIGAND GATED ION CHANNELS.

The receptor-channel complexes for acetylcholine (nicotinic, nAChR), GABA (A receptor) and glycine form a second channel "superfamily" (3,9,14,15,45,62,68,69). The general characteristics include an organization of several homologous subunits which form the receptor-channel complex. The two functional components are the receptor site for the physiologic ligand, borne on one of the subunits, and the ion channel proper, composed of the transmembrane helices of the several contributing subunits. Subunits within a single receptor are homologous and significant homologies exist between the subunits from different receptor types. Each subunit has a large hydrophilic N-terminal extracellular domain followed by three hydrophobic membrane spanning regions, M1 to M3 and a further hydrophobic domain M4 located near the C-terminus. The hydrophobic transmembrane sequences show the greatest homology and the least homology exists for the extracellular N-terminal area and the large cytoplasmic loop joining M3 and M4.

The nicotinic acetylcholine receptor - Although the nAChR receptor is classically associated with skeletal muscle, it is now quite clear that it is the product of a family of genes expressed in both peripheral and central nervous systems as well as in skeletal muscle (70-74). The pentameric complex, $\alpha_2\beta\gamma\delta$, 50 to 60 kDa each (total, ~300 kDa) exhibits a quasisymmetrical organization with pentagonal symmetry around a central axis. A "barrel stave" arrangement of subunit helices is proposed to form the channel. Each complex contains two nonequivalent ACh binding sites located on the α- subunits.

Molecular biology approaches have focussed on defining the sites of drug action and of mapping the regions concerned with channel gating properties (9). The four types of subunits are necessary for full functional channel expression and the different subunits contribute to the several functions of the receptor-channel complex. Replacement of the γ-subunit of fetal tissue by the ε-subunit of adult tissue is associated with an increased conductance, from 40pS to 60pS, and a decrease in current duration (75). Similarly, denervation of skeletal muscle is accompanied by the appearance of the γ-subunit (76). The subunits from Torpedo and calf are highly homologous and can be mutually substituted to generate hybrids with the same uniform conductance (77). However, the gating behavior of the native channels differs dramatically: the average current duration in the calf is some ten times that of Torpedo and the construction of hybrids from calf and Torpedo by insertion of calf α- and δ -subunits gave current durations intermediate and comparable to the calf respectively. Presumably sequences in the calf δ-subunits determine channel open time and a sequence in the α-subunit determines channel opening or ACh dissociation.

The ACh binding site is associated with cysteines 192 and 193 on the extracellular domain of the α-subunits (78,79) and functional regions associated with channel permeation, gating and drugbinding have also been identified (9,80). Amino acid replacements in the α-subunit alter ligand binding, channel properties, and the expression of other subunits (81). Replacement of the N-glycosylation site 141 (asparagine) by aspartic acid disrupts the assembly of nAChR and there is little expression of functional activity. Similarly, conversion of cysteines 128 and 142 to serine alters the expression of the α-, γ -and δ -subunits and virtually abolishes functional receptor activity. Under

conditions of low ionic strength, the conductance of the nAChR from Torpedo is higher than the bovine system. Construction of chimeric δ-subunits from Torpedo and calf permits the localization of this difference to the residues of the M2 region and its vicinity (82). Point mutation studies establish three rings of negatively charged and glutamine residues, at the external channel opening and in the interior and cytoplasmic regions of M2, as important determinants of ion transport (Figure 4): an inverse relationship exists between conductance and residue charge (83). The importance of these charges is also indicated by the presence of two more negative charges in the mammalian ε- than in the γ-subunit, probably underlying the higher conductance of the $\alpha_2\beta\gamma\varepsilon$ than the $\alpha_2\beta\gamma\delta$ complex (75). Similarly, replacement of serine residues of the α- and β-subunits by alanine in the interior of the M2 segment decreases outward conductance, although leaving other channel properties essentially unchanged and produced a progressive decrease in the affinity of QX-222, a quaternary ammonium local anesthetic believed to interact at the open channel state (84). These results suggest an increased barrier to ion permeation near the cytoplasmic side of the channel (84).

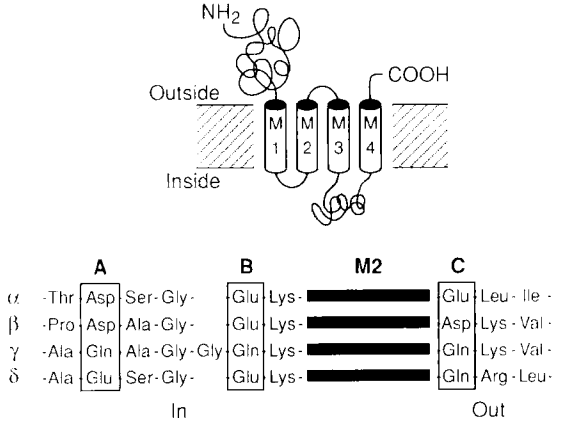

Figure 4. The conduction pore of the nAChR depicted as composed of the M2 domains (above) of the several contributing subunits with the three rings of charge shown boxed as A, B and C. Reproduced with permission from Miller, Neuron 2: 1195, 1989.

Other ligand-gated channels - The primary structures of the $GABA_A$ receptors (bicucculine and benzodiazepine sensitive) and glycine receptors were defined in 1987 (69,85): the GABAA receptor remains the focus of considerable attention (68,86,87). The originally defined α- and β-subunits, 429 residues and 48.8 kDa and 449 residues and 51.4 kDa respectively, form functional GABA-gated Cl^- channels and it was proposed that an $\alpha_2\beta_2$ tetrameric complex represented the receptor-channel complex with GABA, bicucculine, picrotoxin and barbiturate sensitivity (88). Individual subunits of the $GABA_A$ receptor can form functional channels. Although, these are relatively inefficient channels (87,89,90), their formation supports the notion derived from homology that all subunits have a common ancestral origin and have arisen through gene duplication and mutation. At least four distinct α- and three distinct β-subunits exist and it is likely that in any combination they can reconstitute Cl^- channels (87,91-95). A differential brain distribution of the mRNAs coding for the α- and β-subunits occurs, with granule cells expressing both, but Purkinje cells expressing only the α-subunit (96). Furthermore, there exists a differential neuronal expression of α-subtypes (97).

The critical benzodiazepine sensitivity of $GABA_A$ receptors is not, however, provided by the α- or β-subunits, but rather by the more recently discovered γ_2 subunit (87,98-100). The γ_2 subunit, sharing some 40% homology with the other subunits, has mRNA prominently localized in the CNS and when coexpressed with the α_1- and β_1-subunits forms GABAA receptors with high affinity benzodiazepine binding and with the GABA responses appropriately potentiated by the BZ agonists diazepam and flunitrazepam, reduced by the inverse agonist DMCM (methyl-4-ethyl-6,7-dimethoxy-β-carboline-3-carboxylate), and blocked by the antagonist Ro15-1788 (98). Distribution of the γ_2 subunit parallels that of the benzodiazepine receptor, but that of the δ-subunit, sharing some 35% sequence homology with the other subunits, resembles that of high affinity muscimol sites that lack benzodiazepine binding (99). A γ_1 subunit found in glial cells awaits definition (99).

The GABA/benzodiazepine receptor subtypes may be due to their differential expression, localization and combination of the different subunits. Thus, the functional combination $\alpha_1\beta_1\gamma_2$ has higher affinity for CL 218 872, β-CCM and 2-oxoquazepam, and exhibits the temperature-dependent affinity modulation shown by these compounds and a pharmacological profile consistent with the dominant type I benzodiazepine receptors implicated in anxiolytic states. In contrast combinations with the α_2 and α_3 subunits were more typical of type II receptors associated with the hippocampus, striatum and spinal cord and mediating ataxia and depression (87,100).

The postsynaptic glycine receptor of the spinal cord contains two subunits of 48 and 58 kDa (101). The lighter subunit has been cloned and expressed to yield glycine- and strychnine-sensitive Cl^- channels (85,101-103). This homo-oligomeric assembly is characteristic of this channel class; both subunits (or more) exist in vivo and have substantial homology (101). Heavy and light mRNAs are isolable from spinal cord, exhibit developmental regulation and appear to express electrophysiologically distinguishable glycine receptors (104).

It is likely that excitatory amino acid receptors will also fall into the ligand-gated channel class. Glutamate receptors are classified according to pharmacologic criteria into four major classes - N-methyl-D-aspartate (NMDA), kainate, quisqualate and 2-amino-4- phosphonobutyrate (105). A cDNA clone from rat brain was expressed in Xenopus to yield a kainate binding protein, 99.8 kDa, with the appropriate pharmacologic and electrophysiological properties. The hydropathy profile reveals four transmembrane regions, but there is very little sequence homology to other members of the ligand-gated channel family. Smaller kainate binding proteins, 48 and 49 kDa, have also been isolated but functional properties are not known (106,107). They possess the characteristic 4 transmembrane domains and also show little homology to other ligand-gated channels. They may represent a second ligand-gated channel family (108).

OTHER ION CHANNELS

Intracellular Ca^{2+} channels critical to the release of Ca^{2+} from sarcoplasmic and endoplasmic reticulum have now been characterized. The ryanodine-sensitive channel from skeletal muscle is an extraordinarily large protein with a 565 kDa monomer assembling as a tetramer (109). This protein organizes in the sarcoplasmic reticulum membrane with a 500 residue C-terminal sequence forming 4 transmembrane α-helical segments and the large remaining N-terminal region forming the cytoplasmically located foot structure that communicates with the 1,4-dihydropyridine channel/voltage sensor (110-112). Little homology exists to other channel sequences save for the M2-M3 region which resembles that of the nAChR.

The IP_3 receptor of endoplasmic reticulum serves to release Ca^{2+} through the intermediacy of the receptor-linked phospholipase C system (113). The IP_3 receptor, 250 kDa, previously known under other names including the P400, PCPP-260 and GP-A proteins (114), shows fragmentary sequence and topologic homology to the ryanodine receptor (115,116). The IP_3 receptor possesses a large terminal foot and several transmembrane domains in common with the ryanodine receptor and likely functions also as a tetramer, but lacks the putative nucleotide binding domains and homology to the nAChR.

The cGMP gated channel of rod photoreceptors is a relatively non-selective cation channel, blocked by divalent cations and by l-cis-diltiazem. It thus demonstrates the opposite pharmacologic stereospecificity to the L class of Ca^{2+} channel. The primary structure reveals a 690 residue sequence with 4 or 6 transmembrane α-helices, but with no extensive sequence similarity to other channels, transporters or to G protein coupled receptors (117). However, a C-terminal region of some 80 residues shows similarity to nucleotide binding domains of c-GMP dependent protein kinase. It remains to be established whether these channels define new families.

Regulation of Ion Channels - Membrane effectors are currently considered as existing in three major classes - the G protein coupled receptors, the ligand-gated ion channels and the voltage-gated ion channels - each a sequentially and topologically homologous group of transmembrane proteins (15,23,45,60). Important similarities may, however, exist between these several effector classes (Figure 3). Some ion channels are regulated by direct interactions with G proteins (19,20); the cardiac Ca^{2+} channel is regulated by Gs (119) and neuronal K^+ channels by Go (120). Thus, both receptors and ion channels must contain cytoplasmic domains that mediate specific G protein interactions. The properties of specific ligand recognition or defined ion translocation may not

uniquely indicate a specific effector class. Thus, the 5-HT_3 receptor may be a gated ion channel rather than a G protein receptor (121); K^+ channels may belong to both channel families.

Ion channels are presumably regulated in similar fashion to other membrane proteins, and may be subject to short-term biochemical regulation and channel sequestration and long-term down regulation as well as effects on mRNA levels and posttranslational processing (122,123). Ca^{2+} channels are subject to a number of regulatory influences (122). The numbers and function of voltage-gated Ca^{2+} channels are up-and down-regulated by chronic exposure to antagonist and activator drugs (122,124-126). They are altered by thyroid and estrogen treatment (127,128), they are up-regulated during chronic alcohol administration (129) and they are increased clinically in cardiomyopathy (130). Channel changes occur during disease states: the lethal recessive absence of voltage-dependent Ca^{2+} channels in murine muscular dysgenesis is overcome by microinjection of an expression plasmid carrying DNA encoding the muscle 1,4-dihydropyridine receptor (131).

Overactivity of ligand-gated excitatory amino acid receptors is important in a variety of neurodegenerative disorders (132). Cystic fibrosis, a genetic defect carried by 5% of the Caucasian population (133), is associated with defective protein kinase phosphorylation of Cl^- channels (or associated proteins) in epithelial cells (134,135). It is likely that channel regulation will be seen increasingly as an important contributor to disease states and to the choice and selectivity of drug action during these states. G proteins are important and ubiquitous coupling proteins, are altered during disease states (136), and are associated with ion channels. It is probable that ion channel defects will, like receptor defects, also be associated with changes in G protein-channel coupling.

Drug Action at Ion Channels and The Impact of Molecular Biology - Two major classes of drugs acting at ion channels have been distinguished according to their sites of action. Drugs acting at classically defined receptor sites of ligand-gated channels and for which physiologically based ligands exist fall within the first category. The second category includes drugs which interact at sites associated with the channel proper: physiologically equivalent ligands likely do not exist (but see 118). This second class of agents interacts at sites associated with the permeation and gating processes of the channel and their activity both determines and is determined by the functional state of the ligand- or voltage-gated channel - resting, open or inactivated. Formal treatments of these drug-channel interactions are available in the modulated- and guarded-receptor mechanisms according to which the affinity or the access respectively of the receptor varies according to channel state (137,138). Such non-competitive drugs as chlorpromazine, phencyclidine and local anesthetics interact at sites in the M II transmembrane domain of the nAChR subunits (80,84,139). MK 801 similarly serves as a non-competitive antagonist at the NMDA subclass of glutamate receptor (140). At voltage-gated channels this class of drugs includes the Ca^{2+} channel ligands, activators and antagonists, the local anesthetics acting at Na^+ channels and the quaternary ammonium ions at K^+ channels (1-3,137). These state-dependent interactions complicate interpretation of structure-activity relationships, since biological assays may measure the average of drug interactions at several discrete states.

Molecular biology provides a further definition of drug-channel interactions. Ultimately, it will be possible to study directly drug-channel complexes through X-ray crystallography and thus to define directly the drug binding sites (141). A candidate channel for such an approach may be the ryanodine or IP_3 sensitive Ca^{2+} release channels since they contain much of their molecular mass in non-membrane environments and may thus well be easier to crystallize (112). Currently, these molecular biology approaches provide structural correlates of channel function including the pore region of the nAChR (82-84) and the voltage-sensing S4 segments of voltage-dependent channels (14,23), provide sequence-based definitions of channel subclasses analogous to that provided for G protein-linked receptors (142), and generate rationalizations for the cross-interactions of ligands between channels of the same family. Additionally, these approaches map the differential cellular localization of channel subclasses including the $GABA_A$ (96,97) and Na^+ channels (143,144) and provide the beginning definitions of drug binding sites including noncompetitive antagonists at the nAChR (80,84,139), scorpion toxin at the Na^+ channel (65,66) and 1,4-dihydropyridines at the Ca^{2+} channel (14). Finally, the chromosomal localization of the genes coding for ion channels is of importance to defining associations with genetically determined diseases. Localization of the cystic fibrosis gene and identification of the dominant mutation as a deletion of three bases coding for phenylalanine at position 508 of the 1480 residue protein is a major triumph (145-147). More speculatively, the gene coding for the human α_3 subunit of the $GABA_A$ system is localized to the Xq28 region thus making it a candidate defect for the X-linked form of manic depression (148).

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Chapter 25. Cell Adhesion Molecules

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Introduction - Cell-cell interactions play critical roles during embryogenesis and in homeostasis of mature organisms. These interactions are important in fundamental processes such as inductive events, tissue modeling, immune responses and lymphocyte homing, the orderly migration and differentiation of cells such as those derived from the neural crest, and in the formation of the specific connections of the nervous system. A corollary of this notion is that pathological alterations in such interactions would have profound consequences: Failures in cell-cell interaction are suspected to be key players in tumor cell detachment and invasion, in some defects of immune responsiveness, and in birth defects associated with aberrant crest cell migration and embryonic lethality. Several reviews on aspects of these topics have appeared (1-9).

Major progress has been achieved through the use of in vitro assays to identify and characterize cell surface glycoproteins that function as cell adhesion molecules, or CAMs. It is now clear that there are several basic families of CAMs. The detailed structures of many CAMs have been determined, and in several cases the molecular binding mechanisms are known in broad terms. During embryogenesis, these CAMs are expressed in dynamic patterns that are consistent with the idea that they may play key roles in developmental processes. Despite this progress, specific, detailed knowledge as to the precise roles played by these molecules in vivo is generally lacking. Elucidation of signalling pathways associated with the interactions of CAMs is certain to provide fresh insights into the control of development and regulation of tissue functioning.

Definition of Cell Adhesion Molecules (CAMs) - CAMs have been defined empirically as molecules that mediate cell-cell adhesion in highly artificial in vitro assays. As a result, the properties of the assays define the kinds of molecules that may be identified. Typically, such assays involve proteolyticdissociation of a tissue into a suspension of individual cells. After a period of recovery to allow the regeneration of surface components, these single cells are generally capable of aggregating rapidly. The aggregation can be quantitated using electronic particle counters. The surface molecules that mediate this aggregation have invariably been identified by immunological means. In the most direct approach, monoclonal antibodies are raised against a spectrum of cell surface components and are then screened to identify antibodies that bind to and block the major active CAM. Because this approach detects antibodies that block the bulk aggregation of cells, only CAMs that are present on all or most of the cells will be identified. Any CAMs that mediate binding among specialized subsets of cells will be overlooked.

To be convincingly identified as a CAM, a candidate molecule must satisfy several criteria: the CAM must be expressed in tissues and, during development, in a distribution that is consistent with its presumed adhesive role; antibodies to the CAM should produce perturbations of development or function in vivo that may be readily interpreted as the result of disrupting adhesion; the purified CAM should display a binding activity to the target cell; transfection of control cells with CAM sequences should confer adhesive ability; and finally, mutations that alter CAM expression or function should produce direct effects on adhesion. As discussed below, the many CAMs that have been identified by this general approach have fallen into a small number of families or classes.

N-CAM and Other Immunoglobulin Superfamily CAMs - The first CAM to be identified and characterized was N-CAM (neural cell adhesion molecule), so-named because it is found on all neurons throughout development (10,11). However, N-CAM is not restricted to nerve tissue, but rather is expressed by a variety of cell types in tissues derived from all three germ layers (12). N-CAM mediates calcium-independent aggregation of nerve cells (13) and binding between neurons and myotubes (14). Increased N-CAM expression is one of the earliest consequences of

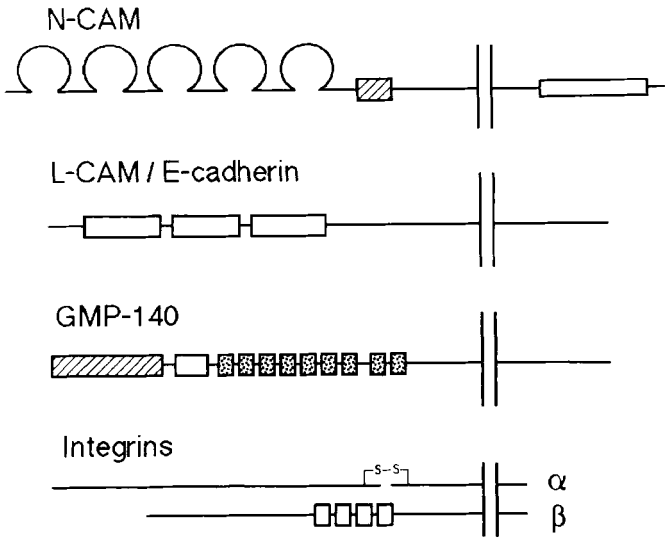

Figure 1. Representative members of CAM families. Each structure is shown with the extracellular region to the left and the cytoplasmic domain to the right of the plasma membrane (vertical lines). The first of the immunoglobulin-domain CAMs to be characterized was N-CAM, which contains five immunoglobulin-like domains (loops) which extend away from the cell surface. The figure shows the Mr = 180,000 N-CAM polypeptide. The Mr = 180,000 and 140,000 polypeptides contain a common hydrophobic segment that spans the cell membrane, but they differ in the extent of their cytoplasmic domains. The Mr = 180,000 chain, which is found only in neural tissue, contains a unique region (open box) coded by a single large exon that is included via a tissue-specific splicing event. In contrast, the Mr = 120,000 chain does not include this membrane spanning domain, but instead incorporates a different hydrophobic-rich segment that is attached to the membrane by a phosphatidylinositol linkage (not shown). The structure of L-CAM/E-cadherin is representative of all the cadherins. Thus far, only a single polypeptide form of each of these molecules has been detected. The large extracellular domain contains three repeated homology units that are unrelated to immunoglobulin domains. The polypeptide spans the membrane, and contains a cytoplasmic domain of approximately 150 amino acids. The structure of GMP-140 illustrates the elements common to the lectin-CAMs: an amino-terminal lectin domain (diagonal hatching), an EGF-related sequence (open box) and 9 repeats (checkered boxes) similar in sequence to elements that regulate components of the complement system. A typical integrin structure is shown at the bottom of the panel. Integrins consist of two non-covalently associated subunits. The α-subunit consists of two disulfide-bonded polypeptides derived from a precursor by proteolytic cleavage. The β-subunit contains four repeated regions (open boxes) that are particularly rich in cysteine. The cytoplasmic domains of both the α-and β-subunits are targets for phosphorylation, which changes the binding activities of the integrins.

neural induction (15). Overexpression of N-CAM had no effect on neural tube formation or closure, but did cause striking disruptions of somite formation (16). Antibodies to N-CAM disturb the interaction of retinal ganglion cell axons with glial endfeet in the optic nerve (17) and lower the precision with which these fibers map onto the tectum (18). Coordinate regulation of N-CAM and Ng-CAM (see below) expression appears to be important in establishing appropriate patterns of innervation in developing chick muscle (19).

N-CAM is a large and abundant surface glycoprotein (Figure 1). About one-third of the mass of N-CAM is carbohydrate and the molecule is particularly rich in sialic acid. N-CAM consists of three major polypeptides with MWs of 180,000 Da, 140,000 Da, and 120,000 Da (20,21). These polypeptides are derived from alternatively spliced transcripts (22-24) of a single gene located on human chromosome 11 (25). The extracellular portion of these polypeptides is largely identical,

although each of the three major polypeptides is made up of subpopulations that differ in slight respects due to minor alternative splices (26-29). The major differences between the three forms are due to alternative splices that alter the size and structure of the carboxyl-terminal portion of the molecule. The two largest forms span the plasma membrane and differ only in the size of their cytoplasmic domain. The smallest of the three forms does not span the membrane, but is attached via a phosphatidylinositol linkage (24,30,31). In addition, analysis of an N-CAM cDNA derived from human muscle mRNA implies the existence of a secreted form of N-CAM (32). In this mRNA, an alternatively-spliced exon introduces a termination codon, resulting in a form of N-CAM that does not contain membrane attachment regions.

The binding region of N-CAM is located in the amino-terminal portion of the molecule (24,33). There is strong evidence that N-CAM binding is homophilic, that is, N-CAM on one cell binds to another N-CAM on an adjacent cell (34-36). In contrast to the immunoglobulin genes, there is no evidence for amino acid sequence heterogeneity that could change the specificity of binding. One modification that affects the rate of binding is glycosylation. N-CAM contains a large amount of sialic acid, linked in an unusual, polymeric form (37). The content of sialic acid decreases during development (21,38), leading to a change in binding, measured in vitro. The adult, or low-sialic acid, forms of N-CAM mediate binding at four times the rate of the embryonic, high-sialic acid forms (39). This developmental decrease in sialic acid might therefore serve to stabilize transient connections, although there is no direct evidence for this view. N-CAM is also phosphorylated, sulfated, and acylated (40), but little is presently known about the effect of these modifications on binding activity. There is evidence that multiple N-CAM polypeptides on the same cell may associate to form a more efficient binding structure (41,42). N-CAM also is capable of binding heparin and heparan sulfate (43,44), but the role or significance of this interaction in vivo has not yet been determined. One recent report suggests that N-CAM alone does not mediate binding, but only facilitates binding mediated by Ng-CAM (45), but this conclusion is opposed by other recent experiments (35,46).

The functional significance of the variations at the carboxyl end of N-CAM is unknown. One exciting possibility is that these variations could mediate different cellular responses after cell-cell binding. For example, it is known that the binding of ligands to surface N-CAM leads to reduced levels of inositol phosphates and intracellular pH, while elevating intracellular Ca^{2+} (47). Such changes are likely to alter cellular function or gene expression. Different forms of N-CAM could produce different cellular responses. Another possibility is that the different cytoplasmic domains could direct specific forms of N-CAM to particular cellular locations. For example, it is known that the Mr=180,000 form is preferentially expressed on axon processes (23,48). Recent evidence suggests that spectrin binds specifically to this form (49), and such an association could be a mechanism for selective localization.

Interestingly, the extracellular domain of N-CAM consists of five segments that are homologous to each other and to members of the immunoglobulin gene superfamily (24,27,50,51) (see Figure 1). Molecular cloning studies demonstrate that other neural CAMs, including Ng-CAM (52), contactin (53), myelin-associated glycoprotein (MAG) (54-56), Thy-1 (57), and P_0 (58) are structurally related to N-CAM and to other immunoglobulin superfamily molecules. The overall extent of homology is low and only a small number of key residues are conserved. These residues lead to formation of the compact immunoglobulin domain structure, which consists of back to back β-pleated sheets, connected by a disulfide bond. The conserved residues presumably determine the three-dimensional backbone, while the sequence variations mediate distinct binding specificities for each CAM. The immunoglobulin superfamily CAMs have been reviewed (4).

Ng-CAM (59), also termed L1 (60) or NILE (61), contains six immunoglobulin domains and two fibronectin Type III repeats (52). In contrast to N-CAM, Ng-CAM is restricted to cells of the nervous system, including post-mitotic neurons and Schwann cells (62,63). Ng-CAM is preferentially expressed on axons (62,64) and appears to play a major role in the collection of axons into nerve fascicles (65,66). In addition, Ng-CAM is involved in the migration of cerebellar granule cells along Bergman glial fibers (67,68) and in interactions between Schwann cells and axons. Antibodies to Ng-CAM partially block the initial binding of Schwann cells to axons, reduce extension of neurites from neurons plated onto Schwann cell monolayers, and block Schwann cell myelination of axons (69-71). There has been a lively controversy surrounding the mechanism of Ng-CAM mediated adhesion. All groups now agree that Ng-CAM can mediate homophilic, or Ng-CAM to Ng-CAM, binding (72-74). Some recent experiments suggest that such binding is enhanced when a complex between Ng-CAM and N-CAM on one cell binds to Ng-CAM on a second cell (45). This would represent an "assisted homophilic" mechanism of binding. There is also strong evidence (72) that Ng-CAM can mediate binding via a second, heterophilic, mechanism, although this mode of

binding has not yet been universally accepted. The ligand recognized by Ng-CAM in such a heterophilic mechanism has not yet been identified.

The adhesive role of other neural immunoglobulin superfamily CAMs is less well established. MAG may play a role in Schwann cell/axon interactions, in particular in establishing close apposition between the Schwann cell and axon membranes (75). Contactin was originally identified based on its tight linkage to the cytoskeleton (76). Although its localization (concentrated in fascicles and at synapses) is consistent with an adhesive function, there is no direct evidence for such a role. P_0 has been proposed to mediate compaction of myelin sheaths (58).

Many cells are known to express two or more CAMs simultaneously. For example, Schwann cells express N-CAM, Ng-CAM, N-cadherin, MAG, and integrins. N-CAM, N-cadherin, and Ng-CAM are all expressed on axons as well, so many parallel adhesive interactions are possible. In fact, antibodies to Ng-CAM, N-cadherin, and integrin each partially block the extension of neurites by neurons plated onto Schwann cell monolayers (70,71). Antibodies to N-CAM had no effect. The functional reason for several parallel adhesive systems is not known, nor is it clear why N-CAM, present on both cells, does not contribute to the binding. Such findings underscore the need for new assays that determine the consequences of cell-cell binding as well as adhesion.

Cadherins - The binding activity and structure of several CAMs are dependent on calcium (77-80). This property of calcium-dependence is diagnostic of a class of related adhesion molecules, the "cadherins" (81,82), which have been the subject of a recent review (2).

The first of these molecules to be extensively characterized was the epithelial CAM variously termed E-cadherin (80), L-CAM (77), uvomorulin (78), cell-CAM 120/80 (79) or Arc-1 (83). Like N-CAM, L-CAM/E-cadherin is a large and abundant surface glycoprotein. However, the complete sequence of L-CAM/E-cadherin, deduced from cDNA clones (84-86) shows no similarity to immunoglobulin domains. All of the cadherins do show three internally repeated homology units (84-86). Each of these units contains sequence motifs that may be involved in calcium binding (86,87). L-CAM/E-cadherin appears to exist as a single, invariant polypeptide. L-CAM/E-cadherin has little or no associated sialic acid, but is phosphorylated and acylated (88).

L-CAM/E-cadherin is expressed in virtually all epithelia derived from ectoderm or endoderm, with the exception of some terminally differentiated cells such as lens epithelium (2). L-CAM/E-cadherin is expressed in mouse blastomeres at the cleavage stage (89) and antibodies to this CAM dramatically delay compaction of 8-cell stage morulas (78,79,90). Morulas that are incubated continuously in the presence of these antibodies do eventually compact, but they fail to differentiate properly (90), suggesting that L-CAM/E-cadherin mediated interactions are necessary for some early differentiation signals. Antibodies to L-CAM/E-cadherin directly prevent cell-cell communication between teratocarcinoma cells, as indicated by dye transfer experiments (91). Cell-cell contacts mediated by L-CAM/E-cadherin appear to be necessary for the establishment or maintenance of gap junctions inasmuch as expression of L-CAM/E-cadherin in L-cell fibroblasts from transfected cDNA constructs caused induction in these cells of functional gap junctions (92). If gap junctions convey cell-cell signals affecting development, then differential expression of L-CAM/E-cadherin may be a decisive controlling event in embryogenesis.

Two other cadherins have been identified, from placenta (P-cadherin) (93,94) and from neural tissue (N-cadherin) (87,95,96) which appears to be the same as A-CAM (97). The structures of all of the cadherins are very similar (87), indicating that they form a family of evolutionarily related adhesion molecules that is distinct from the N-CAM family. Surprisingly, comparison of the various cadherin structures shows that the region with greatest sequence conservation is the cytoplasmic domain (87). The conservation of this sequence implies selective pressure to conserve some essential function. This conclusion is supported by recent experiments that determined the effect of deletions in the cytoplasmic domain on adhesion. Even small deletions completely abolished the ability of E-cadherin to mediate adhesion (98). One possible interpretation of these results is that alterations in the cytoplasmic domain may have altered the conformation of the extracellular binding domains, abolishing binding activity. Another possibility is that the cytoplasmic domain must interact with cytoskeletal or cytoplasmic components in order to localize correctly or to activate the extracellular binding domain.

N-cadherin and P-cadherin are expressed in dynamic patterns during development and in a wide variety of adult tissues (2, 93-96, 99, 100). N-cadherin is first expressed at gastrulation, and is found in neural tissue from the time of neural plate invagination. Although initially expressed

uniformly, different neural regions eventually express varying amounts of N-cadherin. N-cadherin is also found in skeletal and cardiac muscle, in lens epithelium, and in nephric primordia (95,96). P-cadherin first appears in extraembryonic membranes of implantation stage embryos (93). This cadherin is not expressed in non-pregnant uterus, but is expressed at high levels in uterine decidual cells (2). later in embryogenesis, P-cadherin is expressed in basal cells of epidermis (100), in pigmented epithelium of the retina, and in mesothelium (93). In several cases, tissue rearrangements, such as the separation of the neural tube from overlying ectoderm, are accompanied by corresponding changes in the distribution of distinct cadherins (2). Such observations provide the basis for the suggestion that differential adhesion mediated by distinct cadherins could play a causative role in cell layer separation (2,95). It is interesting that reduction in E-cadherin expression has been correlated with enhanced invasiveness of carcinoma cells (101).

Lectin-like CAMs - Recently, several adhesion molecules that are expressed on vascular or lymphoid cells have been cloned and sequenced, defining a new class of CAMs (8,102-105). These molecules contain an amino-terminal domain that shares appreciable sequence similarity with several defined lectins, molecules that specifically bind particular carbohydrate moieties. The implication that the binding mechanism of this family of CAMs involves specific recognition of carbohydrate bearing receptors is supported by some evidence from binding studies.

One of these CAMs, MEL-14, is the "lymphocyte homing receptor", involved in the circulation of lymphocytes between lymphoid organs and the vascular and lymph systems (106). Lymphocytes enter the spleen, peripheral lymph nodes, and Peyer's patch regions of the gut by passing through blood vessels at specialized sites called high endothelial venules or HEVs. Previous evidence suggested that these specific interactions were mediated in part through recognition of specific carbohydrate moieties. B-lymphocytes bind preferentially to Peyer's patch HEVs while T-lymphocytes show preferential binding for HEVs of peripheral lymph nodes. It is now clear that these binding specificities are achieved by different sets of receptor-ligand pairs. One key mechanism, involving a lectin-like CAM, is described here. Other mechanisms are discussed later.

Monoclonal antibody technology was used to identify the murine T-cell ligand, termed MEL-14, that mediates binding to peripheral node HEVs (9). Competition binding experiments indicate that MEL-14 specifically recognizes phosphomannosyl-related residues associated with the HEV receptor. Recent cloning and sequencing of MEL-14 (102, 103) provided strong evidence supporting this picture: the amino terminal 125 amino acids of MEL-14 comprise a domain that is highly homologous to several previously characterized mammalian lectins. In addition, MEL-14 contains an Epidermal Growth Factor (EGF) motif, and a series of segments that show sequence similarity to components that regulate the complement system (See Figure1).

A second member of this family is the "endothelial-leukocyte adhesion molecule" ELAM-1 (104). ELAM-1 is expressed on endothelial cells that have been activated by inflammatory agents such as interleukin-1, tumor necrosis factor, and bacterial endotoxin. Like MEL-14, ELAM-1 contains an amino-terminal lectin-like domain, an EGF motif, and six seqments showing similarity to complement regulatory components (104). ELAM-1 appears to be involved in the binding of neutrophils (107) and thus may play a key role in both the positive effects of inflammation and acute inflammatory disease states such as adult respiratory distress syndrome.

A third lectin-CAM which has been described is the granule membrane protein GMP-140, which is localized in granules of platelets and endothelial cells. Granule-localized GMP-140 is rapidly released at the cell surface in response to treatment with thrombin or other platelet activators. GMP-140, also known as PADGEM, mediates binding of neutrophils and monocytes to endothelial cells (108). Analysis of cDNA clones indicates that GMP-140 consists of a lectin-domain, an EGF-like domain, and nine segments related to complement regulatory proteins (105). Although lectin-CAMs have thus far been found only in cells of the vascular and immune systems, it remains possible that related CAMs may function in cells of solid tissues.

Integrins - Integrins are a family of cell-surface receptors (5) that bind components of the extracellular matrix and therefore are generally thought of as mediating cell-substrate or cell-matrix interactions. In this light, their structure and function have been extensively reviewed (5,6).

Integrins consist of two subunits, an α and a β polypeptide, that are both transmembrane proteins (5). The α-subunit consists of two polypeptides that arise from a precursor by proteolytic cleavage, but remain associated via a disulfide linkage. All integrin α-subunits contain several segments that are homologous to Ca^{2+}-binding sites in proteins such as calmodulin. The β-subunit

contains four segments that are very rich in cysteine. The α- and β-subunits show no sequence similarity. Subfamilies of integrins share a common β-subunit, while possessing distinct α-subunits.

Integrins show considerable diversity in their ligands (5,6). The integrin derived from human osteosarcoma cells binds fibronectin, as does the human hepatocyte integrin, while the human platelet integrin gpIIb/IIIa binds fibronectin, vitronectin, fibrinogen, and von Willebrand factor. In general, the common feature of these ligands that is recognized by the integrins is a tripeptide sequence, Arg-Gly-Asp, or RGD (109). While the RGD sequence is necessary for each integrin/ligand interaction, it is not sufficient, inasmuch as integrins have different specificities.

The importance of the integrins stems from their transmembrane signalling capability. The cytoplasmic domains of integrins are linked to the cytoskeleton via binding to talin (110). The cytoplasmic domains of both the α- and β-subunits contain tyrosine residues that can be phosphorylated by tyrosine kinases (111). Tyrosine phosphorylation uncouples binding to talin, but also blocks binding of ligands to the extracellular domains of the integrin chains (112). These observations suggest that integrins could serve as a pathway for signalling across the cell membrane. Binding of ligands to integrins can trigger changes in the expression of collagenase and stromelysin genes (113). This transmembrane signalling ability takes on greater significance in light of the findings, discussed below, that integrins on lymphoid cells can mediate interactions between cells, as well as cell-matrix interactions.

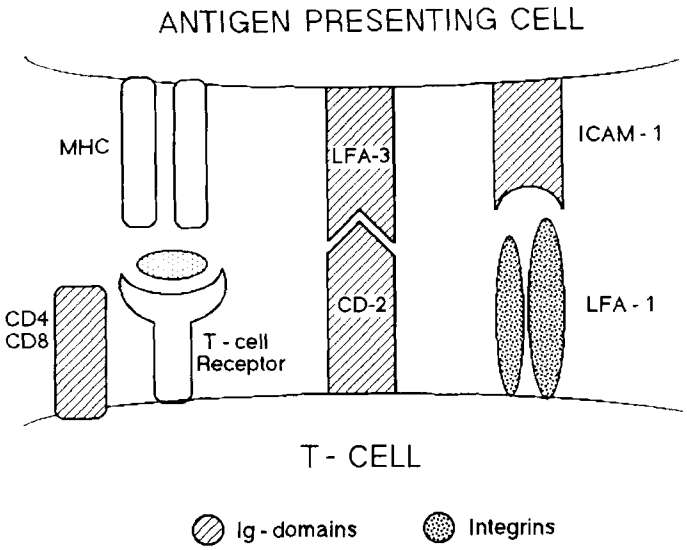

Figure 2. CAMs mediating cell-cell interactions during immune responses. The diagram shows parallel sets of adhesive interactions between a T-lymphocyte (bottom) and an antigen presenting cell (top). In this figure, antigen (light stippling) associated with molecules of the major histocompatibility complex (MHC) binds to the T-cell receptor, which is associated with a group of invariant proteins collectively termed CD-3 (not shown in this figure) and with CD-4 (on helper T-cells) or with CD-8 (on cytolytic T-cells). A second adhesive interaction occurs between LFA-3 and CD-2, and a third binding interaction takes place between ICAM-1 (or, on some cells, ICAM-2) and LFA-1. Blocking any of these three adhesive events interferes with the interactions between the two cells. CAMs that are composed of immunoglobulin domains are filled with diagonal slashes and the integrin (LFA-1) is filled with heavy stipples.

Adhesion Molecules on Lymphoid Cells - Lymphoid cells are highly specialized for cell-cell interactions. In addition to the lectin CAMs described previously, other adhesive systems participate in lympohocyte homing and in T-cell/target cell and T-cell/effector cell interactions. These interactions have been reviewed recently (7-9).

Several of the cell-cell interactions that occur during immune responses are specific for particular antigens. These are enhanced or strengthened by additional adhesive interactions that are not antigen-specific (see Figure 2). For example, many interactions among lymphocytes, monocytes, natural killer cells, and granulocytes involve the lymphocyte-function associated (LFA) antigen, LFA-1. LFA-1 is a member of the leukocyte subfamily of integrins that also includes Mac-1 and p150,95 (5, 114). These three components share the same integrin β-subunit.

One ligand for LFA-1 is ICAM-1, which is expressed constitutively on some cells, but is induced on others during inflammation (115). Many ligands that bind to integrins contain a common RGD sequence, but molecular cloning of ICAM-1 (115, 116) revealed that it does not contain any RGD sequences. ICAM-1 contains an RVE sequence (in the same relative location as an RGD sequence in the myelin-associated glycoprotein, MAG) and an RGEKE sequence, but synthetic peptides containing these sequences have no effect on cell-cell binding. Surprisingly, ICAM-1 is a member of the immunoglobulin superfamily of receptors, showing sequence homology to N-CAM and MAG. ICAM-1 mediates an additional adhesive interaction of significance: it is the major human receptor for rhinoviruses (117, 118). Several lines of evidence indicate that LFA-1 can also bind to a second ligand, ICAM-2, which is an integral membrane protein containing two immunoglobulin domains (119, 120). Like ICAM-1, ICAM-2 does not contain any RGD sequences.

These unexpected findings were the first demonstration of specific nteractions between members of the integrin and immunoglobulin superfamilies (121). A second example of binding between integrins and CAMs of the immunoglobulin superfamily has recently been described. VCAM-1, an immunoglobulin superfamily member that is expressed on endothelial cells in response to cytokines (122), binds to the leukocyte integrin VLA-4 (123). It is not yet known whether such interactions will prove to be widespread among cells outside of the immune system.

Two other binding events contribute to T-cell/antigen-presenting cell interactions. First, the CD-2 receptor on the T-cell binds to LFA-3 on the target cell (7). Both molecules are members of the immunoglobulin superfamily (51, 124). Antibodies to either CD-2 or LFA-3 inhibit T-cell/target cell interactions, and in addition to stabilizing cell-cell adhesion, binding between these receptors appears to trigger the release of lymphokines from the antigen-presenting cell. A second interaction occurs between CD-4 or CD-8 on the T-cell with MHC class II or MHC class I antigens, respectively (7). These events are associated with the antigen-specific interaction of the T-cell receptor/CD-3 complex with MHC molecules on the target cell, but they are not antigen-specific, and simply serve to strengthen or stabilize binding.

Lymphocyte homing also appears to involve several adhesive systems opering in parallel. The murine MEL-14 lectin-CAM discussed previously mediates specific binding to peripheral node HEVs. In addition to the MEL-14 antigen, human molecules defined by the Hermes series of monoclonal antibodies are also involved in lymphocyte binding to HEVs, but to a broader spectrum of targets, including HEVs of Peyer's patches, peripheral nodes and synovia (9). Cloning of the Hermes antigen indicates that its structure is distinct from other CAMs, containing a region that shows sequence similarity to proteoglycan link proteins (125, 126). Other molecules, the endothelial addressins, may serve as receptors for the MEL-14 or Hermes receptors (127, 128). Finally, LFA-1 also appears to play a non-specific, stabilizing role in lymphocyte homing (9).

Multiple adhesion systems - The existence of multiple binding systems on lymphoid cells operating in parallel is similar to the participation of several adhesive systems in the interactions between extending axons and Schwann cells noted previously. While parallel systems may simply provide redundancy that reduces the likelihood of catastrophic errors in development, a more intriguing possibility is that these different "adhesive" systems serve distinct signalling or regulatory functions.

Methods of genetic analysis have been used to analyze adhesive systems in Drosophila. Homologs of immunoglobulin superfamily CAMs, of cadherins, and of integrins have been identified in Drosophila (129-133). Suggestive evidence that parallel systems operate even in this simple organism was provided by the surprising results of experiments in which particular CAMs were genetically ablated. Complete ablation of different neural-specific immunoglobulin superfamily CAMs caused only subtle effects on development of the nervous system; the implication was that, in each case, parallel adhesive systems could compensate (130, 131, 134).

A major strength of the Drosophila system is that conventional genetic methods can be used to identify all members of these parallel systems. One step forward in this direction was provided by a recent study suggesting that abl and fasciclin I may be key elements in parallel signalling pathways

(134). Fasciclin I is a novel adhesion protein whose vertebrate counterpart, if any, has not yet been identified. Fasciclin I is expressed on a small number of axons as they pioneer a nerve pathway in early development. Although its pattern of expression suggested that it might play a role in guidance of these axons, null mutations of fasciclin I produced no obvious effects on neural development (134). However, double mutants of fasciclin I and the tyrosine kinase abl (which is also expressed preferentially in growth cones of the same axons) showed severe disruption of neural development. The most likely explanation for these results is that fasciclin I is a component of one signalling pathway, and abl is a component of a second, redundant pathway. In this case, disruption would only be observed if both pathways were blocked, as in the double mutant. Because the only comparable method for analyzing such pathways in higher organisms is the laborious creation and analysis of transgenic mice, the most exciting new discoveries in this arena are virtually certain to emerge from work on Drosophila.

Conclusion - Many CAM's have been identified, characterized, and classified into families. The CAM's are widely distributed among various tissues and their structure and binding activities are conserved across the animal kingdom from Drosophila to man. Recent evidence suggests that many of the CAM's may play active roles in cell-cell communication as well as adhesion. Individual cells may express several CAM's and these CAM's may play roles in parallel signalling pathways. Such a key role implies that alterations in CAM expression or function may accompay or cause severe pathologies, but this possibility has only begun to be explored. Finally, the involvement of CAM's in key signalling processes together with their accessibility at the cell surface may allow the development and administration of specialized ligands or antibodies that could interact with the cell surface and produce decisive intracellular effects on cell differentitaion or function.

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Chapter 26. Action of Neurotrophic Factors on Central Nervous System Neurons

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Introduction- A neurotrophic factor is a molecule produced by various cell types that can affect neuronal growth. Agents that affect neuronal survival, the size of the cell body (hypertrophy), neurite (axon or dendrite) elongation, and neurotransmitter synthesis can be considered as neurotrophic factors (1). Although some neurotrophic factors influence many aspects of neuronal growth, others have a limited effect (i.e. maintaining survival or promoting neurite outgrowth only). The functions of four neurotrophic factors are discussed in this chapter with major emphasis on nerve growth factor. The theme of the chapter will be restricted to the effects of the described agents on central nervous system (CNS) neurons during development and after injury in the adult.

NERVE GROWTH FACTOR

Structure - The best characterized neurotrophic factor is undoubtedly nerve growth factor (NGF). NGF, isolated predominantly from homogenates of adult male mouse submaxillary glands, has a molecular weight of approximately 130 kDa and a sedimentation coefficient of 7S (2). The NGF molecule is composed of three subunits, designated α, β, and γ. The stoichiometry of the subunits is $\alpha_2\beta\gamma_2$. The α subunit, whose function has yet to be determined, has a molecular weight of ~26 kDa. The γ subunit, with a molecular weight of ~26 kDa, is a peptidase closely resembling bovine trypsin and may be involved in processing the 7S complex of NGF to its biologically active form (3). The neurobiological activity of NGF is mediated solely by the ß subunit (2). This subunit consists of two 118 amino acid monomers each with a molecular weight of ~13.3 kDa (Figure 1). There are six cysteines and three disulfide bridges within the peptide. The sedimentation coefficient of the ß subunit, 2.5S, is commonly used when referring to the active NGF molecule. Mouse 2.5S NGF cDNA clones have been isolated and indicate that the NGF gene spans 45 Kb (4-6). The primary structure of mouse ßNGF has been deduced from the cDNA clones, as has the structure of human, bovine, rat, chick, and snake ßNGF. Comparison of the molecules indicates a high level of conservation across these species.

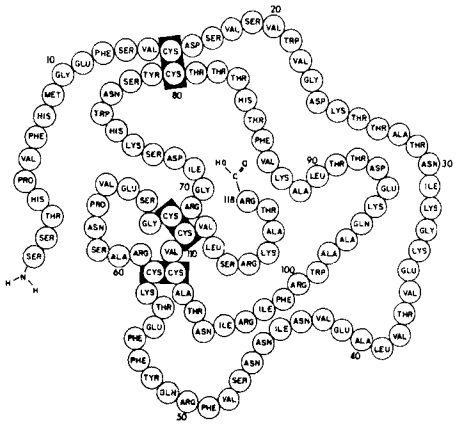

Figure 1: Amino acid sequence of a monomer of βNGF (from R.H. Angelleti and R.A. Bradshaw, Proc. Natl. Acad. Sci. USA, 68, 2417 (1971)).

Distribution of NGF and NGF-Sensitive Neurons - The vast majority of NGF-sensitive neurons in the CNS are cholinergic (7); however, some evidence now suggests that embryonic GABAergic neurons are also NGF-responsive (8). Cholinergic neurons within descrete nuclei (clusters of neurons) of the basal forebrain exhibit many of the characteristics indicative of NGF dependence, i.e. NGF receptor immunoreactivity and biochemical and morphological changes in response to NGF administration. Of these neuronal populations, the cholinergic neurons within the septum (including the medial septum and the vertical limb of the diagonal band of Broca) are the best studied. Although there are several reasons why this particular area of the brain has been widely used in NGF studies, the major advantage is that septal cholinergic neurons can be easily estranged from their target tissue (the putative source of NGF). In a similar manner, cholinergic neurons within another frequently studied nuclei (the nucleus basalis of Meynert) can be isolated from its target (the cerebral cortex) by cortical ablation. Severing the fiber tract connecting the medial septum with its target has the added advantage that rats that have undergone this treatment exhibit cognitive deficits closely resembling those observed in aged rats. Cholinergic neurons in both paradigms undergo cell shrinkage followed by death of the neurons .

Most of the cholinergic neurons within the septum project to their target, the hippocampus, via a well defined fiber tract, the fimbria-fornix. Several cell populations associated with the hippocampus manufacture NGF which is utilized by the septal neurons (this will be discussed further in the next paragraph). Severing the fimbria-fornix in adult rats isolates the cholinergic neurons from their supply of NGF. If this condition persists, cholinergic neurons in the septum eventually degenerate (9,10). Thus, fimbria-fornix transection is a useful model whereby the effects of an exogenous application of NGF on injured cholinergic neurons can be studied. Non-injured neurons can be obtained from fetal brain tissue and the effects of NGF on these neurons studied in culture.

In order for NGF to act as a neurotrophic agent in the CNS, it must first be produced by cells indigenous to the CNS, such as those in the hippocampus. In situ hybridization studies have demonstrated that mRNA coding for NGF is found in pyramidal and granule neurons of the hippocampus and dentate gyrus, respectively (11). The notion that neurons within the hippocampus and dentate gyrus are producing NGF is supported by observations that neurons isolated from the hippocampi of embryonic mice secrete NGF into the culture medium (12). It should be noted that cholinergic neurons within the septum are devoid of NGF mRNA. An alternative source of NGF may be provided by non-neuronal cells, i.e. astrocytes, also in the target area. Although in situ hybridization studies have not detected any NGF mRNA in glial cells, studies have shown that astrocytes prepared from the hippocampus, or cortex, secrete NGF or a NGF-like molecule into culture medium (12). Whether astrocytes are a normal source of NGF in vivo remains contentious.

NGF and Cholinergic Neurons - In the peripheral nervous system (PNS) NGF acts as a neurotrophic factor for noradrenergic neurons found in sympathetic and dorsal root ganglia. The effects of NGF on these neurons have been well documented and are extensively reviewed elsewhere (13). In the CNS, the primary target cells of NGF are cholinergic neurons. One way to monitor the effects of NGF on cholinergic, CNS neurons is to measure the level of choline acetyltransferase (ChAT) in the cells. This enzyme is critical to the formation of acetylcholine by these neurons. Embryonic neurons isolated from brain areas rich in cholinergic neurons (eg. medial septum) can be cultured and the influence of NGF on ChAT activity measured. The addition of NGF to the culture medium increases ChAT levels in these cultures (14,15). Increases in ChAT activity can be mitigated by the simultaneous addition of antibodies specific to NGF into the culture medium. Moreover, recent evidence suggests that the elevation in ChAT activity is accompanied by an increase in the amount of intracellular acetylcholine produced by cultured rat septal neurons (16). NGF is also capable of modulating ChAT activity in vivo. Intraventricular administration of NGF into a newborn rat will significantly increase the level of measured ChAT activity; however, a similar elevation in ChAT activity is not evident when NGF is administered to adult rats (17,18). In contrast, in another study which used similar techniques and sources of NGF, the continuous infusion of NGF elevated ChAT levels in septal neurons of normal adult rats (19). Recent in vitro studies suggest that NGF may modulate ChAT levels during particular developmental windows, with the ChAT enhancing effects of NGF dissipating as normal development of the cholinergic neurons proceeds (20,21). This study demonstrated that neurons obtained from the septum of postnatal day 1 rats will show an increase in ChAT activity when cultured in the presence of NGF. NGF had no affect on cell number. In contrast, septal neurons derived from postnatal day 14 rats exhibited enhanced cholinergic neuronal survival in the presence of NGF. ChAT activity in these cultures was not significantly different from controls.

The influence of NGF on ChAT levels can be monitored immunohistochemically. During normal development, cholinergic neurons in the septum become immunoreactive for ChAT by embryonic day 17 (22). Immunoreactivity continues to increase until postnatal day 23 when staining begins to decline to adult levels. Comparatively, NGF receptor immunoreactivity is present in the

septum by embryonic day 15 (23). Receptor mRNA levels remain fairly constant well into the second postnatal week (24). Therefore, septal fibers possess NGF receptors well before they encounter NGF. The increase in ChAT immunoreactivity corresponds with a concomitant increase in the levels of NGF expression by the hippocampus and NGF protein in the septum (25). The accepted hypothesis is that NGF levels in the hippocampus correspond to the amount of cholinergic innervation. Indeed, cholinergic neurons may modulate NGF mRNA expression in the hippocampus (26). The effect of NGF on ChAT immunoreactivity is presumably due to the retrograde transport of NGF from the hippocampus to the septum. Thus, it appears that NGF may modulate the activity of the enzyme (ChAT) responsible for the manufacture of the neurotransmitter (acetylcholine) produced by septal cholinergic neurons.

In addition to modulating ChAT activity, NGF has been proposed to influence the survival of embryonic cholinergic neurons. The ability of NGF to affect cell survival is still highly speculative. In vitro experiments using dissociated neurons have yielded conflicting results, depending on the density of cells used and the presence or absence of glial cells. For example, early culture experiments using dissociated, high density neuronal or explant cultures failed to demonstrate any cell survival benefits of NGF (27,28). In contrast, subsequent experiments using low seeding densities have shown that NGF can promote the survival (as assessed by ChAT and NGF receptor immunocytochemistry) of septal cholinergic neurons (29). An alternative means of testing whether NGF can influence cell survival is by administering anti-NGF antibodies into a newborn animal. This method has been effectively used to demonstrate the trophic dependence of developing superior cervical ganglion neurons to NGF. Although intraventricular administration of anti-NGF antibodies into newborn rat pups decreases ChAT activity and immunoreactivity, a reduction in cholinergic neuronal number was not reported (30). These results corroborate findings demonstrating that NGF affects ChAT levels, but not neuronal survival, in early postnatal rats (20,21).

NGF and Injury - NGF can sustain ChAT levels in adult cholinergic neurons that have suffered an injury. For example, separating NGF-dependent neurons in the medial septum by transecting the fimbria-fornix reduces ChAT immunoreactivity in cholinergic neurons ipsilateral (on the same side) to the transection (18). Additionally, lesioning the cerebral cortex decreases ChAT activity in neurons residing in the nucleus basalis of Meynert (31). NGF, delivered intraventricularly either by injection or via an osmotic pump, will specifically save septal, cholinergic neurons from degenerating (32,33). NGF need not be immediately administered since delayed infusion weeks after the axotomy can still induce ChAT immunoreactivity (34). In the nucleus basalis, NGF can attenuate the loss of neurons that normally occurs after ibotenic acid injection (35). A recently employed technique uses cells, that have been genetically modified by introducing the NGF gene via retroviruses, as biological pumps for NGF. In a manner similar to their mechanical counterparts, the biological pumps can save septal, cholinergic neurons from death after a fimbria-fornix transection (36). The level of ChAT immunoreactivity in this paradigm is comparable to that obtained using more traditional methods. From these studies, it appears that NGF may be necessary for the maintenance of adult cholinergic neurons in the CNS.

NGF also stimulates the collateral sprouting of axons from non-injured neurons. For example, ablating inputs from the entorhinal cortex to the hippocampus results in the sprouting of cholinergic septal fibers, as visualized by acetylcholinesterase and NGF receptor staining, into the area of the hippocampus normally innervated by axons projecting from neurons located in the entorhinal cortex (37). The increase in fiber density is probably due to the increased availability of NGF in the injured hippocampus. Supporting this assertion, studies have revealed that transecting the fimbria-fornix results in the accumulation of NGF protein in the denervated hippocampus (38). This is probably due to the dramatic decrease in the amount of NGF that is retrogradely transported to the septum. Moreover, recent experiments have indicated that injuring the brain results in the increased expression of neurotrophic factors, including NGF, around the site of injury (39). The ability of NGF to promote fiber growth does not appear to be restricted to sprouting; NGF may also promote the regeneration of axons. For example, after a fimbria-fornix lesion, animals which received transplants of embryonic tissue and NGF infusion showed a greater level of regrowth of fibers from the host septum into the hippocampus as compared to animals that received embryonic transplants or NGF infusion alone (40). This empirical observation is supported by quantitative studies conducted in vitro. In these studies, neurites, emanating from organotypic explant cultures prepared from the septal-basal forebrain of embryonic day 15 rats, were shown to grow faster in the presence of NGF after neurite transection (axotomy) (41). Septal neurons had to be exposed to NGF before and after the axotomy in order to facilitate regeneration. NGF had no effect on the outgrowth of neurites from non-axotomized, embryonic neurons (27, 28). Taken together with in vivo observations, this experiment demonstrates that NGF can influence the regrowth of axons from axotomized septal neurons. Moreover, fiber outgrowth from NGF-naïve neurons, as are found in embryos, is not stimulated by NGF. Thus, NGF may only act as a neurite promoter for neurons that have begun to transport NGF, such as those found in the adult.

NGF Receptors - The interaction of NGF with responsive neurons is mediated by NGF receptors (NGFr). These have been described in detail elsewhere (42). During development, NGFr immunoreactivity is diffusely present in the CNS, including areas of the CNS normally not NGF-dependent (43,44). As expected, neurons within the septum are immunoreactive for NGFr. Staining of these neurons becomes progressively more intense, culminating in the pattern of immunoreactivity seen in adults (22). Areas that are not normally NGF-dependent transiently express NGFr immunoreactivity; however, recent experiments demonstrate that NGFr immunoreactivity in these areas can be induced by tissue damage. For example, cholinergic interneurons within the rat neostriatum lose NGFr immunoreactivity as development progresses. Infusing NGF or phosphate buffered saline (PBS) into the adult neostriatum, or basal forebrain, causes a reappearance of NGFr immunoreactivity , presumably through local damage caused by the infusion of fluid into the brain (45-48). In the neostriatum, the number of NGFr immunoreactive neurons in both NGF and PBS infused animals was similar. However, only animals that received NGF demonstrated somal hypertrophy, an increase in NGFr mRNA, and an increase in ChAT immunoreactivity (46). Thus, it appears that injury causes a re-expression of NGFr by striatal interneurons. This re-expression enables NGF to exert trophic effects on the neurons. A similar phenomenon also occurs in other areas of the CNS. In the spinal cord, the NGFr immunoreactivity of motoneurons is transient (43). Traumatising adult rat motoneurons, by compressing the sciatic nerve, results in an increase in NGFr mRNA by these neurons (49). It should be pointed out that the presence of NGFr, and even the retrograde transport of NGF, does not necessarily indicate that NGF exerts a trophic action on these neurons. Developing rat motoneurons, as described above, are immunoreactive for NGFr and can transport NGF retrogradely (50), however NGF does not elicit any of the classical trophic responses, i.e. hypertrophy, increase in neurotransmitter enzyme, or save the motoneurons from death after axotomy. Thus, although receptor staining is indicative, it can not be used by itself to definitively demonstrate a neurotrophic role for NGF.

FIBROBLAST GROWTH FACTOR

Structure - Fibroblast growth factor (FGF), isolated from pituitary and brain extracts, was initially described as a mitogen for 3T3 cells (51). There are two distinct proteins responsible for the growth activity, designated acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF). Both have molecular weights of approximately 16 kDa (52). As their names imply, the pI of aFGF is acidic (5.6) whereas the pI of bFGF is >9. There is a 55% amino acid sequence homology between the two molecules (53). The bFGF gene has been cloned from a cDNA library prepared from bovine pituitary (54). The most interesting observation from these studies is the lack of an identifiable signal peptide sequence. This suggests that the molecule is not secreted; however, FGF is found in the extracellular environment (see below). How the protein leaves the producer cell is not currently known.

Distribution of FGF - The presence of FGF in the CNS has been confirmed by Northern blot analysis and by immunochemistry. Northern blots have revealed that a 4.6 Kb band exists in extracts prepared from bovine hypothalamus (54). Although this demonstrates that FGF is present in the CNS, it does not distinguish which type of cell actually produces the molecule. Recent *in vitro* experiments, using embryonic neurons isolated from embryonic day 13 rat cerebral hemispheres, have shown that intense bFGF immunoreactivity is present in neurons (55). No immunoreactivity was present in non-neuronal cells. Correspondingly, bFGF staining was found in the cerebral cortices of postnatal day 15-20 rats (56). The intensity and number of neurons exhibiting immunoreactivity decreases with age. Although astrocytes are not FGF immunoreactive *in vivo*, they do produce FGF, mostly bFGF, *in vitro* (57).

An interesting characteristic of FGF is its ability to bind heparin. Heparin binding may reflect an affinity of FGF for the extracellular matrix (ECM) molecules heparin sulfate proteoglycan and the glycosaminoglycans (GAG). Indeed, several laboratories have reported finding bFGF in the ECM (58). The exact advantage of binding to the ECM is not completely understood; however, several observations have been made. For example, heparin bound bFGF is more stable to thermal denaturation and extreme pHs. Binding heparin sulfate or heparin also protects bFGF and aFGF from proteolytic digestion (59,60); however, heparinase or plasmin digestion of FGF-bound ECM can release a bFGF-ECM complex into the surrounding environment (58). This complex is more stable than the uncomplexed bFGF and appears to bind to FGF receptors in a manner similar to that described for free bFGF (59). These observations have led to the hypothesis that the ECM may serve as a reservoir for trophic factor that can be tapped during development or regenerative processes (52).

Trophic Effects of FGF - Unlike NGF, which appears to exert its trophic effects on a specific population of CNS neurons, FGF can affect neurons from many different CNS regions. In vitro, FGF promotes the survival of neurons isolated from the cerebral cortex, hippocampus, striatum, septum and thalamus of fetal rats, postnatal mouse cerebellum, and chick spinal cord (61-64). Moreover, FGF stimulates fiber outgrowth in many of these cultures (63,64). In most cases both forms of FGF can elicit the mentioned responses; however, bFGF typically expresses 10-100 fold more activity than the acidic form (65). Thus, it appears that FGF acts as a neurotrophic factor for cultured CNS neurons.

FGF also apparently possesses neurotrophic activity in vivo. Some neurons in the intermediolateral column of the spinal cord project to the adrenal gland where they synapse on chromaffin cells in the adrenal medulla. Chromaffin cells in the adrenal medulla produce bFGF. Ablation of the adrenal medulla results in a 25% reduction of preganglionic neurons in the ipsilateral intermediolateral column of the spinal cord (66). Neuronal death in the spinal cord can be ameliorated if bFGF-rich gelfoam is placed within the ablation cavity. In the rat forebrain a similar sparing of neurons was observed when FGF-laden gelfoam was placed into the wound cavity created by a fimbria-fornix lesion (67). Additional studies have also indicated a partial sparing of retinal ganglion cells after the optic nerve was transected (68). Thus, it appears that FGF may act in a manner similar to NGF in that it can rescue injured CNS neurons from death after injury; however, FGF appears to influence a broader range of neurons than NGF.

FGF Receptors - Like NGF, the neurotrophic activity of FGF is probably mediated by membrane bound receptors. Currently, two tentative bFGF receptors, with molecular weights of 125 kDa and 145 kDa, have been identified (69). Receptors for aFGF have also been identified (70). aFGF will also compete for binding to the bFGF receptors. The 145 kDa receptor apparently has a higher affinity for bFGF, whereas the 125 kDa receptor has a stronger affinity for aFGF (71). Moreover, recent findings indicate that the hst/K-fgf protein, a third member of the FGF family, may share receptors with bFGF (52).

OTHER TROPHIC FACTORS

Brain Derived Neurotrophic Factor- Brain derived neurotrophic factor (BDNF) is an extremely rare protein that, to date, has only been isolated from porcine brain. An attestment to its rarity is the more than million-fold purification required before BDNF could be isolated to homogeniety (72). The molecule has a molecular weight of ~12.3 kDa and a pI > 10.0 (73). These physiochemical characteristics of the BDNF are similar to those described for the ßNGF monomer. BDNF also appears to share a significant number of amino acid sequence identities with NGF. This assertion has been confirmed by the recent cloning of BDNF from porcine superior colliculi (74). mRNA was prepared from this area of the brain because it is one of the major target sites for the only population of CNS neurons that are currently known to be responsive to BDNF (see below). Fifty one amino acids are common to both BDNF and NGF. Importantly, 6 cysteine residues which are involved in determining the secondary shape of NGF molecules are also present in BDNF. The exact arrangement of the disulfide bridges are not currently known. Several differences between NGF and BDNF do exist, suggesting that the neurotrophic substances, while distinct, are members of the same molecular family.

As mentioned, BDNF has only been prepared from porcine brain extracts. The cloning of BDNF has allowed the construction of sensitive cRNA probes so that the distribution of BDNF in the CNS can be analysed. Northern blot analysis has shown that a 1.4 Kb band, corresponding to the BDNF mRNA, is present in extracts of mouse brain and spinal cord (74). No signal was detected in non-neuronal tissue, i.e. muscle, heart, and lung. This data suggests that BDNF is specific to nervous tissue. To date, the type of cell that is producing BDNF in the CNS has not been elucidated.

Because BDNF and NGF share similar molecular characteristics, it may not be surprising that they also share similar neural binding characteristics. Responsive neurons bind BDNF with a low and high affinity. The only difference between BDNF and NGF binding is that BDNF binds to and dissociates from the low affinity receptor at a rate much slower than that observed for NGF (75). The subsequent sequence of events after ligand binding that triggers neuronal responses is still, as for NGF, highly speculative.

Most neurons that are responsive to BDNF are from the PNS; specifically from neural crest- and ectodermal placode-derived sensory neurons (13). In the CNS, BDNF has been found to affect retinal ganglion cells. For instance, BDNF can save retinal ganglion cells, isolated from embryonic

day 17 rats, from degenerating in culture (76). BDNF did not affect cell number. Adult retinal ganglion cells maintained in vitro responded in a similar manner, i.e. BDNF supported ganglion cell survival and enhanced the rate of regeneration in these cultures (77). To date, no in vivo evidence has been provided to substantiate in vitro observations that BDNF can support the survival of retinal ganglion neurons; however, a recent report has demonstrated that the exogenous application of BDNF in vivo can prevent neurons in the developing PNS from developmentally programed cell death (72). It remains to be seen if BDNF can assert any trophic activity on other populations of CNS neurons.

Ciliary Neurotrophic Factor - Ciliary neurotrophic factor (CNTF) is a distinct molecule found predominantly in the PNS; however, a large amount has been purified from the chick eye (78). CNTF isolated from rat sciatic nerve has a molecular weight of ~24 kDa and a pI of 5.0. In comparison, CNTF purified from embryonic chick eye has a molecular weight of 20 kDa and a pI of 4.8. Thus, the proteins isolated from the two sources appear to be similar. Recently, CNTF has been cloned by two independent laboratories. In one instance, the cDNA library was created from mRNA isolated from rabbit sciatic nerve (79); in the other report, mRNA was prepared from cultured rat brain astrocytes (80). The amino acid sequences and pIs of CNTF deduced from the cDNAs were in agreement with the properties measured for the purified protein. Comparing the amino and nucleic acid sequences to other known neurotrophic factors (i.e. NGF, FGF, BDNF, and purpurin) demonstrated that CNTF is an unique molecule. A major difference between CNTF and NGF is the lack of an amino-terminal signal sequence. This finding, along with the absence of a glycosylation sequence and the presence of only one cysteine residue, indicates that CNTF is a cytosolic protein. This is supported by the observation that cells transfected with the CNTF cDNA expressed the molecule in the cellular fraction, but not in the culture medium (80). However, secretion of CNTF cannot be totally ruled out. Other trophic factors that also lack a signal sequence (eg. FGF) are secreted by unknown mechanisms.

The distribution of CNTF was studied using Northern blot analysis. A ~4.3 Kb band is detected in rabbit sciatic nerve extracts (79); in contrast a ~1.2 Kb band was detected in extracts of rat sciatic nerve and spinal cord (80). No signal was detected in mRNA prepared from liver, muscle, or skin extracts. The type of cell responsible for CNTF production was not elucidated; however, the mRNA used to prepare the cDNA library in one of the studies was isolated from cultured astrocytes. This suggests that astrocytes may produce CNTF in the CNS. Unfortunately, the presence of mRNA encoding for CNTF in the eye was not studied.

CNTF appears to predominantly influence the survival of PNS neurons, particularly parasympathetic ciliary ganglion neurons. In the CNS, CNTF affects neurons in the eye and in the spinal cord. Cultures of chick retinal neurons have demonstrated that a factor secreted by Müller cells, the glial cells of the retina, elevates ChAT levels in Amacrine cells, the cholinergic neurons in the retina (81). The addition of medium containing CNTF elevated ChAT activity to the same extent as medium conditioned by Müller cells. NGF had no effect on cholinergic differentiation. In addition to Amacrine cells, preganglionic neurons found in the spinal cord are also CNTF-responsive (82). These neurons normally project to the inner part (medulla) of the adrenal gland. Lesioning the adrenal medulla results in the retrograde degeneration of preganglionic neurons. These cells can be spared this fate if CNTF-laden gelfoam is transplanted into the cavity created by medullectomy. Thus, it appears that CNTF or a CNTF-like molecule may influence the development of cholinergic (Amacrine) neurons in the chick retina and survival of preganglionic neurons in the spinal cord. CNTF also influences the in vitro differentiation of type 2 astrocytes which are a population of CNS non-neuronal cells (83).

The observation that CNTF is a cytosolic protein has led to the hypothesis that CNTF may be released only after traumatic events whereby lysis of CNTF-producing cells (eg. type 1 astrocytes) could release pent up CNTF into the extracellular milieu (80). This idea has received support from a study in which gelfoam was placed into a lesion cavity created by aspirating a portion of the brain (84). The gelfoam, once removed from the cavity, supported the in vitro survival of dissociated, chick ciliary neurons. This indicates that CNTF or a CNTF-like molecule accumulates around wound cavities created in the CNS.

Other Neurotrophic Factors- The trophic factors described in this chapter are by no means the only ones that are worth pursuing. Several factors that may potentially prove important are gangliosides (85), hormones, such as thyroid hormone (86), the interleukins (87), and potentially, various neurotransmitters (88). These molecules have been shown to influence neuronal survival, cytoarchitecture, and the regeneration of various CNS neuronal populations.

Conclusion- The study of neurotrophic factors is still relatively young. Evidence demonstrating a role for various molecules during regeneration and development suggest a possible clinical application for these types of molecules. For example, it has been suggested that NGF be used as a potential therapy for neurodegenerative diseases that involve cholinergic neurons, such as been described in Alzheimer's. To date, however, it is still to early to employ these drugs therapeutically. Much more basic research, especially on how trophic factors elicit a response from target neurons, is needed.

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Chapter 27. The Structure of the Multidrug Resistance P-Glycoprotein and Its Similarity to Other Proteins

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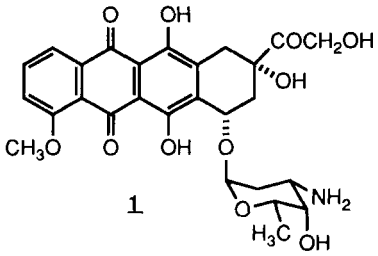
Introduction - The phenomenon of multidrug resistance in cancer cells has been known for over 15 years. The earliest work from Ling's laboratory showed that when cultured cells were selected for their ability to resist the cytotoxic action of one drug, they simultaneously acquired resistance to several other drugs to which they had never been exposed (1). Drugs typically in this group include adriamycin (**1**), colchicine (**2**), vinblastine (**3**), actinomycin D and many other natural products. Perhaps the most striking aspect of this collection of agents is that they do not share a common mechanism of action, suggesting that the basis of resistance must not derive explicitly from the mechanism of pharmacologic action.



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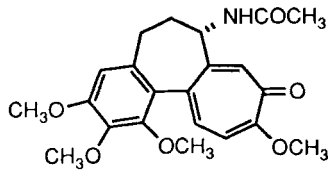

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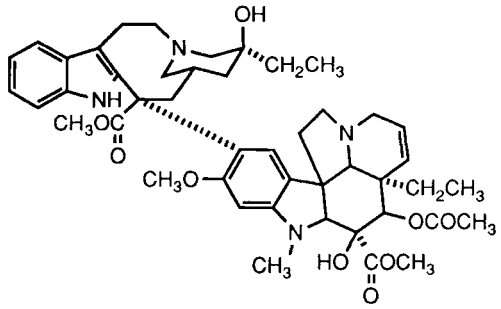


3

Very early in the studies of multidrug resistance it was demonstrated that the plasma membrane of affected cells contained a protein of about 170 kDa that was absent in membranes of sensitive cells (2). This protein is now universally called P-glycoprotein and is known from genetic studies to provide a necessary and sufficient basis for cells to adopt the multidrug resistant phenotype (3,4). In this chapter we will summarize the current state of understanding of the structure of the P-glycoprotein. Since there have been a number of excellent reviews of the biology and molecular genetic basis for multidrug resistance (5-7) these aspects will not be covered, except insofar as they relate to P-glycoprotein structure.

STRUCTURE OF P-GLYCOPROTEIN

Sequence of P-Glycoprotein - Despite the fact that P-glycoprotein was purified from multidrug resistant cells over a decade ago, there is no direct spectroscopic or crystallographic information on its structure. This paucity of information stems both from the fact that P-glycoprotein is an integral membrane protein of fairly large size, and thus difficult to study by conventional solution methods, and from the occurrence of glycosylated forms of the protein which lead to the existence of a variety of heterogeneous isoforms, further complicating attempts at detailed physical characterization. Most of what is known about the structure of P-glcyoprotein (and in fact most membrane proteins) is based on analysis of amino acid sequences.

The modern age of studies of P-glycoprotein began with the discovery that multidrug resistant cells contained amplified DNA sequences (8). Several groups then quickly found that one of the genes in the amplified sequence coded for P-glycoprotein (the gene locus is referred to as *mdr*), thus allowing for the development of molecular probes for the study of the underlying basis of drug resistance. Three independent groups simultaneously announced the sequence of the cDNA for P-glycoprotein in 1986 and analyzed the predicted protein by standard computational methodologies (9-11). The single polypeptide chain contains about 1280 amino acids and has several recognizable structural motifs. The most obvious feature is a division of the sequence into two homologous halves, each of which is again divided into a hydrophobic and a hydrophilic domain. When the sequences of the C-terminal and N-terminal halves are optimally aligned (*i.e.* gaps are allowed in order to achieve the best fit), there is about 43% amino acid homology and an additional 35% with functionally similar amino acids. Thus the two halves do have a certain degree of divergence of sequence, but it is likely that the overall organization arose from an internal gene duplication.

Each half of the P-glycoprotein sequence also has a conserved consensus nucleotide binding region in the hydrophilic domain. It is not known if both sites are functionally the same, although it has been shown that both must be present in order to allow full P-glycoprotein function (12). It is not yet definitively known if hydrolysis of a nucleoside triphosphate is required for activity of P-glycoprotein, or if nucleotide binding serves instead in a regulatory role.

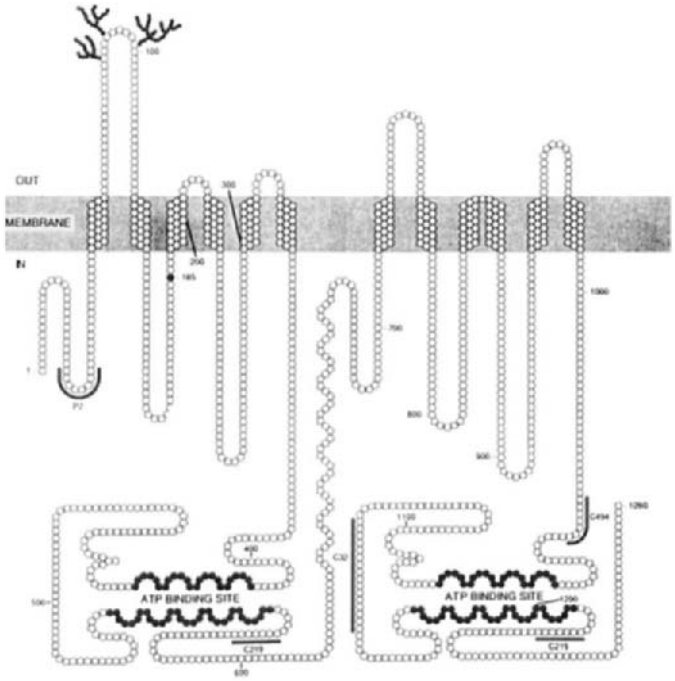


Figure 1. A model for the organization of P-glycoprotein in a membrane bilayer (taken by permission from Juranka et al., FASEB Journal 3, 2583 (1989)

Structural Organization - The two halves of the protein each have six hydrophobic stretches that by hydropathy plots are suggestive of serving as transmembrane regions. It is notable that the *sequence* similarity between the two long hydrophobic stretches is not especially high, only the hydropathy plots. It is tempting to propose that the mature protein has twelve membrane spanning regions, and such a model for P-glycoprotein structure has become widely accepted (Figure 1). The word *model* should be emphasized, however, since there is little direct experimental evidence to support the conclusion drawn by sequence analysis, and in fact there is really no hard structural information on the molecular orientation of the protein in membranes other than the immunochemical localization of the C-terminus and the nucleotide binding domains in the cytoplasm (13). It is instructive to note however, that although different analytical approaches to the prediction of transmembrane stretches often yield very different conclusions about the same sequence (14), in the case of P-glycoprotein both the Kyte-Doolittle (15) and Eisenberg (16) algorithms yield the same twelve domains, supporting the premise that the model structure may be a close representation of the real one.

Glycosylation - Evaluation of the sequence reveals the presence of eight possible N-linked glycosylation sites. The protein is glycosylated and the amino acid sequence only accounts for about 140 kDa of the total 170-180 kDa of functional protein. Most of the carbohydrate consists of high mannose oligosaccharides and the glycosylation is hetereogeneous (17,18). Glycosylation deficient mutants show no obvious difference in the MDR phenotype compared to normally glycosylated cells (19). Therefore, the role of the carbohydrate is still unknown. Heterogeneity of cross-resistance patterns observed in MDR cells may be explained by a structural heterogeneity in P-glycoprotein glycosylation, however this remains to be established.

Phosphorylation - The P-glycoprotein can be phosphorylated (20) and it was suggested that this phosphorylation was capable of regulating the function of the protein (21-23). The kinase responsible for phosphorylating P-glycoprotein is of the serine/threonine variety (*i.e.* not a tyrosine kinase) as judged by phosphoamino acid analysis (24-27); this phosphorylation can be increased by agents that reverse multidrug resistance like verapamil and trifluoperazine (28). Most of the research has concentrated on protein kinase C as the major responsible kinase (29-32), but some evidence for a role of cAMP dependent kinase also exists (33).

SIMILARITY WITH BACTERIAL TRANSPORTERS

Sequence Homologies - Each of the three original studies of P-glycoprotein sequence reported a striking amino acid sequence homology of P-glycoprotein with bacterial transport proteins (Table 1). This structural similarity immediately leads to the suggestion that P-glycoprotein acts as a pump to export drug from resistant cells, and this hypothesis is the dominant one in P-glycoprotein research today. Best-fit sequence comparisons show that the two nucleotide binding regions of P-glycoprotein have a greater than 40% homology with bacterial multi-component, periplasmic transport systems (permeases) such as the oligopeptide permease and histidine systems in *Salmonella typhimurium* and the maltose/maltodextin transport system of *Escherichia coli* (10,34). Each of these systems is composed of four components: two homologous hydrophobic membrane proteins, a peripheral membrane protein, and a substrate binding protein located in the periplasmic space between the outer and inner membranes. The *hisP* and *malK* subunits of the histidine and maltose transport systems, respectively, each contain a hydrophilic sequence and bind ATP at a consensus nucleotide binding region (35-37). Sequence homology between permeases and P-glycoprotein lies within, but is not limited to, the hydrophilic region of these subunits. The extent of this homology is not seen between P-glycoprotein and other nucleotide binding proteins. For example, a consensus sequence seen in $pp60^{src}$, EGF-R, and CAMP dependent kinases (38) is not present in P-glycoprotein. However, the functional significance of similarities between nucleotide binding proteins extending over stretches of only 30 or so amino acids has been questioned (38).

Functional Similarities - P-glycoprotein and the bacterial permeases also have other functional properties in common. Interaction of the substrate binding protein with the two membrane bound proteins, and with ATP bound to its binding protein, is required for active transport of the substrate into the bacterial cell. This complex then assembles into a pore, similar to the structure suggested for P-glycoprotein based on hydropathy plots. Although P-glycoprotein is a single protein, not a multi-component system like the permeases, it too can bind ATP analogues and substrates (39). The genes which code for the permease proteins are closely linked on the same chromosome, and in some cases function as an operon. Through evolution then, it is possible that this multi-gene unit

may have become a single gene encompassing functions of all the subunits and coding for P-glycoprotein.

Although bacterial permeases exhibit substrate binding specificity, the P-glycoprotein binds a number of unrelated compounds. To account for this difference, one could postulate the presence of a carrier protein which binds drugs and presents them to P-glycoprotein. There is no direct evidence to support the existence of such accessory proteins in MDR cells. Moreover, studies using labelled vinblastine have demonstrated direct interaction between the drug and P-glycoprotein; many other drugs bind directly to P-glycoprotein as well since they compete for binding of vinblastine. Alternative explanations for low substrate specificity and cross-resistance include P-glycoprotein heterogeneity, possibly through glycosylation, or differential expression of related P-glycoprotein species.

P-glycoprotein exhibits an even higher degree of similarity to the *E. coli* transport system which extrudes hemolysin, than to the bacterial permeases. Best-fit sequence analysis reveals an almost 60% homology between the amino acid sequence of the *hlyB* gene of the transporter and P-glycoprotein (9). This homology extends over a large area which includes the nucleotide binding domain of P-glycoprotein, as well as portions of its hydrophobic regions. Unlike bacterial permeases, the hemolysin transporter exports its substrate from the cell, instead of bringing it in through the periplasmic space, and thus operates on substrates in the same vectorial direction as P-glycoprotein.

The bacterial hemolysin system is a four component complex composed of: (i) *hlyA*, which encodes the alpha hemolysin molecule; (ii) *hlyC*, which encodes a cytoplasmic protein; and (iii) *hlyB* and *hlyD*, each of which code for membrane proteins that secrete alpha hemolysin (40). Although hemolysin is not a tandemly duplicated protein like P-glycoprotein, hydropathy plots suggest that the hydrophobic region of the hemolysin complex is arranged as six transmembrane segments, in a similar manner to the six regions found in each of the two hydrophobic domains of P-glycoprotein (9).

ATP AND SUBSTRATE BINDING SITE

ATP Binding Site - The bacterial *pstB* gene, which encodes one of the four subunits of the phosphate specific transport system, has an ATP binding site in which a specific glycine and lysine are the key residues. Point mutations at these amino acid sites result in a loss of phosphate transport through the pst system, indicating that the nucleotide binding site on the pst protein is responsible for energy coupling and is required for transport (41). Similarly, site-directed mutagenesis of the lysine residues in both ATP binding sites in *mdr1* transfected cells resulted in complete loss of the MDR phenotype (42). The importance of the ATP binding site was analyzed by amino acid replacement at lysine; this operation does not affect the binding of the ATP analog 8-azido ATP, but the modified protein fails to confer resistance to cells (43). This result suggests that ATP binding *per se* is not sufficient to activate P-glycoprotein, but that subsequent steps (probably hydrolysis, see below) are also needed.

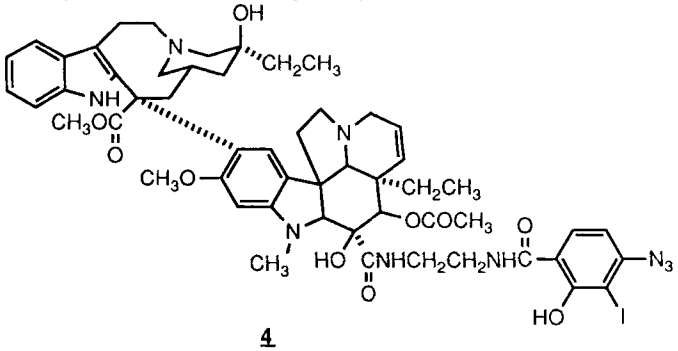
The two putative ATP-binding sites of P-glycoprotein are in the cytoplasmic domain of the protein (13). Several additional studies indicate that these ATP sites are functionally important. Using partially purified membranes from sensitive and multidrug resistant KB carcinoma cells it was shown that the vesicles from MDR cells accumulate ^{3}H-Vinblastine in an ATP-dependent manner while those from sensitive cells do not (44). Vanadate, an ATPase inhibitor, noncompetitively inhibited the transport of vinblastine suggesting that the hydrolysis of ATP is required for transport. Energy dependent transport is also inhibited by daunomycin, actinomycin D, vincristine, verapamil, and quinidine, each of which would be expected to be a competitive inhibitor. Furthermore, kinetic analysis of vinblastine uptake by vesicles indicates that one ATP site is involved in vinblastine transport. These findings are difficult to reconcile with the existence of two ATP binding sites, so further work will be necessary to define the precise roles of these loci. Adenosine 5'-[β,γ-imido] triphosphate, a nonhydrolysable analogue of ATP, could not be substituted for ATP in a transport assay, but is a competitive inhibitor suggesting that ATP hydrolysis is needed for transport (44). Verapamil induces ATP consumption in MDR cells, while failing to do so in sensitive cells, providing further evidence that energy expenditure may be related to the maintenance of the resistance level (45).

The nucleotide binding properties of P-glycoprotein have been assessed by using the labelled photoaffinity reagent 8-azido ATP, an analogue of ATP (39). This reagent, previously used

to study the nucleotide binding properties of hisP (46), bound to P-glycoprotein and competed with both ATP and GTP. Vinblastine, however, did not inhibit this binding showing that vinblastine and ATP bind at different sites.

Mg-dependent ATPase activity was found in P-glycoprotein immunoaffinity purified from adriamycin-resistant human myelogenic leukemia K562 cells (47). GTP and ADP were also hydrolysed by this partially purified preparation but less efficiently than ATP. Verapamil and trifluoperazine, reagents that reverse drug resistance, also increased the ATPase activity of P-glycoprotein. *In situ* in a cancer cell verapamil might serve as a competitive inhibitor of chemotherapeutic agent transport, or could modify the function of P-glycoprotein allosterically, thereby modulating the activity of the pump. None of the experiments reported to date clearly demonstrate that drug efflux and ATPase activity of P-glycoprotein are indispensably linked.

Substrate Binding Site - In addition to an ATP binding site, P-glycoprotein also has a locus for drug binding. Photoaffinity labelling with analogues of vinblastine (**4**) specifically labelled a 150-180 kDa protein in multidrug resistant Chinese hamster lung DC-3F cells (48). The binding was not detected in drug sensitive cells and was inhibited by vinblastine. Using immunological methods, the labelled protein was later identified to be P-glycoprotein (49), and more details on the exact nature of the binding site are expected to be forthcoming from photolabelling studies.



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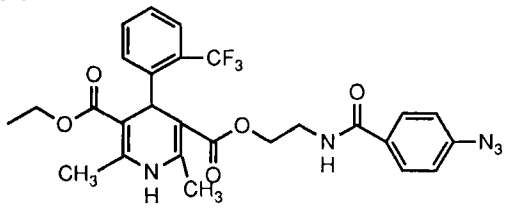
In equilibrium binding studies membrane vesicles isolated from KB 3-1 cells resistant to colchicine bound 8-fold more ^{3}H-vinblastine than sensitive cells (50). This ability to bind ^{3}H-vinblastine was lost in the presence of verapamil. Binding of ^{3}H-vinblastine was also inhibited by unlabelled vinblastine, vincristine and daunomycin, but was not affected by colchicine or actinomycin D. This raises the possibility either that the substrate binding site has several subdomains for different drugs, or that there is more than one distinct substrate site. Tubulin, which is a component of both the mitotic spindle and the cytoskeleton, is also a functional site in cells for vinca alkaloid binding. Binding of vincristine and vinblastine to tubulin is not sensitive to verapamil treatment, so reversal of vinca alkaloid resistance is not likely to be due to target alteration (51).

The binding sites of P-glycoprotein were studied using the photoactive calcium channel antagonist azidopine (**5**) (52). P-glycoprotein was labelled with azidopine, digested with proteolytic enzymes and the fragments characterized by using antibodies directed to P-glycoprotein. The results indicated two binding sites for azidopine, one in the amino half and the other in the carboxyl terminus, of the P-glycoprotein. The functional relationship of these sites is not known but it is plausible that two homologous halves come together to form a single binding site for azidopine in the native protein structure.



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Most of the drugs that reverse multidrug resistance, except chlorpromazine and trifluoperazine, are found to inhibit photoaffinity labelling of P-glycoprotein at the site where azidopine binds (53). Thus, it has been suggested that inhibition of photoaffinity labelling may be an efficient tool to screen compounds that modulate multidrug resistance. However, bleomycin, an antitumor agent that does not belong to the group of drugs in the multidrug resistance family, stimulated the binding of ^{3}H-azidopine (54), so it is difficult at this point to make straightforward correlations between MDR reversal and P-glycoprotein interaction with azidopine. It should also be pointed out that there is no published evidence on a relationship between P-glycoprotein and voltage dependent calcium channels but the six transmembrane structural motif is present in both proteins (see Chapter 24) and the affinity of azidopine is higher towards the authentic calcium channel than towards P-glycoprotein.

A racemic mixture of verapamil isomers reverses chloroquine resistance (55) in malarial parasites and the (+) isomer of verapamil reverses chloroquine resistance without binding to calcium channels. Such chloroquine resistant cells bind ^{3}H-azidopine to a protein of molecular weight 155-170 kDa and the binding is inhibited by chloroquine and azidopine, but not vinblastine (56). Since this pattern is somewhat different than mammalian MDR cells, the exact degree of structural and functional homology between P-glycoproteins of different organisms remains to be established.

SIMILARITIES WITH OTHER PROTEINS

Bacterial transporters are not the only proteins which share similarity to P-glycoprotein; extensive sequence and topographical identity is also observed in a series of eucaryotic proteins (Table 1). Members of this superfamily of transport proteins have been identified by two methods: (1) oligonucleotides of conserved sequences of the human *mdr1* gene have been used to screen genomic and cDNA libraries for P-glycoprotein analogues; and (2) the deduced amino acid sequence of P-glycoprotein has been compared with other sequences in computer databases.

Table 1: Proteins with Similarity to P-Glycoprotein[a]

PROTEIN	FUNCTION
HylB, HylD	hemolysin export
HisP	histidine uptake
MalK	maltose uptake
OppD, OppF	oligopeptide transport
PstB	phosphate trasnport
STE6	**a** factor pheromone export
brown locus	pigment transport
CFTR	Cl^- channel
adenylyl cyclase	synthesize cAMP

[a]Each of the proteins listed has sequence or structural similarity to mammalian P-glycoprotein, and contains both the consensus nucleotide binding sequence in a hydrophilic domain and at least one hydrophobic stretch predicted to span the membrane six times by hydropathy plots. The first five are bacterial transport proteins, STE6 is from yeast, brown locus from Drosophila, and the final two are mammalian. The Table combines information from references 9-11, 57-59, and 61-62.

Homologs of the mammalian P-glycoprotein have been isolated in the unicellular organisms *Plasmodium falciparum* (57,58) and *Saccharomyces cerevisiae* (59). Using oligonucleotides corresponding to the highly conserved nucleotide binding site of mammalian *mdr1*, three major clones were isolated from *P. falciparum*. In one case, a full length sequence was obtained and encoded a protein of 162,000 kDa (57). Approximately 50% of the amino acid residues were conserved between the proteins of the two species. The predicted tertiary structure indicated the familiar P-glycoprotein motif of two tandemly duplicated molecules, each consisting of six hydrophobic regions. The gene is amplified in some chloroquine-resistant lines suggesting that the *P. falciparum* P-glycoprotein may mediate chloroquine resistance (58). Thus the malarial parasite protein appears to share both structural and functional homology with its mammalian counterpart, strengthening the impetus to search for drugs which antagonize P-glycoprotein since these may have relevance in two of humankinds most common diseases, cancer and parasitic infections.

A P-glycoprotein homolog in *S. cerevisiae* has been identified as a product of the STE6 gene (59). Approximately 57% of the amino acid sequence is identical or conserved between the

proteins in the two species and the similarity profile extends along the entire sequence. Like the human P-glycoprotein, the yeast protein is tandemly duplicated, and each dimer is composed of six putative transmembrane domains and a consensus sequence for a potential ATP binding site. Interestingly, the yeast STE6 gene product does not appear to function as a drug resistance protein, even in yeast cells selected for cytotoxic drug resistance. Rather the STE6 protein is active in the export of the hydrophobic lipopeptide, a factor pheromone, whose loss leads to sterility. Thus, although functional homology to P-glycoprotein as a membrane associated transporter is conserved, the uses to which the cell puts such a function can be quite different.

A variation in the direction of transport is observed with another non-mammalian system, *Drosophila melanogaster*, where a P-glycoprotein homolog has been identified (60). It is proposed that the *D. melanogaster* brown and white proteins function as a heterodimer in the import of pteridine pigment precursors into cells. The membrane spatial orientation of this complex is probably an inversion of the P-glycoprotein structure, but the two components join together to form the structural complex analogous to P-glycoprotein.

Finally, two mammalian proteins related to P-glycoprotein, but having no obvious relationship to drug resistance, have been identified. Both adenylyl cyclase (61) and the cystic fibrosis transmembrane conductance regulator (CFTR) (62) share topographical identity to P-glycoprotein. Their predicted structures consist of two repeated domains, each spanning the membrane six times, with a sequence in each domain consisting of a nucleotide binding consensus site. In both cases however identity at the amino acid level is modest. The proposed structure of adenylyl cyclase suggests a potential transport role for this enzyme, possibly in the export of cyclic AMP. Such a transport function of adenylyl cyclase has not been previously suggested by biochemical studies and the authors speculate that the sequence information indicates "possible unappreciated functions of the protein". Conversely, the CFTR is thought to transport material, particularly anions, across the lipid bilayer. Most workers have thought that the function of the CFTR was to serve as a Cl^- channel, rather than a pump. Since a channel does not ordinarily require ATP hydrolysis, it is not clear why the CFTR needs to have a nucleotide binding site. Another property shared by the CFTR and MDR P-glycoprotein is phosphorylation by protein kinase C. Both proteins contain potential substrate sites for this kinase, and as previously described, P-glycoprotein is phosphorylated on serine and threonine residues by the enzyme. In contrast to the MDR P-glycoprotein, the two halves of the CFTR protein do not share sequence identity. Consequently, it is unlikely that the two domains evolved from exon duplication.

CONCLUDING REMARKS

It seems quite clear that a great deal can be learned about protein structure from analysis of predicted or actual amino acid sequences. Such structural information can in turn be used to make inferences about biologic function. It will be enlightening to discover how accurately the structure of P-glycoprotein has been depicted when appropriate molecular structure techniques are applied to the purified protein. Are the transmembrane domains rigidly fixed in the bilayer and does the superfamily of P-glycoprotein-like structures share similarities beyond the merely organizational? Equally intriguing will be an assessment of how correct were the proposals about function when we finally understand the deepest secrets of P-glycoprotein action. Do both proposed nucleotide binding sites simultaneously bind ATP and couple its hydrolysis to drug export? Can the affinity of P-glycoprotein for drug molecules compete with other pharmacologic sites in a way that actually explains the transport anomalies seen in MDR? How does a cell surface protein extrude a drug such as actinomycin or adriamycin whose primary intracellular location is the nucleus? What structural features explain the specificity of P-glycoprotein to recognize and transport a variety of substrates, while excluding interactions with others? One expects fairly rapid answers to these questions because of the sheer number of groups interested in the problem, and it is evident that rapid answers will satisfy not only our intellectual curiosity, but also our ambitions to provide assistance to cancer patients in the form of effective therapy.

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SECTION VI. TOPICS IN CHEMISTRY AND DRUG DESIGN

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Chapter 28. New Directions in Positron Emission Tomography-Part II

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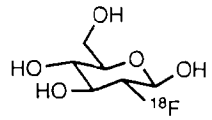
Introduction - Positron Emission Tomography (PET) is a unique tracer method which can be used to measure the spatial and temporal concentration of radioactivity in a volume element of tissue in a living system (1). Because most PET radiotracers are labeled with "physiological isotopes" such as carbon-11 ($t_{1/2}$: 20.4 min), fluorine-18 ($t_{1/2}$: 110 min), oxygen-15 ($t_{1/2}$: 2 min) and nitrogen-13 ($t_{1/2}$: 10 min), there is great chemical flexibility in designing and synthesizing such materials to probe specific biochemical transformations. The method is exquisitely suited to the study of organic molecules such as therapeutic drugs and substances of abuse whose **intrinsic** spatial and temporal distribution are of scientific and clinical interest. The research potential of PET has hardly been tapped by the pharmaceutical industry. PET can be extremely valuable in the quantitative determination of the biodistribution of drugs in man and their short term pharmacokinetics in any body tissue. No other technology is capable of answering these questions as quickly, as safely and as precisely as PET.

Part I of this review described the PET method and its recent use in studies of the brain, metabolism, heart disease and tumors (1). This chapter covers PET studies with therapeutic drugs and substances of abuse, and the rapidly emerging use of PET in clinical diagnosis.

PET STUDIES OF THERAPEUTIC DRUGS AND SUBSTANCES OF ABUSE

Drug Effects on Metabolism and Neurotransmitter Activity - PET has been most widely applied to the study of CNS drugs. The 2-deoxy-2-[^{18}F]fluoro-D-glucose (**1**, ^{18}FDG) (and also [1-^{11}C]2-deoxy-D-glucose (**2**, ^{11}C-2DG)) method is commonly used to measure regional brain glucose metabolism and the oxygen-15 water method to measure regional brain blood flow (2,3). These tracers have been used to study the effects of acute as well as chronic drug administration on regional brain metabolism and blood flow. For example, the influence of neuroleptics (4,5), amphetamine (6), alcohol (7-10), azidothymidine (11,12), barbiturates (13) and cocaine (14-16) on glucose metabolism have all been measured. In one particularly dramatic example, regional brain glucose metabolism in brain was found to parallel neurologic and immunologic improvement associated with azidothymidine treatment, demonstrating the ability of PET to provide a direct measure of drug response (11,12).



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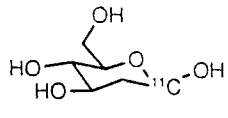

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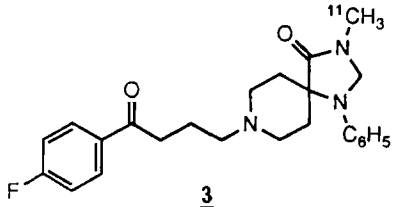
PET has also been used to study the neurochemical effects of substances of abuse such as cocaine. Acute doses of cocaine (40 mg) had no effect on D_2 receptor availability as measured by [^{11}C]N-methylspiroperidol (**3**) (17) whereas chronic cocaine produced a down-regulation of D_2 receptors (18). Dopamine metabolism, as probed by 6-[^{18}F]fluoro-L-DOPA (**4**) is also reduced in chronic cocaine abusers (16). These PET studies along with more classical methods strongly implicate the dopaminergic system in the reinforcing and addicting properties of cocaine (19).



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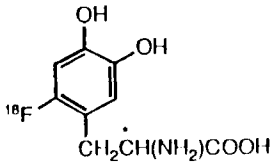

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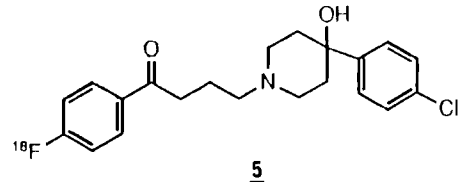
PET studies of cocaine abusers have also revealed significant derangements in brain blood flow as measured by oxygen-15 water, an observation probably related to the drug's vasoactive properties which can lead to strokes and hemmorhage (20).

Drug Binding Sites and Pharmacokinetics - PET has proven useful in identifying and characterizing drug binding sites, and in examining the relationship between the binding and kinetics of a drug and its behavioral effects in a quantitative manner. For example, the regional distribution and pharmacokinetics as well as the binding sites for cocaine have been determined using carbon-11 labeled cocaine in human and baboon brain (21). Cocaine is rapidly taken up and cleared from the striatum. Pretreatment with the dopamine reuptake inhibitor, nomifensine, significantly reduced binding in this region, thus implicating the dopamine reuptake site as the locus of cocaine's action. Moreover, the time course in the striatum parallels the temporal pattern of presentation of the "high" experienced after iv use of cocaine (22). In this case, the use of the labeled drug (cocaine) itself allows the neurochemical profile for binding and kinetics to be related to its behavioral action. PET permits, through a direct observation of drug binding, the evaluation of therapeutic interventions which could counteract the reinforcing properties of cocaine. The same strategies can be applied to other drugs of abuse.

The binding sites for haloperidol, the most widely used antipsychotic drug, have been determined using [^{18}F]haloperidol (**5**) and PET (23). While it is known that haloperidol binds to dopamine D_2 receptors, the distribution of [^{18}F]haloperidol in the brain is widespread and includes the cerebellum which is devoid of D_2 receptors. Thus, other binding sites, such as the sigma receptor as well as non-receptor sites, may be partially responsible for the pharmacological profile of the drug (24).



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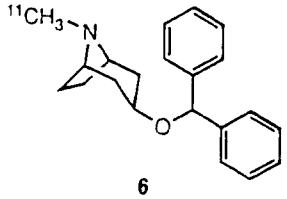
Carbon-11 labeled cogentin (**6**) has been used to map the binding sites for this anticholinergic drug in human and baboon brain (25). Drug distribution in brain parallels the distribution of cholinergic receptors. Even though this drug is known to bind to the dopamine reuptake site, its binding is not altered by the dopamine reuptake inhibitor,



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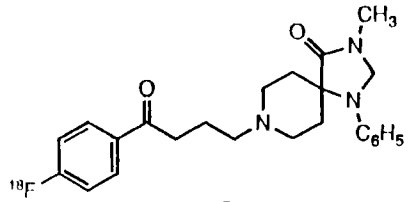
nomifensine. Interactions between the cholinergic and the dopaminergic systems are currently being probed using ^{18}F-N-methylspiroperidol (^{18}F-NMS) and [^{11}C]cogentin (26).

In Vivo Radioreceptor Assay - Plasma drug levels are commonly taken as a measure of the amount of drug binding to target receptors. PET and radiotracers which selectively probe a particular neurotransmitter receptor can be used to examine this relationship directly. For example, the question of whether receptor occupancy by haloperidol parallels the concentration of drug in the plasma was addressed with PET using a serial study protocol with [^{18}F]N-methylspiroperidol (^{18}F-NMS, **7**) (using the ratio index as a measure of occupancy) (27). It was found that receptor occupancy increased with increasing plasma concentrations of the drug, leveling off at the 5-15 ng/ml, the initial phase of therapeutic levels for haloperidol. However, at higher plasma drug concentrations there was no further increase in receptor occupancy (28). This finding supports a growing clinical consensus that there is little added benefit in increasing plasma haloperidol levels above 20 ng/cc. Similar results were obtained with [^{76}Br]bromospiperone (29). Serial PET studies have also been used to measure the clearance time of antipsychotic drugs from the D_2 receptor (or the return of receptor availability to normal values by some other mechanism such as receptor synthesis). D2 receptor availability approaches normal values after a 1 week drug free interval (27,28) However another PET study using [^{11}C]raclopride (**8**) as the tracer showed a relatively constant, high receptor occupancy for many hours after the withdrawal of sulpiride or haloperidol (30).



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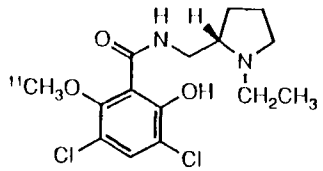

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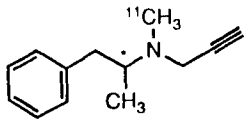
^{18}F-NMS has been used to address the question of whether schizophrenic patients who respond to antipyschotic drugs have a higher receptor occupancy by drug than nonresponders (31). It was found that receptor blockade by haloperidol occurs to the same extent in responders and non-responders. Thus the treatment of non-responders with high neuroleptic doses in order to increase receptor occupancy is not warranted.

While the studies described above use PET to measure receptor availability, absolute receptor density (Bmax) has also been measured using this technology. Two different PET methods have been used to measure Bmax for D2 receptors in schizophrenia. An elevation in Bmax has been observed in post mortem brain tissue from individuals with schizophrenia, supporting the hypothesis that an overactivity of dopamine may play a role in this disease (32). When PET was used to measure Bmax in vivo, one group of investigators reported a significant elevation in Bmax (using [11C]]N-methylspiroperidol as a tracer) (33) and another group reported no deviation from normal values (using [11C]raclopride) (34). The resolution of this apparent discrepancy is now being addressed by a detailed examination of the behavior of the tracers and the models used to calculate receptor density, as well as the patient population (35). One possible explanation for the results of the measurements with [11C]raclopride is that competition of intrasynaptic dopamine for the labeled raclopride may be significant since dopamine and raclopride have similar Kd's (36). The current controversy over what constitutes an accurate measurement of Bmax for the D2 receptor illustrates the complexities that can arise in PET work, but is also an example of where solutions can and will be found.

Drug Interactions - The effects of drug combinations on the pharmacokinetics of a specific drug is a relevant question in drug research. For example, neuroleptic drugs are frequently given in combination with anticholinergic drugs to prevent or alleviate extrapyramidal symptoms. PET is now being used to examine the effects of anticholinergic drugs such as cogentin on the dopamine D_2 receptor (26). Future work in this area may provide detailed information on the neurochemical consequences of drug interactions and offer scientific criteria for the use of drug combinations.

Duration of Drug Action - The duration of action of drugs directly at their target sites has been measured with PET. L-Deprenyl is a monoamine oxidase B (MAO B) selective suicide enzyme inactivator which has been shown to alter the natural history of Parkinson's disease (37). Carbon-11 labeled L-deprenyl (**9**) acts as a tracer for MAO B activity in the brain (38). The duration of MAO B inhibition after a single dose of L-deprenyl has been measured in baboon brain over a 111 day time interval using **9** as a tracer (39). The recovery of the enzyme activity in vivo is remarkably slow with a half life of 30 days. Since (-)-deprenyl presumably acts by covalent attachment to MAO which results in the destruction of the enzyme, the recovery of MAO activity is related to the synthesis of enzyme.



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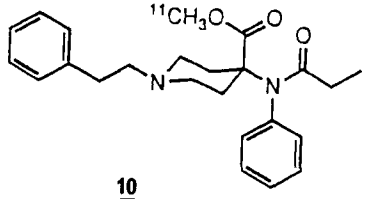

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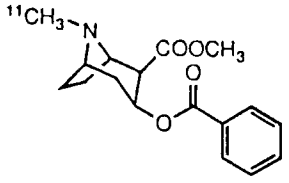
The duration of occupancy of mu-opiate receptors by naltrexone, an orally administered opiate antagonist has been measured using the mu-specific PET ligand, [^{11}C]carfentanil (**10**), and an external detector. The half-time of blockade by naltrexone in the brain ranged from 72 to 108 hours which corresponds to the half-time of the terminal plasma phase of naltrexone clearance (40).

These studies, as well as the in vivo radioreceptor assays described above illustrate the power of in vivo imaging and detection, not only to probe the target sites for a drug, but also the duration of its pharmacological action. They may be of particular

importance in the case of drugs which act by irreversibly inhibiting an enzyme or other biological substrate.

Brain Uptake of Drugs - A drug's ability to cross the blood-brain barrier, as well as its absolute uptake and time course are important properties which are difficult to determine in vivo. However, PET is an excellent method for rapid measurement of brain uptake. PET studies of the stereoselectivity of cocaine binding provide a simple example (41). (-)-Cocaine is the behaviorally active natural product: behavioral research with (+)-cocaine showed low or no behavioral activity in monkeys (42). However, comparative PET studies with carbon-11 labeled (-)- and (+)-cocaine (**11** and **12**) showed that the (+)-enantiomer is not taken up in the brain, a very straightforward explanation of its behavioral impotency but one which had not been previously considered (42). Following this observation, it was shown that the (+)-enantiomer is metabolized in plasma so rapidly that there is essentially nothing available to cross the blood-brain-barrier (41). Thus biological activity of the (+)-enantiomer can only be assessed through direct administration assuming that brain metabolism of the compound is negligible.



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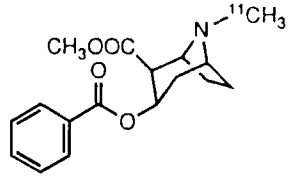

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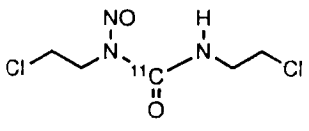
The influence of factors such as diet on brain uptake of therapeutic drugs has also been addressed with PET. The tracer for dopamine metabolism, 6-[^{18}F]fluoroDOPA, was used to determine whether dietary amino acids can interfere with brain uptake (and therapeutic actions) of L-DOPA in Parkinson's disease. The study concluded that a two to three-fold rise in plasma concentration of large neutral amino acids (phenylalanine, tyrosine, tryptophan, leucine, isoleucine, methionine, valine, and histidine) is sufficient to significantly compete with brain uptake of drug (43).

Drug Delivery to Target Tissue - PET has been used to directly compare intravenous versus intraarterial delivery of chemotherapeutic agents [carbon-11 labeled 1,3-bis-(2-chloroethyl)-1-nitrosourea (**13**) and nitrogen-13 labeled cisplatin (**14**)] to malignant brain tumors (44,45). The drug delivery advantage of intraarterial chemotherapy was demonstrated and, in the case of **13**, there are indications that this method may be of value in predicting clinical response. The accumulation of fluorine-18 labeled 5-fluorouracil (**15**) in colorectal carcinoma was measured with PET in order to assess the effect of blood flow (as measured with O-15 labeled water) on its delivery to tumor (46). This study demonstrated that PET can be used to identify patients with low 5-fluorouracil uptake who will probably not respond to standard chemotherapy with this agent.



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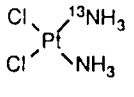

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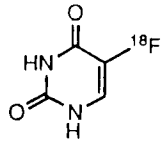


15

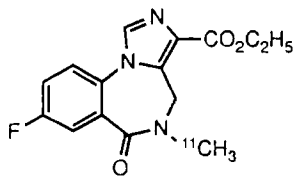
CLINICAL PET

Clinical PET is just now beginning to emerge as a medical specialty. One can base a clinical PET program purely on Rb-82, ^{18}FDG, the oxygen-15 labeled tracers, nitrogen-13 ammonia and fluorine-18 labeled fluoromethane (47,48). These materials are easily prepared with automated or robot controlled equipment and can be produced on demand. The many other labeled probes under study today will be synthesized and used routinely once their efficacy has been clearly demonstrated. PET research has its niche in the development of new probes for the study of basic problems in physiology, human biochemistry and medicine.

A number of unique clinical diagnostic applications have emerged from PET research. These include the identification of salvageable myocardium, the location of an epileptic focus for surgical removal and the differentiation of recurrent brain tumor from radiation necrosis (49-52).

Coronary Artery Disease and Salvageable Myocardial Tissue - The patterns of metabolism in the human heart have proven to be powerful aids in the diagnosis of heart disease (53). PET can be used to identify patients with coronary artery disease with 95 % sensitivity. PET has much higher specificity and sensitivity than thallium imaging in determining coronary artery stenosis, myocardial ischemia and viability, and can minimize the need for invasive procedures such as coronary angiography (54). Myocardial perfusion is most commonly measured with generator produced rubidium-82 (half-life = 75 seconds), or cyclotron-produced nitrogen-13 ammonia, or oxygen-15 water. These tracers are used in a rest-stress serial study protocol. With sequential PET maps of regional myocardial perfusion and regional myocardial glucose metabolism (with ^{18}FDG), the viability of the myocardium can be determined. This information has been a valuable predictor of the outcome of coronary bypass surgery (55). More recently, [^{11}C]acetate has been used to provide an estimate of myocardial oxygen consumption; this method should facilitate the evaluation of therapeutic interventions designed to enhance the recovery of jeopardized myocardium (56). Newer probes for other aspects of myocardial metabolism have been described in Part I (1).

Epilepsy - PET and ^{18}FDG have been used successfully to locate the epileptic focus in patients whose seizures are inadequately controlled by medication and for whom surgery is anticipated (57-59). Since CT (x-ray computed tomography) and NMRI (nuclear magnetic resonance imaging) reveal no anatomical abnormalities in most of these patients, the location of the focus for surgery has required the placement of depth electrodes which in itself is accompanied by risks from the surgical procedure. ^{18}FDG images have been used to guide surgeons in the location and extent of the areas to be resected with very good preliminary results. In a recent report, the accuracy of the location of an epileptic focus with PET was increased when [^{11}C]carfentanil (**10**), a radiotracer specific for the mu opiate system, was used in conjunction with ^{18}FDG (60). In the future, the use of (**10**) as well as other neurochemical probes such as [^{11}C]RO 15-1788 (**16**) may be of value in identifying subpopulations of patients which may respond to specific drugs (61). Additionally, this specific neurochemical information may help limit the size of the resection.



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Brain Tumors - Newer tracers for probing specific aspects of tumor metabolism were described in Part I (1). To date, the major clinical application of PET has been in grading brain tumors and in differentiating recurrent tumor from treatment-related necrosis using ^{18}FDG as a tracer for glucose metabolism (62,63). However, the ability to relate ^{18}FDG uptake or metabolism to tumor grade has been recently questioned (64-66). There is also a recent report that the lumped constant (a constant which takes into account the different affinities of 2-deoxy-D-glucose and glucose for transport across the blood brain barrier and phosphorylation by hexokinase) in glioma differs significantly from that in normal brain tissue (67) in the rat. If this is also the case in human glioma, then calculation of the value of the tumor metabolic rate is directly affected. This is clearly a complex issue whose resolution could optimize the use of PET in the clinical management of cerebral malignancy. The value of PET in early detection and staging of different types of cancer is currently being investigated in a number of PET centers.

Emerging Applications - Other applications with diagnostic potential are emerging. With PET, it is possible to directly correlate cognitive function, metabolism and structural integrity in the brain. In Alzheimer's Disease, PET has been used to identify characteristic deficits in brain metabolism which, in certain cases, have been shown to precede clinical presentation (68). In the future, when the metabolic abnormality is better characterized, PET may be used to differentiate normal aging from early Alzheimer's Disease. Also, PET can be useful in differentiating among the different types of dementias. Similarly, in neurodegenerative disorders such as Parkinson's disease, 6-[^{18}F]fluoro-L-DOPA (4), the previously mentioned probe for dopamine metabolism, is providing valuable new information (69). In Huntington's disease, PET complements clinical symptomology in establishing an early diagnosis (70). For example, a strong positive relationship was found between neuropsychological measures of verbal learning and memory and caudate metabolism, as measured by 18FDG (71). This was not found in the normal control group.

PET has also been used to study psychiatric diseases such as schizophrenia, affective disorders, obsessive compulsive disorders, anxiety disorders, violence and substance abuse (72,73). Using PET and the 18FDG method, it now appears that bipolar and unipolar depression are two different disorders (73). Clinical research in psychiatry has been limited to the evaluation of brain pathology in difficult-to-diagnose patients. Although there still is no PET procedure of proven diagnostic or prognostic utility in psychiatric illness, this initial work is starting to identify abnormal patterns of brain metabolism and neurotransmitter activity associated with the different diseases. Future work in the characterization of the neurochemical and functional brain pathology of mental diseases could enhance the development of better therapeutic interventions.

Other organs such as the lung are amenable to study with PET although the potential of this particular application is largely untapped. For example, pulmonary blood flow, ventilation, vascular permeability and metabolism are all measurable with PET and appropriate radiotracers (74).

PET is coming of age and entering into routine clinical use. As we have described in this review, PET can be used to probe biochemistry and physiology noninvasively in humans and animals, in both normal and pathological states. It has virtually unexplored potential as an aid to drug design. PET will continue to stimulate basic research in multiple peripheral areas which bodes well for this exciting and growing field.

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Chapter 29. Irreversible Ligands for Drug Receptor Characterization

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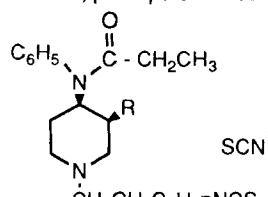
Introduction - The design and synthesis of highly selective affinity labels are critical for progress in the area of receptor pharmacology. These compounds are designed to interact covalently with a specific binding site and are thus tools for the study and characterization of receptors. Irreversible ligands first recognize a specific binding site and then form a covalent bond at or near that recognition site, resulting in irreversible attachment (1). For the purpose of this review, all ligands discussed possess a reactive chemical moiety which is capable of forming a covalent bond. These groups may be intrinsically active and are generally electrophilic in nature, or they may require a photoactivation step that leads to a reactive species. Compounds are classified according to the receptor system with which they interact and are further categorized by reactive functionalities, beginning with electrophiles and ending with the photoactivated agents.

The successful design of highly receptor selective irreversible ligands, containing an electrophilic moiety, is dependent on receptor affinity and selectivity of the ligand, location of the electrophile on the ligand, and the reactivity of the electrophile (1). Electrophilic moieties most commonly used include isothiocyanates, Michael acceptors, haloacetamides, aldol esters and nitrogen mustards. Placement of the electrophilic moiety within the parent ligand generally takes into account ease of chemical synthesis, avoidance of a known site of ligand-receptor interaction, and it is hoped that a nucleophile is on or near the binding site which will be in a position to covalently bond. Because biological tissues possess a multitude of reactive nucleophiles, the opportunity for nonspecific covalent attachment is great.

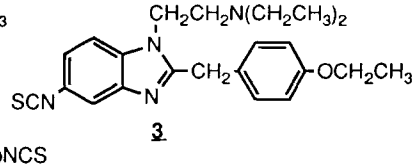
Photoaffinity labels are ligands that have an inherent affinity for a binding site and may function as agonists or antagonists. They contain a photosensitive functional group which, when activated with light, generates an intermediate species which is capable of forming a covalent bond at or near the binding site (2). Unlike electrophilic affinity labels, their association with the recognition site is reversible until photolysis is initiated (2). Due to the high reactivity of the species generated by photolysis, relative to electrophiles, it is generally easier to design a photoaffinity label, since the location of the photoactivated group is not as crucial for covalent attachment to the binding site (1). The chemical mechanism for photoaffinity labeling has been reviewed (3). Caution must be taken to assess the possible effects of irradiation on the tissue being studied (2). For example, UV-radiation damage to the opiate receptor has been reported (4-6). Another limitation of the photoaffinity labels is that they are only useful under conditions that permit photolysis which at the very least eliminates in vivo work.

Opiate Receptors - The CNS opiate receptor subtypes, μ, δ, and κ, have been characterized with several structurally diverse, irreversible ligands. This work has provided insight into the relationship of these subtypes to one another and their physiologic roles (1). The isothiocyanate function has proven to be highly versatile as an electrophilic moiety for irreversible ligands due to its ease of synthesis from primary amines, known rapid reactivity with amino and sulfhydryl groups and low reactivity toward water and hydroxyl functions (7). The fentanyl isothiocyanate FIT, **1**, is a highly selective acylator of δ receptors (8,9). [^{3}H]FIT (10) was first used to identify and partially purify a glycoprotein subunit of the opiate receptor (11). The (+)- and (-)-cis-3-methyl derivatives of FIT were the first enantiomeric pair of irreversible ligands (12). The (+)-enantiomer, SUPERFIT, **2**, is 10 times more potent than **1** in the acylation of δ-receptors and 50 times more potent than its (-)-enantiomer (12). [^{3}H]SUPERFIT labeled the same 58,000-dalton subunit of the opiate receptor as **1** (12) and was used to purify this subunit to homogeneity, as well as to characterize functional coupling of opiate receptors to GTPase (13-15). The trans-isomers of **2** were prepared in order to obtain further insight into the steric requirements of the δ-receptor (16). The benzimidazoleisothiocyanate BIT, **3**, related to the extremely potent opiate agonist etonitazene, is a high affinity irreversible ligand for μ-

receptors (8,9). A new δ-selective ligand, naltrindole-isothiocyanate, **4**, has been reported (17). The first κ-receptor selective irreversible ligand, **5** , is an isothiocyanate-derivative of the more active (1S,2S) enantiomer of the κ-selective compound, U50,488 (**6**) (18). The racemic mixture UPHIT, **7**, and its 1S,2S and 1R,2R-trans-enantiomers are potent displacers of [^{3}H]U69,593 and weak displacers of [^{3}H]bremazocine, and are thus possible probes for identifying κ-receptor subtypes (19). A 300-fold enantioselectivity was demonstrated and the 1S,2S enantiomer of **7** was marginally more potent than **5** in vitro. Racemic **7** was a potent and selective κ-acylator in vivo, whereas **5** was inactive, perhaps because of increased reactivity toward nucleophiles (19,20).



1, R=H
2, R=CH_3



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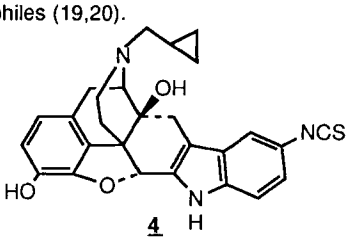

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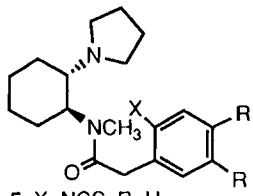
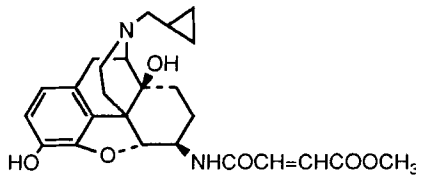


5, X=NCS, R=H
6, X=H, R=Cl
7, X=NCS, R=Cl



8

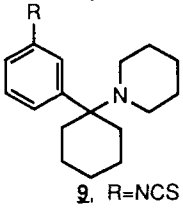
β-FNA, **8**, has been reported to interact reversibly with both κ- and δ-sites, and irreversiblly with μ-sites (21-23). Selective antagonism of μ-receptor-related activities has been observed after systemic administration of **8** (24, for review).

Photoaffinity ligands have not proven to be as useful as electrophilic-type irreversible agents in opiate receptor research, primarily due to the reported UV-inactivation of the receptor (4-6). However, azido-derivatives of the opiate peptides, DAGO and DTLET, have been described as binding to μ- and δ-receptors, respectively (25,26). Recently azido-derivatives of the δ-selective peptide, DPDPE, and μ-selective peptide, CTP, have been synthesized as potential photoaffinity probes (27).

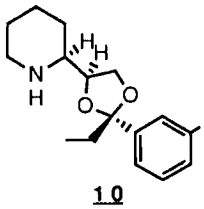
NMDA Receptors/PCP Binding Sites - PCP binding sites appear to be allosterically coupled to the N-methyl-D-aspartate (NMDA)/L-glutamate macromolecular complex, and PCP and related compounds have been shown to block the increases in neuronal discharges induced by NMDA (28,29). The probability of interaction between PCP and NMDA receptors is further supported by the striking similarity in distribution of these receptors in the brain (30). The meta-isothiocyanate derivative of PCP, metaphit, **9**, was the first electrophilic irreversible ligand to selectively label PCP sites in rat brain (31). The metaphit isomer with the isothiocyanate function in the 4-position of the piperidine ring did not irreversibly inhibit [^{3}H]PCP binding, indicating structural specificity for the acylation of these receptors (31). Metaphit caused long term inhibition of PCP-induced stereotypy, in rats (32) but did not antagonize stereotyped behavior induced by the sigma ligands (-)-cyclazocine or (+)-SKF 10,047, suggesting selective acylation of only the PCP sites (33). However, in pigeons and monkeys, **9** induced PCP-like catalepsy, ataxia and convulsions and did not inhibit these PCP-induced behaviors (34,35). In vitro, **9** caused a concentration dependent inhibition of acetylcholine release evoked by NMDA, which paralleled its inhibition of [^{3}H]TCP (thienylcyclohexylpiperidine) binding in striatal tissue and suggests that **9** has long term PCP-like effects (36). Autoradiographic studies reveal that metaphit inhibited most of the PCP-induced increases in glucose metabolism in selected areas of rat cortex (37); microelectrophoresis studies in rat spinal neurones showed a decrease in NMDA-induced excitation by **9** (38). Etoxadrol-meta-isothiocyanate, **10**, is the first chiral electrophilic affinity ligand that displaces [^{3}H]TCP from PCP sites in rat brain, with four times higher affinity than **9** (39).

Photoaffinity labeling of rat brain PCP receptors with [^{3}H]azido-PCP, **11**, revealed five polypeptides which were unevenly distributed in the brain (40,41). Competition studies with [^{3}H]PCP and dexoxadrol suggest that the high affinity PCP site correlates best with the 90,000 dalton peptide (40,41); the 33,000 dalton peptide is suggested to be a low affinity PCP site (41,42).

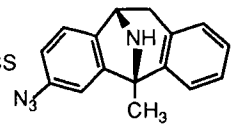
MK 801 potently inhibits binding of [^{3}H]TCP to PCP sites and weakly inhibits binding of [^{3}H]SKF 10,047 to sigma sites (43). (+)-[^{3}H]-7-Azido-MK 801, **12**, displays reversible binding and a pharmacological profile characteristic of interaction with PCP binding sites (44). Photoactivation of **12** in guinea pig membranes resulted in isolation of a single radioactive band of 115,000 daltons. This band was absent from lanes that had been preincubated with PCP which suggests that it is a PCP component of the NMDA-receptor complex (44).



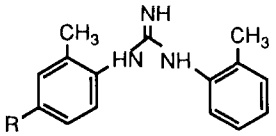
9, R=NCS
11, R=N_3



10



12



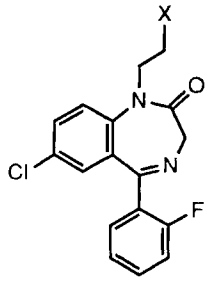
13, R=NCS
14, R=N_3

Sigma Receptors - Many compounds that bind with high affinity to sigma sites exert behavioral effects similar to PCP and bind to PCP sites as well (45). The striking similarities between the actions of the PCP and sigma-drug classes led to the concept that there was only one receptor system involved, however, radioreceptor binding studies and different rank order of potencies of drugs that interact at both sites implicate two distinct drug recognition sites (46). In addition, autoradiography studies show that the anatomical distribution of these sites is very different in rodent brain (47). Metaphit (**9**) was shown to displace [^{3}H]DTG (1,3-di-ortho-tolyl-guanidine) and [^{3}H](+)-3-PPP (3-(3-hydroxyphenyl)-N-(1-propyl)piperidine) from sigma sites in guinea pig brain in an irreversible and competitive manner (48). An isothiocyanato-derivative, DIGIT, **13**, of the selective sigma receptor ligand, DTG, has been prepared (49). Sigma receptors are selectively and irreversibly modified by **13**, whereas PCP receptors are not affected. A radiolabeled azido-derivative of DTG, **14**, recently was used to isolate a 29,000 dalton polypeptide that may represent the intact sigma receptor complex (50).

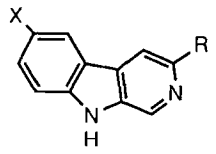
Benzodiazepine Receptors - Central/GABA and Peripheral Types - The benzodiazepine receptors in the CNS are associated with GABA receptors and a chloride ion channel, and are responsible for the mediation of anxiolytic, anticonvulsant and muscle relaxant properties of this class of drugs (51). The first isothiocyanato-benzodiazepine to bind noncompetitively and irreversibly to central benzodiazepine sites was irazepine, **15** (52). When administered intracerebroventricularly to mice, **15** afforded long term protection against pentylenetetrazole-induced seizures accompanied by a decrease in [^{3}H]-diazepam binding in forebrain. (53). The primary amino-precursor of **15** was used to prepare an affinity column for the purification of anti-benzodiazepine antibodies (54). A related bromoacetamide derivative, kenazepine, **16**, appeared to interact reversibly with one population of benzodiazepine receptors and irreversibly with another (7,53). More recently, an isothiocyanato-analog, **17**, of the inverse agonist β-CCE (**18**) has been prepared (55).

When irradiated, the potent centrally active benzodiazepine, flunitrazepam, **19**, irreversibly binds to the benzodiazepine receptor, labeling a protein with Mr 50,000 (56). In contrast to opiate receptors, benzodiazepine receptors are not damaged by irradiation used for photoaffinity labeling (56). Heterogeneity of the benzodiazepine receptors has been reported in subsequent studies using **19** (57,58). [^{3}H]Clonazepam, **20**, can also be photoactivated and binds to benzodiazepine receptors irreversibly, but with 5-fold lower specific activity compared to **19** (59,60). The partial inverse agonist, [^{3}H]Ro 15-4513, **21**, instead of labeling only 25% of the benzodiazepine sites, as **19** does, is reported to photolabel all binding sites (61,62). The first β-carboline photoaffinity ligand, **22**, produces a 42% decrease in maximal binding of β-CCP (3-propyl-β-carboline carboxylate) sites, with essentially no effect on flunitrazepam sites (63). These results provide evidence that the β-carbolines and the benzodiazepines bind to discrete sites on the central benzodiazepine receptor (63).

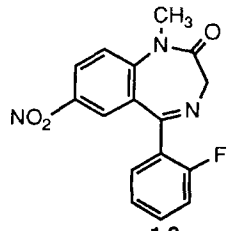
The related GABA-regulated chloride ionophore has been treated with **23** and **24**, derived from the cage convulsant, TBOB (t-butylbicycloorthobenzoate) (64). These compounds are specific, site-directed acylating agents for sites on or near GABA-gated chloride channels labeled by [^{3}H]TBOB and [^{35}S]TBPS (t-butylbicyclophosphorothionate) (64). Compound **23** inhibited binding of these radioligands with an affinity comparable to the parent ligand, whereas **24** was more than one order of magnitude less potent (64).



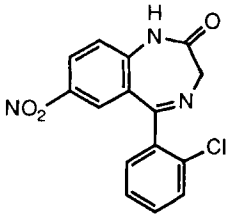
15, X=NCS
16, X=NHCOCH$_2$Br



17, R=NCS, X=H
18, R=$CO_2CH_2CH_3$, X=H
22, R=$CO_2CH_2CH_3$, X=N_3



19



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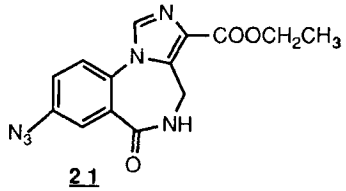

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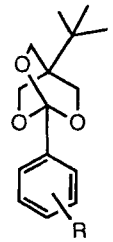
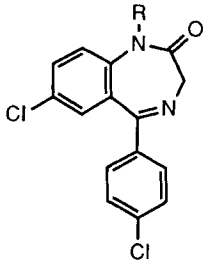
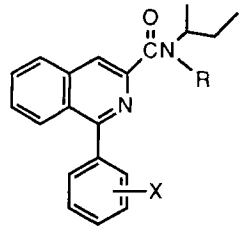


23, m-NCS
24, p-NCS



25, R=CH_3
27, R=CH_2CH_2NCS



26, R=CH_3, X=2'-Cl
28, R=CH_2CH_2NCS, X=2'Cl
29, R=CH_3, X=2'-F, 5'-NO_2

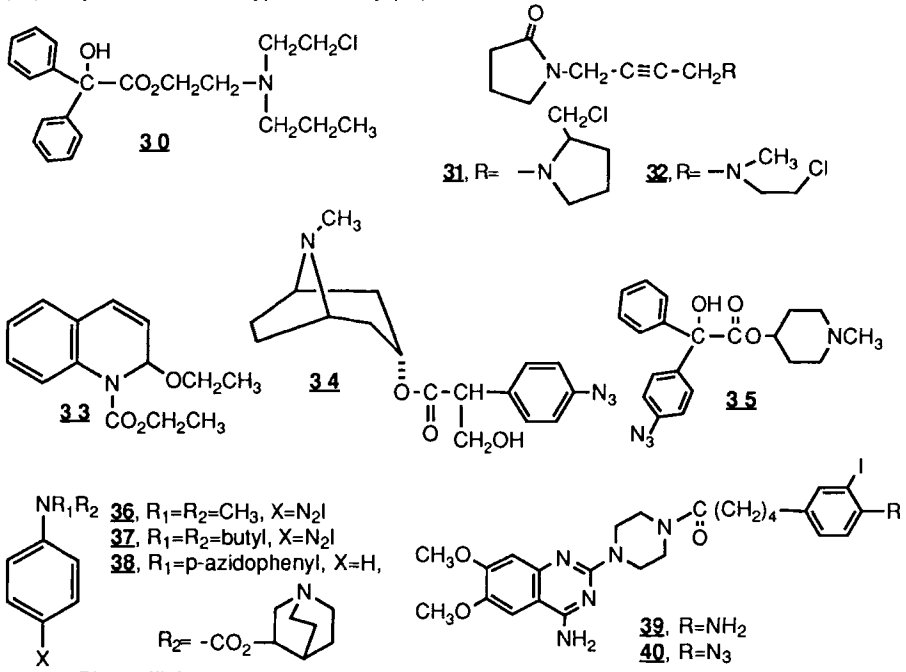
[^{3}H]Muscimol extensively photolabels the $GABA_A$ receptor in rat cerebellar synaptosomal membranes (65). Ultraviolet irradiation did not adversely effect the GABA receptor and the two components isolated were proteins with Mr >250,000 and 52,000 (65). [^{3}H]Muscimol and [^{3}H]flunitrazepam have been used in a large scale purification of the GABA-benzodiazepine receptor protein from rat brain (66). A series of aryl-diazocompounds based on muscimol have also been described as potential photoaffinity ligands for the GABA receptor (67).

In comparison to central type benzodiazepine receptors, very little is known about the function and molecular organization of recognition sites for benzodiazepines found outside the CNS. The benzodiazepine, Ro 5-4864, (**25**) and the isoquinoline, PK 11195, (**26**) have been used to characterize the peripheral type benzodiazepine receptors since they have high affinity and selectivity for these sites and do not possess any of the pharmacological characteristics of classical centrally acting benzodiazepines. The ethylisothiocyanato-derivatives of these compounds, AHN 086, **27**, and AHN 070, **28**, respectively, have proven to be potent, selective and irreversible ligands for these receptors (68,69). Compound **27** has been used to characterize the association between peripheral benzodiazepine receptors and the calcium channels in guinea pig atria and ileal

longitudinal smooth muscle (70), and [^{3}H]AHN 086 has been used to label the peripheral benzodiazepine receptor protein, Mr 30,000, in rat pineal gland (71).

Although peripheral benzodiazepine receptor sites are recognized by flunitrazepam, **19**, they cannot be photoaffinity labeled by this agent (72). However, photoaffinity labeling of the peripheral benzodiazepine receptors has been achieved with PK 14105, **29** (73-78). Photoactivation of **29** results in the covalent coupling of this ligand to an approximately 18,000 dalton protein in cardiac, adrenal, brain, and kidney membranes (73-77). Recently, **29** has been employed to study the distribution of peripheral benzodiazepine sites in immune system organs of rats which suggests a preferential labeling of T- and monocytic cells consistent with a proposed immunomodulatory role for some of these ligands (78).

Muscarinic-Acetylcholine Receptors - Recent uses of the propylbenzilylcholine mustard (PrBCM), **30**, one of the first potent muscarinic irreversible antagonists, include: 1) the selective labeling of muscarinic receptors coupled to phospholipase C or adenylate cyclase in 1321N1 human astrocytoma cells and NG108-15 neuroblastoma x glioma cells, respectively (79); and 2) immunoprecipitation of the affinity-alkylated fragment of the muscarinic receptor with an anti-ligand monoclonal antibody which will allow the immunopurification of the muscarinic receptor that is at or near the ligand binding site (80). Protein-chemical and peptide mapping studies reveal that [^{3}H]PrBCM alkylates an acidic residue in the third membrane helix of the muscarinic receptor (81). A 2-chloromethylpyrrolidine analog **31**, of the muscarinic agent oxotremorine, cyclizes to the reactive aziridinium ion, which alkylates muscarinic receptors (82). The (+)- and (-) enantiomers of **31** both exhibit peripheral and central muscarinic actions and binding affinities, but significantly differ in their rates of alkylation (83). Another oxotremorine analog, **32**, is also susceptible to attack by a nucleophile on or very near the active site of the muscarinic receptor (82-84). Because **32** cyclizes slowly the parent compound crosses the blood brain barrier and alkylates central muscarinic receptors (85,86). Recovery rates of muscarinic receptors from alkylation in cell culture have been studied (87,88). Successful subtype-selective electrophilic affinity labels for the muscarinic receptor have thusfar remained elusive, although the dihydroquinoline **33**, which displays irreversible antagonism at various monoaminergic receptors (89-91) as well as muscarinic receptors (92), may show some subtype selectivity (93).

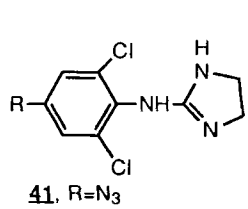


Photoaffinity labeling of the muscarinic receptor (Mr 75,000)has been achieved with **34** (94). A review of the use of **34** and **35** has appeared (95). A variety of new photoaffinity probes, **36-38**, based on N-substituted anilines have been described (96). Comparison of azido-

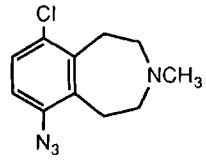
derivatives and diazonium salts has led to speculation regarding the spatial arrangement of these agents at the acetylcholine binding site (96,97).

Adrenergic Receptors - It has been generally accepted that there exist at least two subtypes of mammalian α-adrenergic receptors, specified as α_1 and α_2 (98). [^{125}I]-A44345, **39**, is a selective, high affinity α_1-adrenergic agent which, when cross-linked with a heterobifunctional reagent, is covalently incorporated into the binding site (99). The major peptide labeled by cross-linked-**39**, in rat hepatic membranes, exhibited Mr 82,000, with minor peptides at 50,000 and 40,000 (99). Subsequently, the azido-derivative of **39**, ([^{125}I]APDQ, **40**) was prepared as a photoaffinity probe that displayed high specific radioactivity, and high affinity and selectivity for the α_1-adrenergic receptor in several mammalian and non-mammalian tissues (100,101). Another arylazido-prazosin analog has been used to photoaffinity label α_1-adrenergic receptors in rat heart (102), and in intact DDT_1 MF-2 smooth muscle cells and smooth muscle cell membranes (103).

Difficulties with nonspecific binding by electrophilic irreversible agents, led to the exploration of photoaffinity probes for the α_2-adrenergic receptor. An azido-derivative of clonidine, **41**, binds with high affinity and selectivity to brain α_2-adrenergic receptors (104,105). SKF 102229, **42**, was used to covalently label these binding sites in human platelets and rabbit kidney (106). Although a number of proteins were nonspecifically labeled, a specifically labeled protein with Mr 64,000 was reported to be the α_2-adrenergic receptor (106).



41, R=N_3



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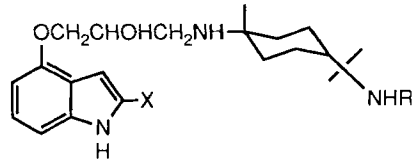
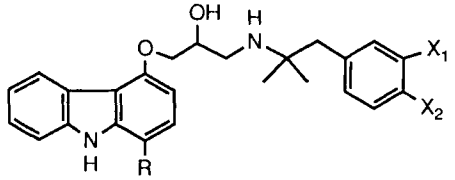
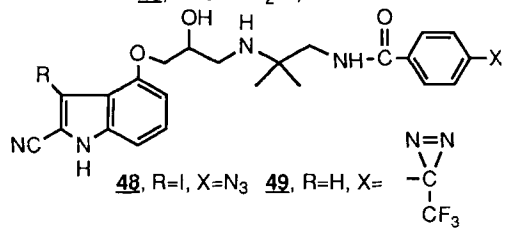


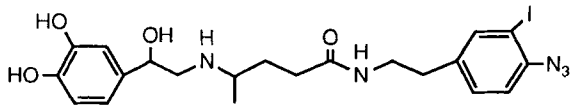
44, R=$COCH_2Br$, X=H
45, R=$COCH_2Br$, X=CN



43, R=I, X_1=H, X_2=$NHCOCH_2Br$
46, R=H, X_1=I, X_2=H
47, R=H, X_1=I, X_2=NH_2
50, R=H, X_1=I, X_2=N_3,



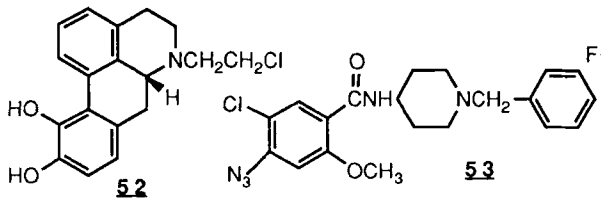
48, R=I, X=N_3 **49**, R=H, X=



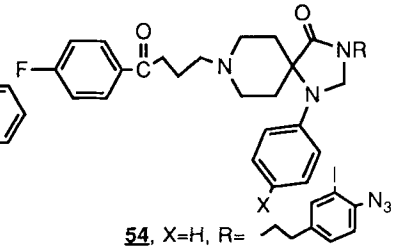
51

Compound **43** binds with high but equal affinity to β_1 and β_2 receptors (107). Compounds **44** and **45** also bind irreversibly and with high specificity to β-receptors (108). As in the case of α-selective agents, photoaffinity probes have proven more valuable than the electrophilic type for studying β-adrenergic receptors. Several radioiodinated photoaffinity agents have been prepared including the [^{125}I]pABC, **46**, [^{125}I]AMBC, **47**, CYP-azide, **48**, and CYP-diazirene, **49** that bind specifically and with high affinity to β-adrenergic receptors (109). In the β_2-system (frog erythrocytes), **50**, the azido-derivative of **47**, labels a broad band of Mr 58,000, whereas in the β1 system (turkey erythrocytes) two bands of Mr 39,000 and 45,000 were labeled (110). More recently, an iodoazido derivative of norepinephrine (**51**, NAIN) has been reported to stimulate adenylate cyclase activity in guinea pig lung membranes; its action was inhibited by (-)-alprenolol (111). [^{125}I]NAIN photolabeled β-receptors in guinea pig lung membranes in the presence of $MgCl_2$ and in the absence of guanyl nucleotide (111).

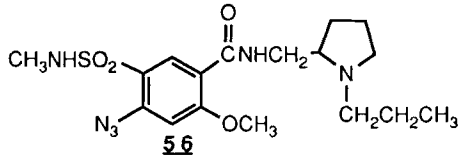
Dopamine Receptors - Tritiated **52** has been used to characterize dopamine receptors and has been suggested to bind selectively to the D_2-sites (112). The first selective striatal D_2-photoaffinity label was an azido-derivative of clebopride, **53** (113-115). Replacing the 5-chloro group of **53** with ^{125}I, resulted in a D_2-selective photoaffinity label with high specific radioactivity (116,117). The [^{125}I]azidoaryl-derivative of spiroperidol, **54**, covalently labels a peptide of Mr 94,000 in rat striatal membranes which has been suggested to be the D_2-receptor (118). An azidomethyl-derivative of spiperone, **55**, covalently labels a peptide of Mr 92,000 in canine striatal membranes, although several minor lower molecular weight proteins were also labeled and this agent had a much lower specific activity than **54** (119). Azidosulpiride, **56**, was used to photolabel D_2-receptors in rat striatal, anterior pituitary and olefactory bulb tissues (120). The radiolabeled receptors from the three tissues were concentrated in a single band at Mr 85,000, with very low nonspecific incorporation (120). Azidohaloperidol, **57**, selectively photolabels D_2-receptors in bovine striatum and has been used to identify the photolabled D_2-receptor binding subunit (Mr 94,000) recognized by antibodies specific for haloperidol (121).



52 **53**



54, X=H, R=
55, X=N_3, R=CH_3



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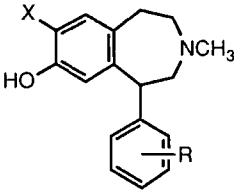
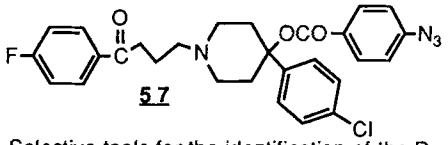


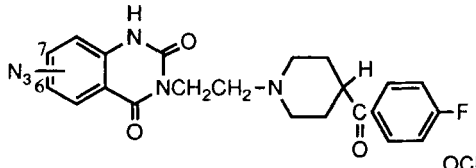
58, R=m-NH_2, X=Cl
59, R=p-N_3, X=I



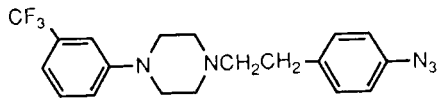
57

Selective tools for the identification of the D_1-receptor protein have more recently become available. Radioiodinated SCH 23390, **58**, binds selectively and with high affinity to D_1-receptors (122). After crosslinking **58** with the heterobifunctional reagent N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (SANPAH), the resulting compound was covalently incorporated into a peptide of Mr 72,000, representing the D_1-receptor subtype (122). A closely related azido-analog, **59**, was effectively used to photolabel D_1-receptors in canine, bovine and porcine striatal membranes and covalently incorporated into a peptide with Mr 75,000, as well as minor bands at 62,000 and 51,000 (123,124).

Serotonin Receptors - Serotonin receptors have been identified in various areas of the brain and have been classified into three major subtypes 5-HT_1, 5-HT_2 and 5-HT_3. These subtypes have been further divided into subclassifications but thusfar, irreversible ligands have only been developed for the 5-HT_2 and 5-HT_{1A} subtypes. The 6- and 7-azido derivatives, **60** and **61**, respectively, of the 5-HT_2 receptor selective antagonist, ketanserin, have been prepared as photoaffinity probes for 5-HT_2 and histamine receptors (125,126). Incorporation of an azido-moiety in either the 6- or 7-position of ketanserin increased the affinity of both compounds for the histamine receptors without diminishing affinity for the 5-HT_2 receptors; however compound **61** photolabels both receptors whereas **60** did not (126). The previously described metaphit, **9**, has also been reported to deplete 5-HT_2 sites as well as PCP binding sites (127).



60, 61



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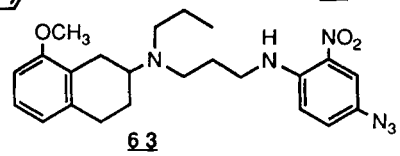

63

The para-azido-derivative **62**, photolabeled a polypeptide (Mr 55,000) in rat hippocampal membranes suggested to be the 5-HT_{1A} receptor or a subunit thereof (128). An electrophilic chloroamino-derivative of the 5-HT_{1A} selective agonist, 8-hydroxy-diisopropylaminotetralin, displayed irreversible but low affinity binding to 5-HT_1-sites (129,130). The arylazido-derivative of this agent, **63**, selectively and irreversibly blocked 5-HT_{1A} sites in rat hippocampal membranes, exhibiting nanomolar potency (131). Tritiated **63** photolabels a P_1 protein in hippocampal microsomal membranes with Mr 63,000 which is believed to be the 5-HT_{1A} site (132). A protein of similar molecular weight has also been isolated from human hippocampus and frontal cortex using photoaffinity labeling and immunoprecipitation techniques with the previously described azido-spiperone derivative **55** (133).

Conclusions - Electrophilic and photoactivated agents have proven to be useful as receptor selective irreversible probes. Novel affinity ligands are allowing the isolation and purification of many receptor systems. Future preparation of more selective, high affinity irreversible ligands will aid in receptor characterization and ultimately in improved drug design.

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Chapter 30. Progress in the Characterization of Peptide Receptors

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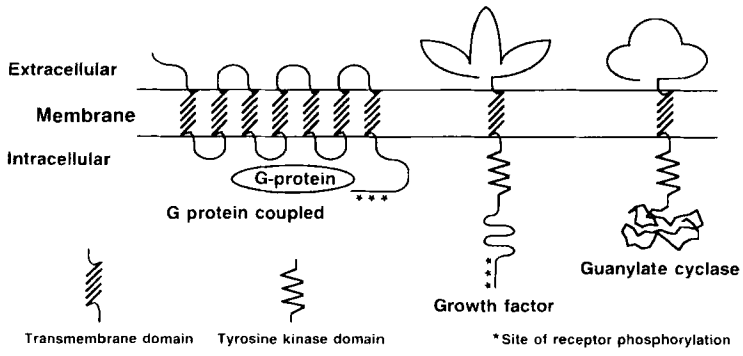
Introduction — While the importance of peptides as transmitters and modulators of cellular function has been appreciated since the discovery of insulin, the characterization of their receptors has awaited recent advances in protein isolation and characterization, especially the advent of molecular genetics. This review covers selected recent research on receptors for peptides of medicinal interest, focussing mainly on G protein-coupled receptors and growth factor receptors/tyrosine kinases. Recombinant DNA technology has been a ground-breaking tool in these efforts; it has been used for: 1) the production of large quantities of protein for characterization studies; 2) site-specific mutagenesis (a technique whereby specific amino acid subtitutions/deletions can be introduced to study structure and function); 3) the generation of chimaeric receptors by fusing of the complementary domains from two related receptor genes; 4) preparation of mRNA probes to locate sites of receptor expression; 5) isolation of homologous receptors; and 6) generation of primary amino acid sequence data (1). It is important, however, not to underestimate the contributions of protein biochemistry, which has provided crucial information for subsequent cloning work. Frequently used biochemical techniques include: 1) the cross-linking of photo- or affinity labelled ligand to its receptor (allowing separation and molecular weight determination); 2) solubilization and chromatography methods; 3) generation of antibodies against purified receptor (for screening expressed protein from cloned genes); 4) characterization of responsiveness to G nucleotides or tyrosine kinase activity; and 5) characterization of other proteins associated with the receptor. The marriage of molecular biological and biochemical techniques is nicely illustrated by the recent dramatic advances in our understanding of the structure and function of the β-adrenergic receptor (2).

GROWTH FACTOR/LYMPHOKINE RECEPTORS

By virtue of their primary amino acid sequence, growth factor receptors are members of the tyrosine kinase 'superfamily' (3). A recent review categorizes these peptides into three families, headed by 1) insulin, 2) epidermal growth factor (EGF), and 3) platelet-derived growth factor (PDGF) (4). While all growth factor receptors possess independent outer, ligand-binding, and inner, tyrosine kinase/regulatory, domains connected by a short transmembrane region, they differ in important ways. For example, only the insulin and EGF families' outer domains contain the cysteine-rich repeat structures characteristic of the immunoglobulin superfamily, and only the insulin family has been shown thus far to use disulfide bonds in holding together its tetrameric assemblage of receptor chains.

Figure 1. Schematic drawing of receptor families



Insulin — The structure and function of the insulin receptor are the subject of two excellent reviews (5,6). Both the initial cloning (7) and subsequent protein work (8,9) established the heterotetrameric structure, composed of two α and two β chains,which is responsible for high affinity insulin binding. More recent work, relying on a photoaffinity/biocytinyl insulin analogue (10) and site-directed mutagenesis (11), has indicated that two of the insulin binding sites in the α chain are widely separated, suggesting the α chain is extensively folded during ligand recognition. Cross-linking studies have shown that insulin binding to the α chain induces a conformational change in the β chain, which may stimulate the next event, the autophosphorylation cascade (12). Once three tyrosine residues in the protein kinase domain have been phosphorylated, by a presumed interchain mechanism, the receptor is fully activated (13,14). The extent of phosphorylation may regulate the ability of the receptor to subsequently phosphorylate a number of protein substrates, among them calmodulin (15) and a phosphatidylinositol kinase (16), which carry on the signal transduction process. In addition, considerable effort has gone into determining the second messenger for insulin (17,18), with recent work suggesting both a novel glycolipid which regulates cAMP levels (19) and possible involvement of a G protein (20). Finally, recent work on the internalization (21,22) and degradation (23) of the insulin/receptor complex suggests that both events are independent of the signal transduction process.

Insulin-like growth factor (IGF)-I/II — These two factors together with insulin form a small subfamily; though each peptide binds to its own specific receptor, the IGF's also bind, albeit with lower affinity, to the insulin receptor, and insulin binds weakly to the IGF-I receptor (24). This latter phenomenon was clarified by the cloning of the gene for the IGF-I receptor, which showed its considerable homology to the insulin receptor sequence (25). Differences from the insulin sequence in the carboxy terminus, where tyrosine phosphorylation plays a regulatory role, may subserve the differential biological activities of the two peptides. As in the case of insulin, high affinity binding of IGF-I leads to formation of an $\alpha_2\beta_2$ receptor complex (26), though in this case the process requires formation of disulfide bonds (27). Cloning of the receptor for IGF-II (28) showed that the receptor consists of a cysteine rich extracellular domain, with, unlike the insulin receptor, a very short cytoplasmic tail; it was subsequently shown to be identical to the receptor for mannose-6-phosphate, although the two ligands bind at different sites. Recent reports have summarized receptor characterization work at the protein level (29), as well as evidence of coupling to a G_i protein (30).

EGF — Antibody work subsequent to cloning of the EGF receptor gene (31) has begun to define residues important for EGF binding, for example, in the region between Ala-351 and Asp-364 (32); CD and fluorescence measurements are being used to probe the conformational changes in the receptor induced by EGF (33). The use of mutant EGF receptors, available from the cloned receptor gene, has clarified the function of the various regions in the intracellular domain (34). Specifically, the first region beyond the kinase domain is responsible for receptor internalization and termination of the signal; the next region masks the kinase domain and, upon phosphorylation, is removed to expose kinase activity (35). Recent evidence confirms the role of the high-affinity, dimeric receptor in signal transduction (36), as well as the location near the carboxy terminus of the tyrosine residues which regulate kinase activity depending on their state of phosphorylation (37,38). Other studies indicate that tyrosine phosphorylation occurs via an interchain mechanism while the receptors are still at the cell surface (39,40). Finally, tyrosine kinase inhibitor work suggests that phosphorylation of phospholipase C may be an important part of the signal transduction process (41), while a recent report presents evidence for the role of a G protein (42).

PDGF — Results of an extensive molecular genetics study have demonstrated two forms for both PDGF (A and B) and its receptor (α and β) (43-45); each dimeric form of PDGF binds to the corresponding dimeric form of its receptor (46). PDGF binding induces a conformational change in the receptor which can occur only if tyrosine kinase activity is intact (47). Studies using cloned α and β PDGF receptor genes separately expressed in naive cells indicate that all three receptor forms can mediate the biological effects of PDGF (48). Although its structure is largely homologous with the receptors for insulin and EGF, the PDGF receptor contains an insert in the tyrosine kinase domain which mediates the mitogenic effects of PDGF, with all other responses to PDGF being normal in a mutant form of the receptor lacking this insert (49). Subsequent work with this mutant receptor has shown that the insert region directs association with a phosphoinositol kinase activity, which thus may be responsible for mitogenesis (50). This kinase activity may also account for the novel phosphoinositides produced by PDGF-stimulated mitogenesis (51). Other aspects of the signal transduction process, the subject of a recent extensive review (52) include physical association of

the receptor with phospholipase C (53) and possible involvement of a G protein (54). The ability of PDGF receptors to phosphorylate the GTPase-activating protein associated with $p21^{ras}$ suggests yet another route for mitogenic signalling by PDGF (55).

Nerve growth factor (NGF) — The sequence of the cloned gene for the human NGF receptor was shown to code for an extracellular domain similar to those of the other growth factor receptors, but lacking an internal tyrosine kinase domain (56,57). This single chain mediates both low and high affinity binding of NGF (58) and its biological responses, suggesting the involvement of an additional cellular component (59) to compensate for the lack of a tyrosine kinase domain. Despite the structural dissimilarity of the NGF receptor to the insulin receptor, its second messenger mechanism involves a glycolipid, similar to that observed in insulin signaling, and protein kinase C (60). Receptor internalization may be involved in biological effects such as long-term memory (61,62).

Colony stimulating factor (CSF-1) — The CSF-1 receptor gene was originally cloned as the c-fms proto-oncogene using the v-fms oncogene, the transforming gene of the McDonough strain of feline sarcoma virus, as a probe (63). In order to delineate the structural basis for the unrestricted proliferation induced by v-fms, which contrasts with the tightly regulated proliferation induced by the CSF-1 receptor, a chimaeric gene comprised of the extracellular domain of the CSF-1 receptor and the intracellular domain of the v-fms gene was constructed (64). Even though v-fms contains substantive modifications of its intracellular domain (in comparison with the CSF-1 receptor), the hybrid receptor exhibited ligand-induced down-regulation and no transforming ability, demonstrating the functional significance of at least part of the extracellular domain of the CSF-1 receptor. Once again, the involvement of polyphosphoinositides in signal transduction is suggested by recent work showing that CSF-1 stimulates phosphoinositol-3 kinase via activation of its receptor (65).

Interleukins — The only homology displayed by the protein sequences determined from the cloned genes for the interleukin receptors is found between the extracellular domains of the IL-1 and IL-6 receptors and the immunoglobulin superfamily; the other external and all internal domains show no homology with known proteins and thus their function, and especially their signal transduction mechanism, remains obscure (66-68). Some insight into the function of the outer domain of the IL-2 receptor has been derived from proteolysis work (69); more information should be available from ongoing X-ray studies of p55, the 55,000 M.W. α-subunit of the receptor co-crystallized with IL-2 (70). The cloning of the gene for p75, the IL-2β receptor subunit, clarified the origin of the low (p55), intermediate (p75), and high (p55/p75 complex) affinity receptors (71). In addition, it has been found that the p75 subunit alone is responsible for promulgating the tyrosine kinase activity, which presumably effects signal transduction (72). A restricted region of this chain is responsible for the growth signal, as shown by work with mutant p75 proteins (73). Additional proteins presumably involved in signal transduction have not yet been isolated. Proteins analogous to p55 have been characterized for other interleukin receptors: a 43kD protein shows IL-1 binding affinity (74), in addition to the 80 kD IL-1 receptor protein, and a 130 kD glycoprotein associates with the 80 kD IL-6 receptor upon ligand binding (75). Finally, the recent cloning of the gene for the IL-3 receptor revealed homology of both its external and internal domains with receptors for IL-2, IL-4, and IL-6 (76).

G PROTEIN-COUPLED RECEPTORS

The structure and function of receptors in this area have been the subject of intense investigation, with molecular genetics again leading the way. Numerous reviews describe the remarkable progress to date, especially in delineating the seven trans-membrane spanning helices of the β-adrenergic receptor, which comprise the ligand binding domain, and the intracellular loops and carboxy terminal tail, which bind the G protein (77). Agonist binding via ion-pair interactions with essential, conserved aspartate residues within the membrane-spanning domain effects a conformational change in the receptor structure and consequent release of the G protein from the third intracellular loop; the G protein carries out the subsequent events in the signal transduction process, such as stimulation or inhibition of adenylate cyclase. The C-terminus also contains sites for phosphorylation which regulate receptor desensitization (see schematic drawing). Considerably less progress has been made in the characterization of G protein-coupled peptide receptors, but there have been numerous interesting findings, as chronicled in the following sections which are organized according to the tissue with which each peptide is predominantly associated.

Neuropeptide receptors — One of the most intensively studied families of peptide hormone receptors, and the one which has seen the most progress in characterization due to gene cloning, is the tachykinin (neurokinin, NK) group (78,79). The primary amino acid sequences, derived from the sequence of the cloned gene, for the substance K (NK2) (80) and substance P (NK1) (81) receptors (SK-R and SP-R) show them to be structurally analogous to the β-adrenergic receptor, with important differences. For example, one of the essential aspartate residues present in the β-adrenergic receptor is missing in the SP-R and SK-R; in addition, the SP-R contains glutamate in place of one of the other conserved aspartate residues. The three neurokinin receptors are coupled to phospholipase C by a pertussis toxin (PT)-insensitive G protein and to adenylate cyclase by a PT-sensitive G protein (82). Finally, studies using a rat SK-R RNA probe indicate that there are two mRNAs which encode the SK-R and that they are expressed in the GI tract but not in the brain in rat (83).

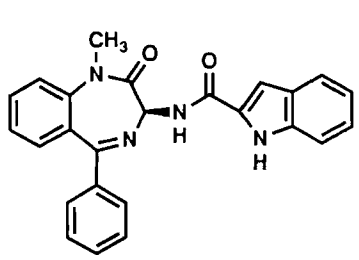
A recent review details the extensive characterization of the opioid peptide receptors, aided immeasurably by the existence of non-peptide ligands (the opiates), which have established the three basic subtypes within this family (84). In addition, three subtypes within the κ-type were recently discovered using compounds in a series of κ-specific agonists (85). The coupling of the opioid receptors with a G protein was confirmed by a study in which a functional μ-opioid receptor-G protein complex was reconstituted (86).

Progress in the characterization of the somatostatin receptor includes a report of immunoaffinity and HPLC purification of the receptor from a human gastric tumor cell line (87) and solubilization from hamster pancreatic beta cells of a complex of 128 kD and 45 kD proteins with a 41 kD G protein (88). Finally, an α-melanotropin analogue bearing an azido group was used to label and characterize the α-MSH receptor in melanoma cells (89).

Gut and metabolic receptors — The exciting discovery of the non-peptide cholecystokinin (CCK) receptor antagonist, **1**, L-364,718, and its analogues has raised the possibility for greatly increased understanding of the structure and function of the CCK receptor (90). For example, recent studies have relied on L-364,718 or its analogues for characterization of the CCK-A (91) and CCK-B (92) receptors. The characterization of the glucagon receptor, apparently a dimer, and its coupling to a stimulatory G protein have been reviewed (93). Separation of the receptor from an associated degrading activity (94), should help in receptor purification. Studies of the receptor for vasoactive intestinal peptide (VIP), which has been solubilized as a 152 kD complex with a G_S protein (95), have shown that protein kinase C and agonist-mediated desensitization and internalization are independent events and thus not sequentially coupled (96).

Cardiovascular receptors — Angiotensin receptors have been the subject of the most exciting developments in this group, both with the finding that the mas oncogene (cloned previously) is a putative receptor for angiotensins I, II and III (97) and with the discovery (see Chapter 6) of the potent non-peptide AII antagonist DuP 753 (**2**) (98). These compounds have proven useful in defining AII receptor subtypes, and may help to unravel the topography of the AII receptor (99). Recent studies have provided additional evidence for two AII receptor subtypes and a separate regulatory site on the receptor which mediates receptor desensitization (100,101). Receptors for the recently identified family of vasoconstrictor peptides, the endothelins (see Chapter 10), have been the subject of several studies, some of which have found two, and others three, forms of the receptor (102-105). Photoaffinity and biotinylated analogues of vasotocin (selective for binding to the V-2 subtype) have been synthesized and should prove useful for V-2 receptor isolation (106). The V-1 subtype has been reported to be isolable as a complex with both a G protein and phospholipase C (107). A caution in the use of photoaffinity probes was noted by workers who, using a photoaffinity analogue of bradykinin (BK), isolated angiotensin converting enzyme (which degrades BK) rather than the anticipated BK receptor (108). Peptidic analogues of BK have been useful in identifying subtypes of the B-2 BK receptor (109).

Reproductive receptors — The recent cloning of the gene for the rat lutropin (LH) receptor revealed an interesting new development in the elucidation of the structure of G protein receptors (110). While the LH receptor possesses a seven-helical trans-membrane domain homologous to the SK-R and β-adrenergic receptors, it also contains a large extracellular domain similar to known leucine-rich glycoproteins (LRG's), which bind substances ranging from thrombin to collagen. Since LH is thought to bind to this extracellular domain, this region apparently also mediates signal transduction to the



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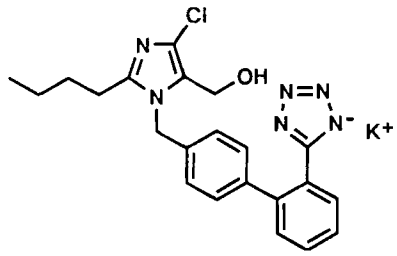

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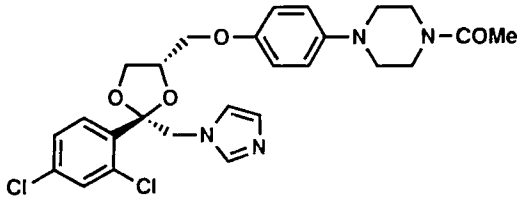

3

transmembrane domain, in part, through recognition of the glycosylated form of LH (deglycosylated LH binds but does not stimulate adenylate cyclase). A family of LH-RH (luteinizing hormone-releasing hormone) receptor antagonists based on ketoconazole, 3, has been reported (111).

GUANYLATE CYCLASES

One of the most exciting developments in peptide receptor structure elucidation is the discovery that the receptor for atrial natriuretic factor (ANF (see Chapter 10)) consists of an extracellular binding domain coupled by a single transmembrane region to an intracellular guanylate cyclase domain which produces cGMP as the second messenger (112). Extensive earlier protein work had established the multidomain structure of the receptor and a conformational change of the receptor on ligand binding which was postulated to trigger guanylate cyclase activity; it also revealed a region between the inner membrane surface and guanylate cyclase sensitive to protease treatment (114,115). Cloning of the ANF receptor gene has provided a firm structural basis for these observations, and revealed that the protease sensitive region shows extensive homology to members of the protein kinase family and may be a regulatory, nucleotide binding, site (116). The subsequent cloning of an ANF receptor subtype from brain has strengthened these conclusions (117). Site-specific mutagenesis using the cloned gene for the third known ANF receptor subtype (ANF-C, involved in ANF degradation) has been used to produce a soluble form of the receptor which still bound ANF (118), showing that the binding and guanylate cyclase domains of the receptors are independent.

Conclusion — In general, regardless of the structural class, receptor functions such as ligand binding, signal transduction, and receptor internalization (regulation) are carried out independently by separate domains of a single chain, or by different receptor subunits or accessory proteins. It has been more difficult to characterize the complete second messenger cascade triggered by ligand-receptor binding, partly because many of the ligands mediate a plethora of effects; an interesting new development is the involvement of G proteins and phosphoinositides with many of the growth factor receptors. Protein and cellular biochemistry are coming to the fore in solving these problems now that molecular genetics has provided much of the necessary information on receptor structure. Another development likely to advance our understanding of receptor structure and function will be the continued discovery of non-peptide receptor antagonists. Such molecules have already provided the basis for important contributions in the CCK and AII areas. New antagonists for the LH-RH receptor, and presumably for other receptors as well, will serve to accelerate the pace of discovery in the field of receptor structure and function.

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Chapter 31. The Impact of Biotechnology on Drug Discovery

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Introduction - Medicinal chemistry has traditionally taken advantage of any opportunity available to aid in the discovery of new therapeutic agents. Both lead identification, the observation of a useful biological property in a novel chemical, and optimization, the acquisition of structure-activity relationships associated with ultimate clinical candidate selection, have rightfully relied heavily on practices, and sometimes prejudices, founded in decades of empirical success (1). Although medicinal chemistry as a discipline has refined optimization into a process without major unidentified obstacles, the challenge to bring the task of lead identification to a comparable state of maturity remains unfulfilled. Lead detection has classically relied upon random screening followed by iterative analog synthesis; more recently, rational design paradigms, made possible by advances in instrumentational and computational methodology, have become increasingly useful (2). Another question, however, remains more problematic: what determines a useful biological property, and how is it measured in the discovery process? The answers to this question can obviously predetermine what is discovered by, and ultimately the success or failure of, a drug discovery program.

The evolution of recombinant DNA technology, from scientific innovation to pharmaceutical discovery process, has occurred in parallel with the development of contemporary medicinal chemistry (3). The products of biotechnology research, many of which satisfy previously unfulfilled markets, share few of the traits characteristic of traditional pharmaceuticals. Biotechnologically-derived therapeutics are large extracellular proteins destined to be, with few exceptions, injectibles for use in either chronic replacement therapies or in acute or near-term chronic situations for the treatment of life-threatening indications (4,5). Their dissimilarity does not end there, however. Unlike most low molecular weight pharmaceuticals, these proteins were developed not because of their novelty of structure, but because of their novelty of action along a relevant biochemical pathway. The discovery process in fact hinged on recognition of a useful biological activity, its subsequent association with an effector protein, be it enzyme, receptor or hormone, and the genetic identification, expression and production of the effector by the application of recombinant DNA technology.

If modulation of biochemical processes by a low molecular weight compound has been the traditional goal of medicinal chemistry, then association of a biological effect with a distinct protein and its identification and production has been considered the domain of recombinant DNA technology. The application of recombinant DNA technology to the identification of proteins and other macromolecules as drug targets, and their production in meaningful quantity as discovery tools thus can provide an answer to at least one of the persistent problems of lead detection. Since a comprehensive review of the genetic engineering of important proteins is well beyond the scope of this volume, this chapter will highlight some novel examples of contemporary advances in recombinant DNA technology with potential applications to the drug discovery process in areas of importance to medicinal chemistry. Throughout this chapter, amino acids are denoted by their one-letter codes; site-specific mutations are represented by the code for the wild type amino acid, the residue number, and the code for the replacement amino acid (6); the symbol ǂ denotes a proteolytic cleavage site.

SITE-DIRECTED MUTAGENESIS AS PROTEIN SAR

Developments in the technique of site-directed mutagenesis over the past few years have created the opportunity to change essentially any amino acid, or even substitute or delete whole domains, in any protein, with the goal of designing and constructing new proteins with novel binding, clearance or catalytic activities (6,7). The concomitant changes in protein folding and tertiary structure, protein physiology, binding affinities (for a receptor or hormone), binding specificities, (either for substrate or

receptor), or catalytic activity (for enzyme active site mutants), are all effects which are measurable versus the "wild type" parent, assuming that expression of the gene, and subsequent proper folding, has successfully occurred. Several surprising observations have been made during the short period that this technology has been available: amino acid substitutions lead in general to highly localized changes in protein structure with few global changes in overall folding; substitutions of residues not involved in internal hydrophobic contacts are extremely well accommodated leading to few unsynthesizable mutants; proteins seem extremely tolerant of domain substitution, even among unrelated proteins, allowing often crude first attempts at chimeric proteins to be successful. The implications of this technology for the discovery of new pharmaceuticals lie in two areas: second-generation protein therapeutics and site- or domain-specific mutant proteins for structure-function investigations.

Second-generation Protein Therapeutics - The cloning and expression, and in fact manufacture, of proteins as therapeutics involves the same problems encountered in the development and successful clinical approval of any drug. Potency, efficacy, bioavailability, metabolism and pharmaceutical formulation challenges presented by the natural protein suggest that second-generation products might be engineered to alleviate the particular problem at hand, producing desired therapeutic improvements. The parent proteins to which this technology has been applied extend across the range of recombinant products undergoing clinical evaluation (8).

As an example, for tissue-type plasminogen activator (t-PA), one of the most studied recombinant products (9), four properties functioning in concert (i.e., substrate specificity, fibrin affinity, stimulation of t-PA activity by fibrin and fibrinogen, and sensitivity of the enzyme to inhibition by plasminogen activator inhibitors (PAIs)) are responsible for the localization and potentiation of the lytic reaction at a clot surface, and are readily analyzed using molecular variants (10). In the absence of crystallographic data, a consensus structure combining the major domains of t-PA has been predicted based on the significant sequence homology with other serum proteins and serine proteases (Figure 1). The complexity of this structure is reflected in its functional multiplicity: efficient production of plasmin by cleavage of the R560-V561 bond of plasminogen; very low binding to plasminogen in the absence of fibrin; moderately high affinity for fibrin; increase in the efficiency of plasminogen activation by 500-fold in the presence of fibrin; rapid inactivation by PAI-1; and rapid hepatic elimination by receptor-mediated endocytosis (11). Point mutations at glycosylation sites (N117Q, N184Q and N448Q) seemed to confirm the hypothesis that the carbohydrate side chains of t-PA exert considerable influence on its clearance via the mannose-specific glycoprotein liver receptor. The mutants, which cannot be N-glycosylated, exhibited reduced clearance and prolonged plasma half-life (12). Later studies with multiple mutants have challenged this clearance hypothesis (10,13) and have implicated structural determinants in the EGF-homologous domain instead (14). Prevention of the proteolytic conversion of single- to two-chain t-PA by mutation at the natural cleavage site (R275Q/G-I276) resulted in mutants with significantly increased fibrin binding (15,16), and a dramatic decrease in specific activity (10). The single chain mutants appear to have the additional advantage of lowered affinity for the PAIs (11). These results are explained by modeling studies which implicate a charge relay system linking the proteolytic cleavage site to formation of the substrate specificity site. Conservative C- and N-terminal truncations result in little change in specific activity, but the C-terminal truncation yielded a 2-fold increase in stimulation by fibrin (10).

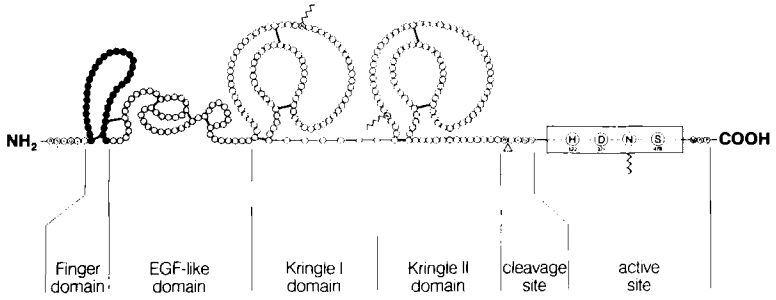

Figure 1: The domain structure of t-PA (10).

Although the systematic changes exemplified by t-PA site-directed mutagenesis studies are the rDNA equivalents of analog synthesis for SAR development, more recent applications of this technology bear a less straightforward resemblance to medicinal chemistry paradigms. For instance, in an effort to overcome the short plasma half-life associated with soluble CD4, the truncated T-cell class II MHC antigen and HIV-1 gp120 receptor under study as an AIDS therapy, chimeric molecules, termed "immunoadhesins" (Figure 2) have been recombinantly constructed from the gp120-specific domains of CD4 and the effector domains of various immunoglobulin classes (17,18). In addition to dramatically improved pharmacokinetics, these chimeric constructs incorporate functions such as Fc receptor binding, protein A binding, complement fixation and placental transfer, all of which are imparted by the Fc portion of immunoglobulins. Dimeric constructs from human (CD4-2γ1 and CD4-4γ1) and mouse (CD4-Mγ2a) IgG, and a pentameric chimera (CD4-Mμ) from mouse IgM all exhibit evidence of retained gp120 binding and anti-HIV infectivity activity. Both CD4-2γ1 and CD4-4γ1 showed significantly increased plasma half-lives, of 6.7 and 48 h respectively, as compared with 0.25 h for rCD4. The former is currently undergoing clinical evaluation as an anti-HIV agent. A more complete discussion of AIDS therapeutics is presented in Chapter 16 of this volume.

Figure 2: Structure of CD4, IgG1 and CD4 immunoadhesins (17).
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Site-directed mutagenesis technology has also been applied to one of the most perplexing problems in structural biochemistry - the nature of the protein-protein interaction. While numerous examples of models of enzyme-ligand complexes have been developed based on active-site modifications, this method is only now being extended to the formidable problem of defining the essential elements of a protein-protein (e.g., a protein substrate to a protease, or a hormone to its receptor) binding epitope. An impressive example of a systematic search for a binding epitope is the recent work used to define the human growth hormone (hGH)-somatogenic receptor interaction (19,20). First, using a technique termed homolog-scanning mutagenesis, segments of sequences (7 to 30 amino acids in length) from homologous proteins known not to bind to the hGH receptor or to hGH-sensitive monoclonal antibodies (Mab) were systematically substituted throughout the hGH structure, using a working model based on the three-dimensional folding pattern found by crystallo-

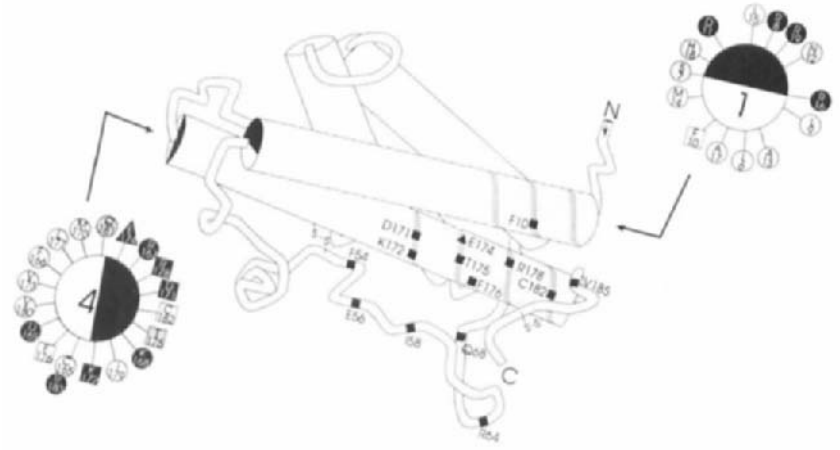

Figure 3: The hGH binding epitope (20). Copyright © 1989;
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graphic analysis of the highly homologous porcine GH (21). Using an ELISA-based binding assay which measures the affinity of the mutant hGH for its recombinantly-derived receptor, swap mutations which disrupted binding were found to map within close proximity on the three-dimensional model, even though the residues changed within each subset were usually distant in the primary sequence. By this analysis, three discontinuous polypeptide determinants - the loop between residues 54 and 74, the central portion of helix 4 to the C-terminus, and, to a lesser extent, the amino-terminal region of helix 1 - were identified. A second technique, termed alanine-scanning mutagenesis, was then applied. Single alanine mutations (62 in total) were introduced at every residue within the regions implicated in receptor recognition. The alanine scan revealed a cluster of a dozen large side chains that, when mutated to alanine, exhibited more than a four-fold decrease in binding affinity. Many of these residues which constitute the hGH binding epitope for its receptor (Figure 3), are altered in close homologs, such as placental lactogen and the prolactins. The overall correct folding of the mutant proteins was determined by cross-reactivity with a single set of conformationally sensitive Mab reagents. The homolog and alanine-scanning mutagenesis techniques should be generally useful starting points in helping to identify amino acid residues important to an interaction (8).

The rDNA site-directed mutagenesis methodology, although advancing rapidly, is still limited to the repertoire of the 20 natural amino acids encoded by DNA. In order to effect more subtle changes in proteins, such as increased or decreased acidity, nucleophilicity or hydrogen-bonding characteristics, without dramatically altering the size of the residue and without affecting the overall tertiary structure, it has been proposed that site-directed mutagenesis using unnatural amino acids might offer the needed advantages. In the past, such changes were accomplished semi-synthetically, on functionalizable residues such as Cys. However, methodology for carrying out such mutations recombinantly has been successfully used. There are four requirements: (1) generation of an amber (TAG) "blank" codon in the gene of interest, at the position of the desired mutation; (2) identification of a suppressor tRNA that can efficiently translate the amber message, but is not a substrate for any endogenous aminoacyl-tRNA synthetases; (3) development of a method for the efficient acylation of the $tRNA_{CUA}$ with novel amino acids; (4) availability of a suitable in vitro protein synthesis system to which a plasmid bearing the mutant gene or corresponding mRNA and the acylated $tRNA_{CUA}$ can be added (22). The first demonstration of this methodology, replacement of F66 with three phenylalanine analogs in RTEM β-lactamase and subsequent determination of the kinetic constants k_{cat} and K_m of the mutants stands as a singular example of the promise of this technology (23).

GENETICALLY ENGINEERED DRUG DISCOVERY TOOLS

Another application of recombinant technology lies not in new protein drug product discovery per se, but in the ability to provide cloned and expressed proteins as reagents for medicinal chemistry investigations. The common practice of in vitro screening using target protein isolated from tissue (usually non-human, and therefore non-target) has begun to give way to the use of solid-phase or whole cell binding assays based on recombinantly produced and isolated, or cell-surface expressed, reagent quantities of the relevant target protein. Sometimes, the differences between tissue isolates and recombinant reagent are small; more frequently, however, the sequence homologies and even functional characteristics can vary greatly, providing a distinct advantage in favor of the recombinant protein. When the possibility of achieving subtype specificity, either because of tissue distribution or differential gene expression, pinpoints a particular isoenzyme as a target for selective drug action, it is of obvious importance to be able to test for the desired specificity. The recently-developed technique called PCR (polymerase chain reaction), an enzymatic method for the in vitro amplification of specific DNA fragments, has revolutionized the search for subspecies (24,25). Classical cloning requires at least a partial sequence for low stringency screening. This method is unlikely to detect cDNAs corresponding to genes expressed at very low levels in the tissue from which the library was constructed. In contrast, the PCR technique can uncover and amplify sequences present in low copy number in the mRNA, and offers a greater likelihood of obtaining useful, full-length clones. The selective amplification afforded by PCR can also be used to identify subspecies present in tissue in especially short supply, offering yet another advantage over classical methods.

Recombinantly-produced reagents of potential application to drug discovery fall into three general categories: isolated or cell-surface expressed enzymes (with catalytic function), receptors (with signal transduction function) and binding proteins (with cellular adhesion properties).

Enzymes - The number of enzymes cloned and expressed in useful quantities for biochemical characterization defies comprehensive cataloguing. Some enzymes of potential value to medicinal chemistry have been extensively cloned for subspecies differentiation and/or access to human isotypes, including the protein kinase C [α, β_I, β_{II}, γ, δ and ε (26-29), and more recently, ζ (30) and nPKC-ε (31)] and the phospholipase A_2 (32,33) families. The ability of rDNA technology to rapidly provide access to quantities of a specific enzyme in a situation where absolute inhibition specificity would eventually be required was no more evident than in the case of the retroviral aspartic HIV-1 protease (HIV-1 PR) (34,35). This enzyme was identified as a viable target for anti-AIDS drugs because mutation of the active site aspartic acid (D25) effectively prevents processing of retroviral polyprotein, producing immature, noninfective virions (36-41). In addition to the DTG at positions 25-27, mutations within the sequence GRD/N (positions 86-88 in HIV-1 PR, a highly conserved domain in the retroviral proteases but not present in cellular aspartic proteases) were found to be completely devoid of proteolytic activity, potentially pinpointing a site critical for design of specific inhibitors capable of recognizing the viral, but not the host, proteases (42). The search for important tertiary structural differences between HIV-PR and known eukaryotic proteases began by determination of the X-ray crystal structure of recombinantly expressed material at 3 Å resolution (43). Subsequent crystallographic studies on both synthetic (at 2.8 Å) and recombinantly expressed (at 2.7 Å) material helped locate side chains and resolved some ambiguities in the dimer interface region (44, 45). From this information, a model of the substrate binding site was proposed (46). Far more useful for inhibitor design purposes, a complex of an inhibitor bound to HIV-1 PR has been solved (47), from which a closest contact map was developed (Figure 4).

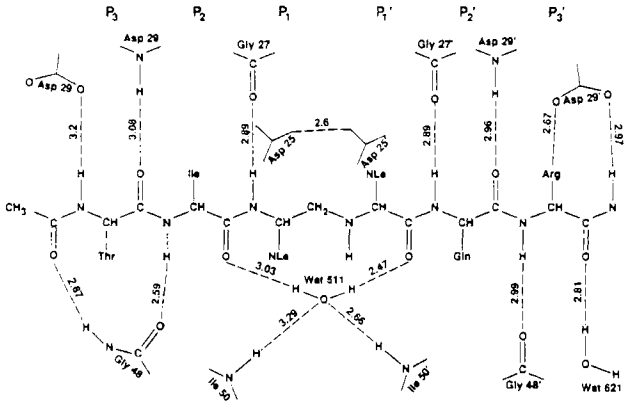

Figure 4: Schematic representation of the interaction of HIV-1 PR with an inhibitor (47). Copyright © 1989; reprinted by permission of American Association for the Advancement of Science.

With the functional role and tertiary structure of the protease determined, additional studies with both recombinant and synthetic material have yielded high-flux assays for screening of chemical libraries, fermentation broths and designed inhibitors using HIV-1 PR cleavage of a synthetic pseudosubstrate. The observation that the antifungal antibiotic, cerulenin, inhibits HIV-1 polyprotein processing (48) sparked the preparation of cerulenin analogs, evaluation of which as HIV-1 PR inhibitors rapidly confirmed their mode of action (49). Natural products such as coriliagin, quercetin and roseolide have been reported to be active at µM concentrations (50). Peptide sequences derived from specific retroviral polyprotein substrates, and inhibition by pepstatin and other renin inhibitors, have identified (S/T)P_3P_2(Y/F)‖P as a consensus cleavage site for HIV-1 PR (51-55). One of the inhibitors derived from such studies, SGN(FΨ[CH_2N]P)IVQ, has been used as an affinity reagent for large-scale purification of recombinant HIV-1 PR (56), while Ac-TI(nLΨ[CH_2NH]nL)QR-NH_2 was used in the co-crystallization studies mentioned above (47). From amongst the peptides identified as HIV-1 PR inhibitors (57-59), only a limited number effectively inhibit viral proteolytic processing and syncytia formation in chronically-infected T-cell cultures (60,61). As with other peptidomimetic structures, their transformation into potential drugs will require additional synthetic work. The short interval from identification of the enzyme as a target from amongst the possibilities presented by the HIV-1 genome to accessing material for assay and structural purposes will hasten the determination of the viability of HIV-1 PR inhibitors as AIDS therapeutics.

Receptors - Even more so than with enzymes, molecular genetics has been primarily responsible for the identification of functional receptor subtypes. The classical tissue binding pharmacological methods which made distinctions on the basis of ligand selectivity have been supplemented, and in most cases supplanted, by further subtyping made possible by cross-hybridization cloning using the known receptor genes. For example, in the case of the muscarinic cholinergic receptors, two subtypes, M_1 and M_2, had been defined pharmacologically by their affinity, or lack thereof, for pirenzepine, and were later confirmed by molecular cloning to be distinct gene products m1 and m2, respectively (62). Three additional muscarinic receptor genes (m3-m5) have since been isolated (63-65). From this work, a subtype-specific heterologous stable expression system in Chinese hamster ovary (CHO) cells suitable for screening potential subtype-specific ligands was developed. Using this assay, pirenzepine, previously thought to bind to m1 only, was found to have only a 50-fold reduced affinity for m2, and an almost equivalent (to m1) binding affinity for m3 and m4, suggesting that studies using pirenzepine on tissue homogenate have failed to distinguish adequately amongst the subtypes (66). These advances have been recently reviewed (67).

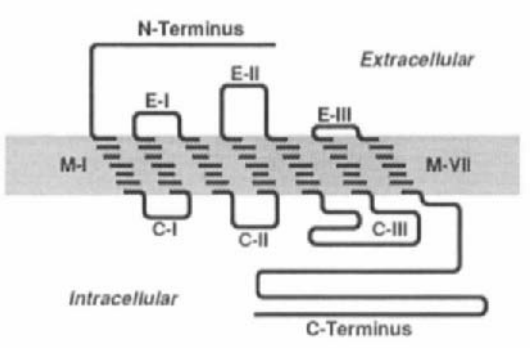

Figure 5: Schematic representation of the G-protein coupled receptors (69). Copyright © 1989; reprinted by permission of The American Society for Biochemistry and Molecular Biology, Inc.

Similar breakthroughs have been realized across the rest of the family of G-protein coupled receptors, since, in addition to the muscarinics, the primary structures of the adrenergic (α_1, α_2, β_1 and β_2), serotonergic, tachykinin, and rhodopsin receptors have been determined (68-71). All of these display the now-familiar homology pattern of multiple membrane-spanning domains packed into anti-parallel helical bundles (Figure 5). Subsequent mutagenesis studies on the β_2-adrenergic receptor have localized the intracellular domains involved in: (1) the coupling of the receptor to G-proteins (72); (2) homologous desensitization by β-adrenergic receptor kinase (β-ARK) (73), itself cloned and a possible target for down-regulation inhibitors (74); (3) heterologous desensitization by cAMP-dependent protein kinase (75); and (4) an extracellular domain with conserved cysteine residues implicated in agonist ligand binding (76).

The complicated biochemical pharmacology of natriuretic peptides (77) has been clarified by the cloning of three receptor subtypes, which revealed the functional characteristics of a new paradigm for second messenger signal transduction. The α-atrial natriuretic peptide (α-ANP) receptor (ANP-AR) and the brain natriuretic peptide (BNP) receptor (ANP-BR) contain both protein kinase and guanylate cyclase (GC) domains, as determined by both sequence homologies and catalytic activities, while the clearance receptor (ANP-C) completely lacks the necessary intracellular domains for signal transduction (Figure 6). This defines the first example of a cell surface receptor that enzymatically synthesizes a diffusible second messenger system in response to hormonal stimulation (Figure 7)(78-83). Since the NPs have differential, but not absolute, affinities for their corresponding receptors (80), and since both agonism (84) and antagonism (85) of the GC activity have been demonstrated in vitro, it seems possible to discriminate among the receptor-GCs to obtain more subtle structure-activity information for the design of selective NP analogs.

Other successfully cloned receptors include epidermal growth factor (EGFR), insulin (INSR), insulin-like growth factor-1 (IGF-1R), platelet-derived growth factor (PDGFR) and related tyrosine

kinases (86), the two subtypes of the $GABA_A$-benzodiazepine receptor complex (87-89), the inositol 1,4,5-triphosphate (IP_3)-binding protein P_{400} (90), the kainate-subtype glutamate receptor (91), and the interleukin-1 receptor (92). The topic of peptide receptors in particular is comprehensively addressed in Chapter 30.

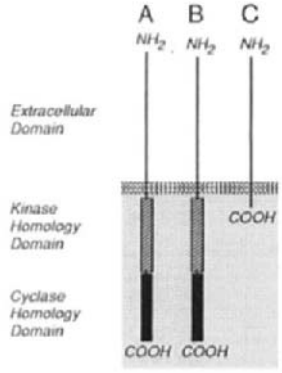

Figure 6: Natriuretic peptide receptor family.

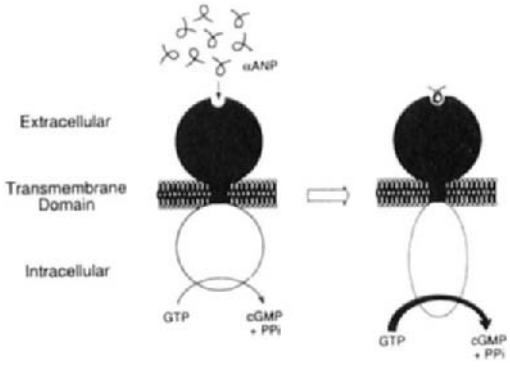

Figure 7: Model for ANP-A and -B receptor function (78) Copyright © 1989; reprinted permission of IRL Press.

Cellular Adhesion Proteins - The understanding of the processes that govern intracellular adhesion and cell localization in various pathologies has been significantly expanded because of the cloning and expression of some of the major cellular adhesion proteins. The interaction in the antigen-receptor cross-linking adhesion of T-cells mediated by the intercellular adhesion molecule (ICAM-1) and the lymphocyte function-associated molecule (LFA-1) was clarified by the cloning and expression of ICAM-1, the major cell-surface receptor for rhinovirus (93-95). LFA-1, a member of the integrin family of adhesion molecules, has also been implicated in HIV-1 infected T-cell syncytium formation (96). Other members of the integrin family have also been successfully cloned (97). Most recently $gpII_bIII_a$, the platelet fibrinogen receptor, was successfully expressed as the functional heterodimer, showing that prior association of the endogenous subunits is necessary to produce the cell-surface complexes (98). The availability of the individual members of this heterodimer superfamily, characterized by gross similarities in structure (Figure 8), function and, in some cases, avidity for RGD-containing peptides, will allow their individual roles in specific disease pathophysiologies to be ascertained. Determination of the subtle structural requirements which differentiate the RGD-containing macromolecular ligands should also facilitate design of specific antagonists for pharmacological studies. The subject of cellular adhesion molecules is discussed in Chapter 25.

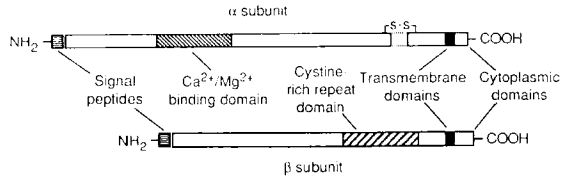

Figure 8: General structure of the integrins (97). Copyright © 1987; reprinted by permission of The American Association for the Advancement of Science.

More recently, but no less exciting, is the discovery of homing receptors (HR), membrane-bound proteins which "traffic" circulating lymphocytes to specialized high endothelial venules within specific lymph node targets (99). Molecular cloning of the murine HR identified as the MEL-14 antibody antigen, revealed that the HR contains a lectin (carbohydrate-binding) domain which appears to be responsible for the binding event (100). The murine clone was then used to identify the corresponding HR, which showed a high homology to the endothelial cell adhesion molecule ELAM (101), subsequently found to be identical to the human pan-leukocyte antigen, Leu-8 (102).

FUTURE PROSPECTS

The power of molecular genetics to provide unique and valuable tools for drug discovery is only now beginning to be exploited. The prospects for uncovering the molecular etiology of a disease state, or for gaining access to a disease-relevant target enzyme or receptor are already being realized. In addition, the possibility of rationally intervening in disease states at other points is becoming more evident. Regulation of inducible or tissue-specific gene expression (103) may become an important method for pharmacological intervention. The tools to monitor such events are only now becoming available, as in the case of the low-density lipoprotein receptor, where tissue-specific up-regulation of receptor population may successfully compete with other cholesterol-lowering agents (104,105). Anti-sense oligonucleotides (106) have, for example, been shown to inhibit viral replication and expression of HIV-1 in chronically infected cells (107,108). The production of catalytic antibodies as designer enzymes has also proven to be a viable technology (109). On the horizon are the numerous opportunities which the mapping of the human genome should provide (110). Both the recent successful identification of the cystic fibrosis gene (111-113), and the acceptance of a "common language" for the actual mapping work (114), have provided new impetus to initiate this decades-long task in earnest.

The development of recombinant DNA technology into a fully integrated component of the drug discovery process, although only beginning, is inevitable (115). In 1987, Nobel Laureate Arthur Kornberg remarked that "...the two cultures, chemistry and biology, [are] growing further apart even as they discover more common ground " (116). However, the broad area of drug development might qualify as one such meeting place for medicinal chemistry and molecular biology where the trend is reversing. The application of genetic engineering techniques to biochemical and pharmacological problems will facilitate the discovery of novel therapeutics with potent and selective actions. The extent of the impact that molecular biology will have on the drug discovery process is, and will be for some time, unknown. However, the reality of recombinant protein therapeutics offers the assurance that this same technology, in conjunction with structural biology, computer-assisted molecular modeling, computational analysis, and medicinal chemistry, will help make possible better therapies for those diseases already controllable, and new therapies for diseases never before treatable.

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Chapter 32: Catalytic Antibodies: A New Class of Designer Enzymes

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Introduction - Catalysis of chemical reactions is an enduring goal of science. However, the unlimited ability to select or program specific catalysis for *any* desired chemical transformation of *any* particular reactant has not yet been achieved. Recently, dramatic progress has been made toward this goal, through the development of a new class of designer enzymes, the catalytic antibodies. The vast repository of potential "catalytic active sites" available via the immune system and clonal selection presents a tantalizing picture. Initial advances resulted from the pivotal integration of the chemists' molecular design ingenuity and the immunologists hybridoma technology. In less than five years, catalytic antibodies have evolved from the idea stage to demonstrated practice, though the full potential of these new catalysts remains largely untapped.

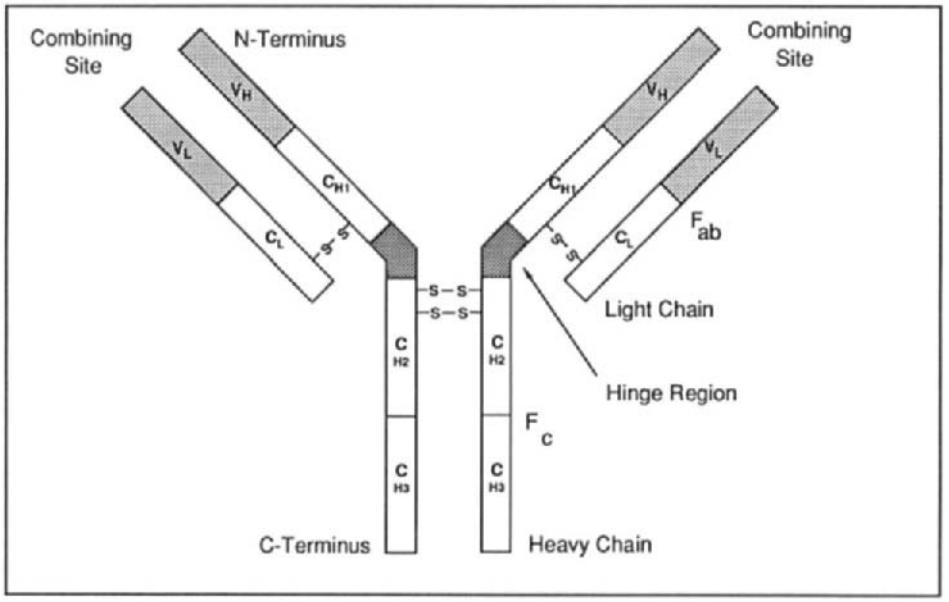
This chapter will describe some of the catalytic processes mediated by antibodies, and will discuss the potential impact that their unique features may bring to bear in the biomedical arena. For a comprehensive survey of this field, the interested reader should consult one of several excellent reviews that have been published recently (1-6).

Background - Antibodies are proteins generated by the immune system to recognize foreign entities and initiate their neutralization or destruction. They are capable of recognizing with high affinity (K_a's as high as 10^{14} M^{-1}), a diverse spectrum of molecules, ranging in size from 6 to 35 Å. A typical IgG antibody (Fig. 1) has a molecular weight of 150,000 kD, with two ligand combining regions. The combining sites are defined by the hypervariable regions of light and heavy polypeptide chains (V_L and V_H respectively), and located in the first 110 amino terminal residues of these polypeptides. More than 10^8 different antibody molecules are available through recombination of V_L and V_H genes, making the antibody pool a particularly rich and attractive source of molecular specificities (7).

The conceptual foundation for catalytic antibodies dates back to Pauling's conjecture that antibodies raised against transition state-like haptens should lower the activation free energy along the reaction coordinate leading from reactant(s) to product(s) (8). Jencks elaborated on this concept, coining the term "abzyme" to describe these antibody-enzyme hybrids (9). It was not until 1975, however, that the first experimental attempts to raise catalytic antibodies were described by Raso and Stollar. They immunized rabbits with a stable Schiff's base intermediate of a tyrosine-pyridoxal transamination reaction, but could detect no catalytic activity among affinity-purified fractions of the polyclonal sera (10,11). This failure was attributed, in large part, to difficulties associated with isolation and purification of their polyclonal antibody preparations.

In 1975, Kohler and Milstein provided an elegant solution to this problem with their pioneering development of immortalized immune cell hybrids that produced monoclonal antibodies (7,12). Since this discovery, hybridoma technology has been used to generate hundreds of thousands of antibodies with distinct epitopic binding specificities. More than a decade passed, however, before the incubation of ideas and technology culminated in successful generation of catalytic antibodies by Schultz and coworkers (13), and Tramantano and coworkers (14). Independently and simultaneously, these two groups reported that mouse monoclonal antibodies raised against tetrahedral phosphate or phosphonate haptens could catalyze the hydrolysis of carbonates (13-15) and esters (16-20). Since these initial studies, reports on amide hydrolysis (21,22), lactonization (23), aminolysis (24,25), lipase activity (26), concerted rearrangements (27-29), β-eliminations (30), cycloadditions (31), photo-cycloreversions (32), and redox reactions (33,34) have appeared.

Fig. 1. Schematic diagram of an IgG molecule.



CATALYTIC ANTIBODIES

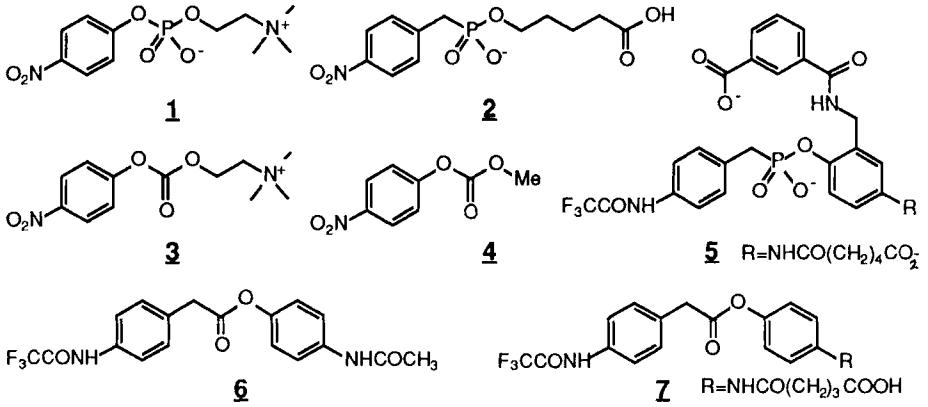
Catalytic Antibody Design -- Principles and Tools - Antibody catalysis of chemical reactions entails two elements - *recognition* and *catalytic capability.* Recognition allows the antibody to select one or a small subset of molecules from all molecules functionally capable of participating in the catalyzed chemical reaction, and this selectable recognition/specificity is a unique attribute of catalytic antibodies. Catalytic capability is the sum of several components that result in a lowered activation energy for the rate determining step of a reaction. As suggested by Pauling (8), one of these components is favorable binding interactions between antibody and reactant to stabilize a high energy conformation approximating that of the reaction transition state. A second component is minimization of unfavorable entropy associated with pre-organization or orientation of reactants. A third aspect of catalysis is the precise placement of essential catalytic functional groups or cofactors to execute the chemical transformation.

Several tools are available in the design of catalytic antibodies, the most important of which is the hapten molecule used to elicit the desired immune specificity (1-6). Hapten design is critical, since hapten structure and functionality will define or dictate the steric and electronic topology of the antibody combining site(s) ultimately selected from the immune reservoir. Thus, effective hapten design involves careful approximation of the transition state geometry of the intended reactant, and incorporation of specific functionality that will result in suitable placement of necessary catalytic amino acid residues at the combining site. The second important design tool is the direct introduction of catalytic functional groups into antibody combining sites, by directed chemical modification. After a desired specificity, perhaps with some level of catalytic capability has been elicited in a particular antibody, ligand-directed chemical modification can be used to incorporate additional catalytic groups or cofactors. A third design tool is site-directed mutagenesis. This technique, widely used for modification of enzyme active sites, will be used extensively in the future to enhance the catalytic capability of antibody combining sites, after analysis of the combining site-ligand structure.

Generation of Catalytic Antibodies - Most catalytic antibodies described to date have been generated by classical hybridoma techniques (7,12), which typically involve four steps: 1) immunization of mice with a hapten-protein carrier conjugate; 2) generation of immortalized hybrid clones by fusion of murine spleen cells with murine myeloma cells; 3) screening of individual clones for specific antibody binding to hapten, and 4) screening of hapten-specific antibodies for desired catalytic activity. This technique works reasonably well, though it is essential to completely purify the catalytic antibodies from endogenous murine enzymes that might be capable of catalyzing the same type of reaction.

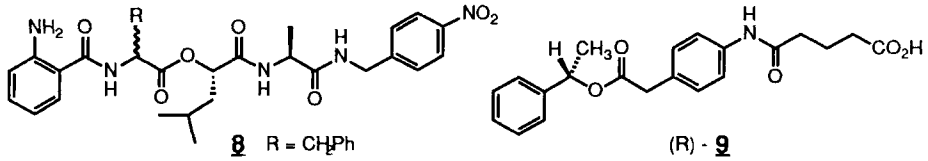
Recombinant DNA methodology is emerging as an effective technology for rapid and efficient generation of catalytic Fab fragments consisting of V_L and V_H chains. The first major advance in this area involved the cloning of the immunological repertoire into *E. Coli* by construction of a cDNA library specific for the heavy chain variable region (35). In an important follow-up to this work, libraries of cDNA for both the light and heavy variable chains were generated from harvested spleen RNA using the polymerase chain reaction (PCR), and inserted into phage λ expression vectors containing specially tailored restriction sites (36). These restriction sites were designed such that when the digested DNA was recombined, the only viable vectors were those capable of expressing combinations of V_H and V_L chains, i.e., Fabs. Using this technique it is possible to screen large numbers of phage to identify those clones that bind antigen. Similar techniques also should be applicable to generating fully intact antibodies.

Antibody Catalyzed Reactions - Hydrolytic and Transacylation Reactions - Hydrolysis of ester or carbonate bonds represented an excellent test of Pauling's transition state argument for catalytic antibodies, since formation of a negatively charged tetrahedral intermediate had been well documented as the rate determining step for the uncatalyzed reaction. Phosphate **1** or phosphonate **2** was used as a hapten to obtain monoclonal antibodies that catalyzed the hydrolysis of carbonates **3** and **4**, respectively (13, 14). Several bis- arylphosphonate haptens were similarly employed to obtain hydrolytic antibodies. Immunization with phosphonate **5** led to an antibody that catalyzed the hydrolysis of ester **6** (14). The catalysis by these antibodies represented rate accelerations of 770, 16,000, and 960, respectively, over the background hydrolysis for these reactants. For each of these antibodies, good discrimination against dissimilar substrate structures was exhibited, and competitive inhibition of hydrolysis by the hapten was observed.

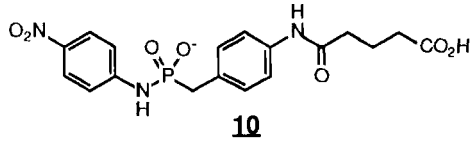


Since these initial reports, faster and more specific hydrolytic antibodies have been described. The most efficient catalytic antibody was capable of a 6.25×10^6 rate enhancement for hydrolysis of the ester **7** (17). Stereospecificity greater than 200/1 was observed in the hydrolysis of the two diastereomers of **8**, by several antibodies (20). These antibodies did not exhibit large turnover numbers; less of the binding energy is derived from interactions with the phosphonate transition state mimic, than from interactions with other substituents along the tripeptide backbone. A stereospecific lipase antibody, catalyzing only the hydrolysis of (R)-**9**, also has been described (26).

It is noteworthy that the diastereomers of **8** also are fluorogenic substrates, due to quenching of the anthranilamide fluorophore by the nitroaromatic group via resonant energy transfer. These substrates permit direct measurement of hydrolysis kinetics, obviating the need for tedious HPLC quantitation of reactants and products. This fluorescence energy transfer assay technique is emerging as a powerful method for evaluation of a broad spectrum of cleavage reactions, and in this context, will facilitate rapid screening and evaluation of large numbers of catalytic antibodies (37-39).



Amide hydrolysis, particularly as it relates to peptide bond cleavage, is an important challenge for catalytic antibodies, since peptidases with tuned sequence specificity would be extremely valuable. However, the inherent stability of amides ($t_{1/2} \approx 7$ yrs) suggests that efficient hydrolysis by antibodies would be difficult. Phosphonamidate **10**, was used to generate an antibody that hydrolyzed **11** with a rate acceleration of 250,000, relative to the background hydrolysis. Inhibition and binding studies with a hapten analog suggested that catalysis by this antibody involved more than simple transition state stabilization: some type of acid-base assistance by residues at the combining site was suspected (21). In an attempt to further accelerate amide hydrolysis, antibodies were raised to hapten **12**, containing a metal-trien cofactor. One of the antibodies obtained catalyzed the hydrolysis of peptides **13** or **14** (3.6×10^{-2} min^{-1}), but only in the presence of the metal-trien cofactor (22). These turnovers are relatively low, and improvements in catalytic efficiency will be a continuing goal.



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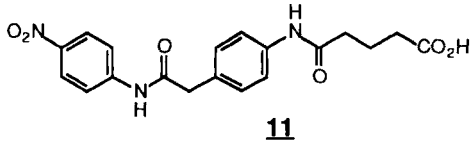

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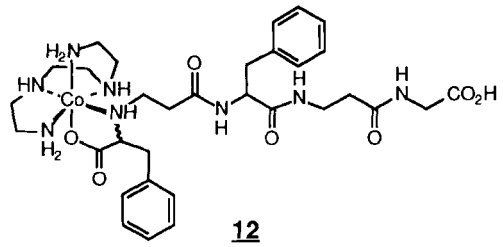

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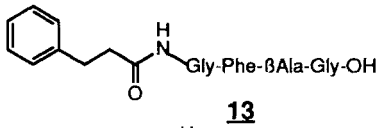

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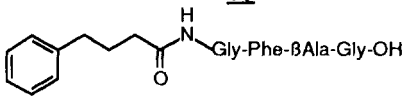

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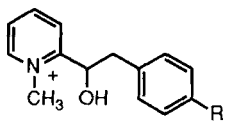
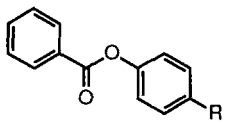
It also has been possible to generate antibodies that catalyze the conversion of esters to amides via transacylation. In these instances, the antibodies were raised using phosphonamidate haptens resembling the transacylation transition state (24,25).

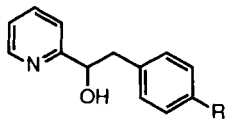
Recently, a different strategy for generating hydrolytic antibodies has been described, in which a charged amino acid was induced at the combining site using the cationic hapten **15**. This strategy, employed initially by Schultz to generate an eliminative antibody (vide infra), resulted in generation of seven antibodies that catalyzed the hydrolysis of **16**. None of the antibodies generated by the uncharged hapten **17** hydrolyzed **16**, indicating an essential role for a charged binding site group in the catalyzed hydrolysis. The pH dependence of k_{cat} for hydrolysis of **16** indicated participation of a dissociable group with a measured pK_a of 6.26 (40).



15 R = $NHCO(CH_2)_3CO_2H$

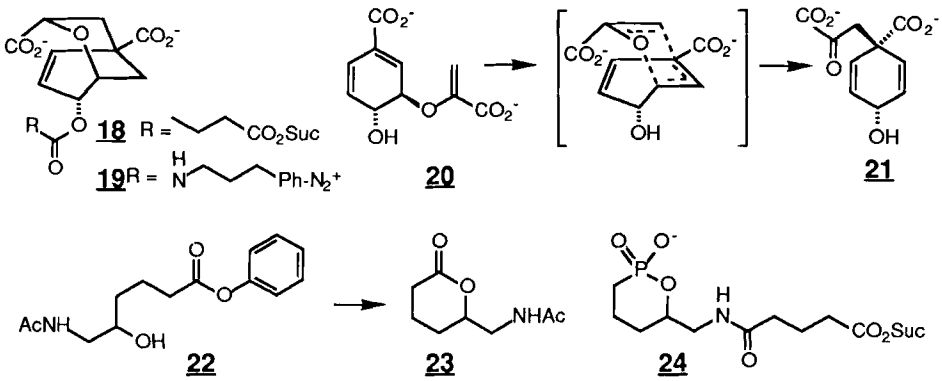


16 R = $NHCO(CH_2)_3CO_2H$

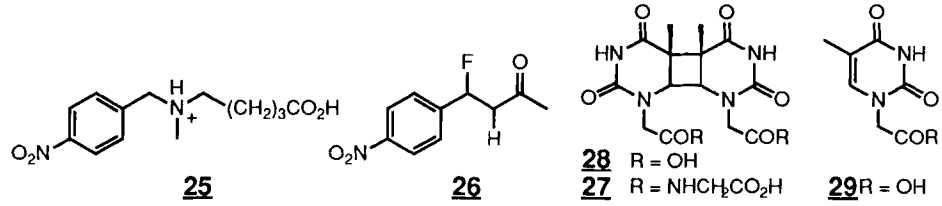


17 R = $NHCO(CH_2)_3CO_2H$

Unimolecular Cyclization Reactions - Unfavorable entropic factors associated with highly ordered transition states can be overcome by favorable binding interactions at an antibody combining site. Antibody catalysis of two different types of unimolecular reactions, a Claisen rearrangement (27-29) and lactone-forming transacylation reaction (23) has relied on antibody binding energy. The bicyclic haptens, **18** and **19**, induced antibodies that catalyzed the chorismate to prephenate Claisen rearrangement (**20**→**21**). One of these antibodies accelerated the rearrangement by 10,000 fold over the uncatalyzed rate, comparing reasonably to a rate of 3.6×10^6 for chorismate mutase from *E. coli*. Formation of a lactone (**22**→**23**) was catalyzed by an antibody raised against the cyclic phosphonate ester **24**. This represented an acceleration of 167 over the uncatalyzed solution rate. The cyclization was stereoselective, generating a 94:6 ratio of enantiomeric lactones from racemic starting material. These two reactions illustrate the powerful capability of antibody combining sites to restrict the degrees of freedom of conformationally mobile reactants, resulting in lowered entropic requirements in the rate determining steps of these unimolecular reactions.



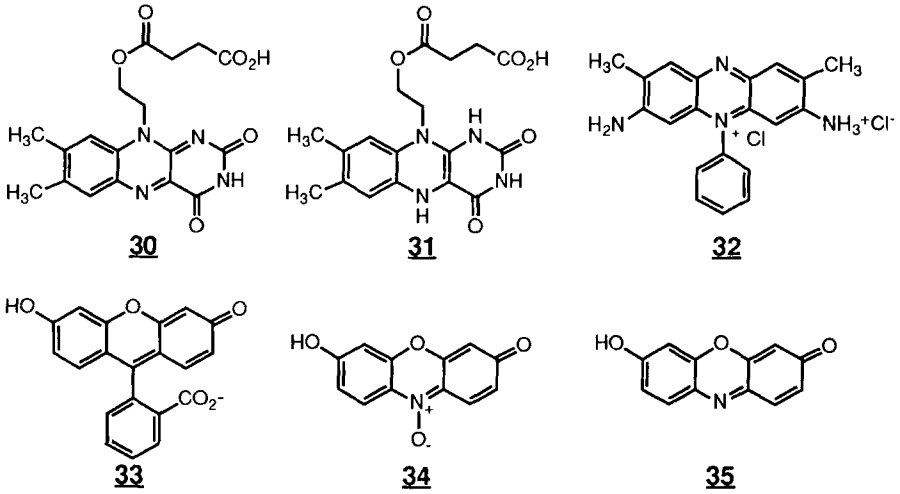
A β-Elimination Reaction - The ability to orchestrate placement of specific catalytic functional groups at an antibody combining site was first demonstrated by the generation of antibodies that catalyzed a β-elimination reaction. The tertiary amine hapten **25**, protonated under immunization conditions, induced antibodies that catalyzed the elimination of HF from **26**. (30). Studies in which antibody catalytic activity was measured as a function of pH indicated that a glutamate or aspartate residue was situated at the combining site in a position to abstract an α-proton from the substrate **26**. The 88,000 fold rate enhancement attributable to the combining site carboxylate for this reaction is comparable to accelerations mediated by carboxylate bases in several other enzymes. This tactic of inducing specific, catalytic functional groups is quite effective in generating catalytic antibodies that cannot be induced simply by imprinting transition state conformations onto the antibody combining site.



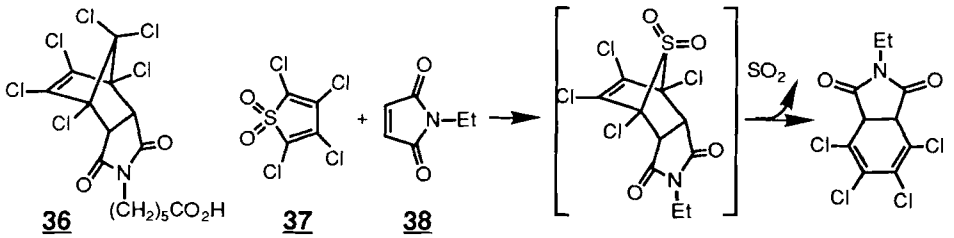
A Photo-cycloreversion Reaction - Another elegant example of catalysis brought about by specific induction of a catalytic amino acid residue at the combining site was recently described (32). It was postulated that a tryptophan residue could be induced at an antibody combining site via π-stacking interactions with a hapten containing a polarized π system. The tryptophan would then be capable of sensitizing a [2+2] photo-cycloreversion reaction. Immunization with hapten **27** resulted in generation of antibodies that bound thymine dimer **28**, and catalyzed the photo-reversion to **29** when irradiated with UV light (>300 nm).

Cofactor Mediated Reactions - Induction of cofactor binding sites and modulation of cofactor reactivity represent other strategies for the the generation of catalytic antibodies (33, 34). The flavin hapten **30** induced antibodies that lowered the reduction potential of **31** by almost 40% (33). These antibodies bound to **31**, more weakly than to **30**, but enhanced the reducing capability of **30** upon binding. Thus, **31**, normally unable to reduce compounds with reduction potentials lower than -206 mV, was capable of reducing Safranine T (**32**) (reduction potential of -289 mV). In this instance, antibodies altered the thermodynamics of a redox process, facilitating a reaction not accessible to the free flavin. In another study, fluorescein (**33**) was

used as a hapten to generate antibodies that accelerated the bimolecular reduction of resazurin by sulfite (34). In this instance, two reactant binding sites were generated by fluorescein -- one for a xanthenoid type ring, the other for the negatively charge sulfite. The binding affinities of **34** and **35** to the antibody were similar, indicating that the rate acceleration was not due to alteration of the redox potentials. The high avidity of antibody for the product limited the turnover for this reaction, since product dissociation was slow.

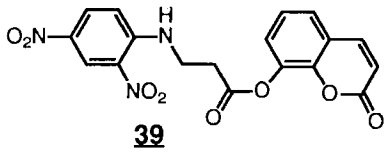


Bimolecular Cycloadditions - A Diels-Alder Reaction - Another bimolecular reaction catalyzed by an antibody was recently reported (31). Using hapten **36**, which resembled a late transition state for the [4+2] cycloaddition of **37** and **38**, several antibodies that catalyzed this Diels-Alder reaction were obtained. In this study, the key factor was selection of a cycloaddition reaction that ultimately generated, after loss of SO_2, a product structurally dissimilar from the hapten or the initial transition state. This strategy provides an excellent solution to the problem of product inhibition, which has limited the turnover capabilities of many catalytic antibodies. The generation of catalysts for multi-substrate reactions will become an important goal, since catalysts or enzymes simply do not exist for many important bimolecular processes.



Modification of Catalytic Antibodies - The introduction of functional groups at the combining site of antibodies is not limited to induction by specifically designed haptens, but can also be accomplished by specific chemical modification or site directed mutagenesis. Schultz described a technique in which thiol groups could be introduced by antigen directed affinity labeling in the vicinity of the combining site (41). These thiol groups were available for subsequent modification by a variety of functional groups (41) or spectroscopic probes (42). In one instance, a

histidine group was introduced at the periphery of the combining site of the MOPC315 Fab fragment specific for 2,4-dinitrophenyl groups. This functionalization imparted hydrolytic capability to the Fab for the coumarin ester **39** (41). Site-directed mutagenesis ($Tyr^{34L} \rightarrow His^{34L}$) of MOPC315 Fab also generated a catalytic antibody that hydrolyzed coumarin ester **40** (43).



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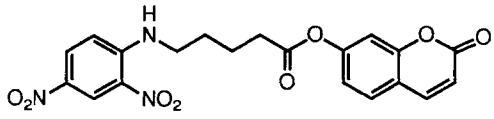

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MEDICAL AND DIAGNOSTIC APPLICATIONS OF CATALYTIC ANTIBODIES

The major promise of catalytic antibodies lies in medical and diagnostic applications that capitalize on the combination of the inherent properties of antibodies and specifically tailored catalytic properties. It has been proposed that antibodies can be generated to recognize tumor-specific antigens and then carry out catalytic reactions to mediate the destruction of those tumor cells (44). This is similar in concept to approaches involving antibody-enzyme conjugates as described by several groups (45). However, catalytic antibodies offer a number of advantages. The pharmacokinetics and distribution of antibodies is better than that of large enzyme-antibody conjugates, and catalytic antibodies are much less immunogenic than the conjugates. Many other therapeutic applications can be envisioned, in which the gross antibody structure simply provides a serum stable framework for a catalytic entity, or in which the specific antigen targeting ability of antibodies is combined with a catalytic function to carry out a desired chemical reaction. An obvious example would be a hydrolytic antibody specific for plasminogen or fibrin, to replace the use of tissue plasminogen activator in the treatment of myocardial infarction.

Enzymes have been used extensively in diagnostic applications ranging from immunoassays to biosensors. It has been proposed that the use of catalytic antibodies or catalytic Fab fragments could be used for a broad range of diagnostic applications, essentially replacing the function of enzymes in these applications (46). The realization of some of these ideas may come to pass, though the catalytic efficiencies of antibodies described to date are too poor to function effectively in these applications. The real benefit of catalytic antibodies in diagnostic applications may arise in situations where a desired catalytic reaction simply cannot be carried out by known enzymes, but can be accomplished by a specifically tailored catalytic antibody. Results obtained during the next several years will begin to establish the scope and limitations of catalytic antibodies in the diagnostic arena.

CATALYTIC ANTIBODIES - FUTURE CHALLENGES

The major challenge for scientists designing catalytic antibodies is the improvement of catalytic efficiency. Ultimately, this aspect of the problem will critically affect the extent to which this methodology is applied to medical and diagnostic problems. Although it was the similarities between antibody combining sites and enzyme active sites that stimulated the development of catalytic antibodies with a broad spectrum of catalytic capabilities, the fundamental differences between antibodies and enzymes must now be overcome. Antibodies

are relatively rigid structures, capable of binding with high affinity to antigens, while enzymes are inherently flexible molecules that permit the facile access of substrates to the active site, and the efficient dissociation of products. Scientists will need to learn how to manipulate antibody structure to impart greater flexibility and higher catalytic efficiency. Many of the tools needed to accomplish this are in hand, including site-directed mutagenesis and selective chemical modification, but an even greater emphasis will be placed on understanding and manipulating antibody structure in order to generate highly efficient catalytic antibodies.

A second challenge is to broaden the scope of reactions that can be catalyzed by antibodies. Based on the progress of the past several years, it is likely that many antibodies with novel catalytic capabilities will be developed in the near future.

A third challenge is the development of more efficient, streamlined methods for generation and production of catalytic antibodies or antibody fragments. This and other challenges presented by catalytic antibodies will demand further creative assimilation of ideas in chemistry, immunology, protein biochemistry and molecular biology.

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Section VII. Trends and Perspectives

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Chapter 33. To Market, To Market - 1989

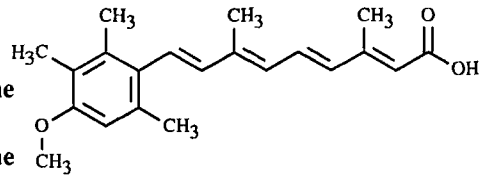
Helen H. Ong and Richard C. Allen
Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ 08876

The new chemical entities (NCEs) for human therapeutic use introduced into the world marketplace for the first time during 1989 totaled 33 in number, compared with 52 in 1988 (1) and 61 in 1987 (2). The continued decline observed in recent years could reflect a change in the world-wide regulatory climate or, to some extent, a shift of basic research emphasis to diseases of increasingly complex etiology.

As in the past, Japan held the lead by introducing 12 NCE's, followed by France and United Kingdom, each with 4 entries. Again, nearly 50% of the new launches in 1989 originated in two countries: 10 in Japan and 6 in the United States, followed by United Kingdom, West Germany, Switzerland and France. It is worth noting that while the cardiovasculars continued to outnumber other categories during 1989, agents for the treatment of CNS disorders have overtaken antiinfectives and antineoplastics as the second-ranked therapeutic class. The NCEs with novel mechanisms of action include the first aldose reductase inhibitor, tolrestat, for the treatment of diabetic complications; the first GABA aminotransferase inhibitor, vigabatrin, for use in epilepsy; and recombinant interleukin-2 for renal cell carcinoma, a previously untreatable malignant disorder.

During 1989, 21 therapeutic agents were approved in the United States, the largest number since 1985 (3). From these, as well as those approved in previous years, a total of 24 reached the marketplace, including six with significant therapeutic gains and one, bupropion hydrochloride, which was also a first world-wide introduction.

Acitretin (antipsoriatic) (4-6)

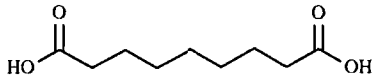


Country of Origin:	**Switzerland**
Originator:	**Hoffmann-La Roche**
First Introduction:	**Australia**
Introduced by:	**Hoffmann-La Roche**
Trade Name:	**Neotigason**
CAS Registry No.:	**55079-83-9**

Acitretin is the free acid form of etretinate useful in the treatment of severe psoriasis and other disorders of keratinization. Although the two compounds have virtually the same efficacy and teratogenic side-effects, acitretin is advantageous for child-bearing women, as its shorter half-life reduces the necessary contraception period from two years to only one month after treatment ceases.

Azelaic Acid (antiacne) (7-9)

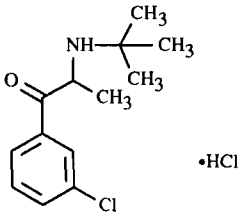
Country of Origin:	**W. Germany**
Originator:	**Schering AG**
First Introduction:	**W. Germany**
Introduced by:	**Schering AG**
Trade Name:	**Skinoren**
CAS Registry No.:	**123-99-9**



Azelaic acid is a topical antiacne agent which exerts its therapeutic action through a myriad of antimicrobial, antiproliferative and cytostatic effects. *In vitro*, azelaic acid has been shown to inhibit DNA polymerases in several tumor cell lines.

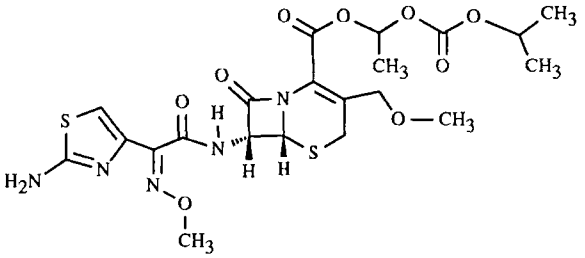
Bupropion Hydrochloride (antidepressant) (10,11)

Country of Origin:	**United Kingdom**
Originator:	**Burroughs Wellcome**
First Introduction:	**USA**
Introduced by:	**Burroughs Wellcome**
Trade Name:	**Wellbutrin**
CAS Registry No.:	**34911-55-2**



Bupropion hydrochloride, an aminoketone structurally unrelated to tricyclics or tetracyclics, is a dopamine uptake blocker with antidepressant activity. Its clinical efficacy is reportedly comparable to that of amitriptyline, yet unlike most conventional antidepressants, bupropion hydrochloride is not associated with orthostatic hypotension or other cardiovascular side-effects.

Cefpodoxime Proxetil (antibiotic) (12-14)

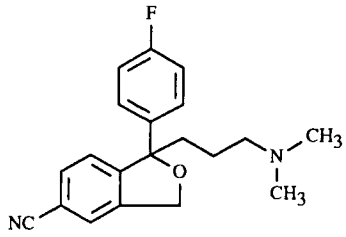


Country of Origin:	**Japan**	Introduced by:	**Sankyo**
Originator:	**Sankyo**	Trade Name:	**Banan**
First Introduction:	**Japan**	CAS Registry No.:	**87239-81-4**

Cefpodoxime proxetil is an orally active, broad-spectrum cephalosporin especially useful in the treatment of bacterial infections in children. It is a prodrug converted by esterases in the GI walls to cefpodoxime, which reportedly has a bacteriostatic spectrum comparable to injectable third-generation cephem antibiotics.

Citalopram (antidepressant) (15-17)

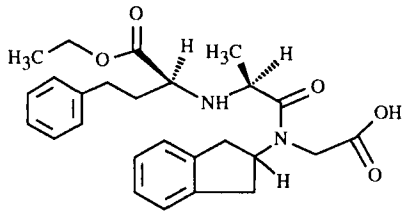
Country of Origin: **Denmark**
Originator: **Lundbeck**
First Introduction: **Denmark**
Introduced by: **Lundbeck**
Trade Name: **Cipramil**
CAS Registry No.: **59729-33-8**



Citalopram is a specific serotonin-uptake inhibitor useful in the treatment of depression. In endogenous depression citalopram was reported to be as effective as amitriptyline and mianserin, while being inferior to clomipramine in both endogenous and non-endogenous depression.

Delapril (antihypertensive) (18-20)

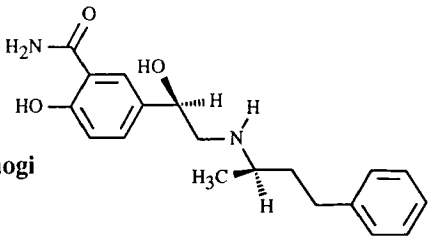
Country of Origin: **Japan**
Originator: **Takeda**
First Introduction: **Japan**
Introduced by: **Takeda**
Trade Name: **Adecut**
CAS Registry No.: **83435-67-0**



Delapril is a new angiotensin-converting enzyme (ACE) inhibitor useful in the treatment of essential hypertension. Orally administered delapril is a prodrug which is de-esterified to its active diacid metabolite. In hypertensive patients delapril significantly decreased systolic blood pressure and ACE activity 24 hours after dosing without affecting endogeneous creatinine clearance.

Dilevalol (antihypertensive) (21-23)

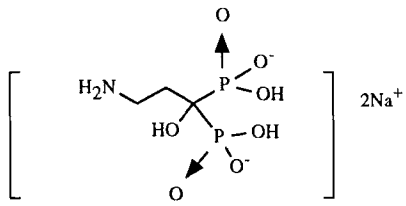
Country of Origin: **USA**
Originator: **Schering Plough**
First Introduction: **Japan**
Introduced by: **Schering Plough; Shionogi**
Trade Name: **Levadil; Dilevalon**
CAS Registry No.: **75659-07-3**



Dilevalol is a "dual-mechanism" antihypertensive reportedly suitable for a wide range of patients. Its partial $beta_2$-agonist action is expected to induce vasodilation, whereas its $beta_1$-antagonist component may protect against stress-induced hemodynamic charges without altering the cardiac output. Dilevalol is the R,R-isomer of labetalol.

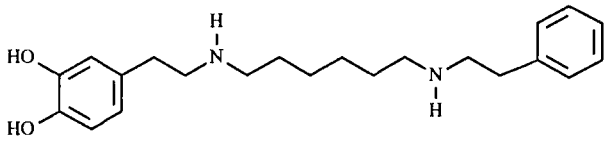
Disodium Pamidronate (calcium regulator) (24-26)

Country of Origin:	**United Kingdom**
Originator:	**Henkel**
First Introduction:	**United Kingdom**
Introduced by:	**Ciba Geigy**
Trade Name:	**Aredia**
CAS Registry No.:	**57248-88-1**



Disodium pamidronate is a calcium metabolism regulator useful in the treatment of hypercalcemia associated with malignancy. It presumably reduces the bone resorption of calcium by inhibiting the formation and attachment of osteoclasts. Other potential uses include osteoporosis and calcitonin-resistant Paget's disease.

Dopexamine (cardiostimulant) (27-29)

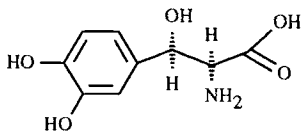


Country of Origin:	**United Kingdom**	Introduced by:	**Fisons**
Originator:	**Fisons**	Trade Name:	**Dopacard**
First Introduction:	**Ireland**	CAS Registry No.:	**86197-47-9**

Dopexamine is a dopamine analog useful in the treatment of acute congestive heart failure. Its biochemical actions include the stimulation of beta$_2$ and peripheral dopamine receptors, as well as the inhibition of neuronal uptake of norepinephrine. The resultant effect is an increase in cardiac output mediated by afterload reduction, increased blood flow and positive inotropism following iv infusion.

Droxidopa (antiparkinsonian) (30-32)

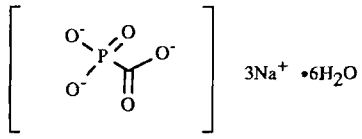
Country of Origin:	**Japan**
Originator:	**Sumitomo**
First Introduction:	**Japan**
Introduced by:	**Sumitomo**
Trade Name:	**DOPS**
CAS Registry No.:	**23651-95-8**



Droxidopa is a synthetic amino acid precursor of (-)-norepinephrine which is absorbed from the gut and metabolized to norepinephrine. In Parkinsonian patients, droxidopa added to existing levodopa/decarboxylase inhibitor therapy produces significant improvements in retropulsion, dysarthria and muscular rigidity refractory to other treatments; however, tremor was unaffected.

Foscarnet Sodium (antiviral) (33-35)

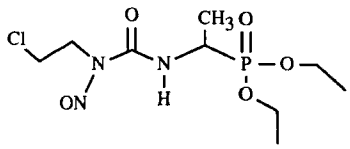
Country of Origin:	**Sweden**
Originator:	**Astra**
First Introduction:	**Sweden, Netherlands**
Introduced by:	**Astra**
Trade Name:	**Foscavir**
CAS Registry No.:	**63585-09-1**



Foscarnet sodium is a new, injectable antiviral agent useful in the treatment of severe cytomegalovirus retinitis in immunodepressed patients. Foscarnet sodium acts by inhibiting viral-specific DNA polymerases; its side-effects include decreased hemoglobin levels and acute tubular necrosis.

Fotemustine (antineoplastic) (36,37)

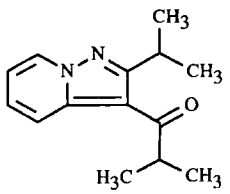
Country of Origin:	**France**
Originator:	**Servier**
First Introduction:	**France**
Introduced by:	**Servier**
Trade Name:	**Muphoran**
CAS Registry No.:	**92118-27-9**



Fotemustine is a new nitrosourea reportedly effective in the treatment of disseminated malignant melanoma, with only mild hematological toxicity. Response rate is generally lower in patients with colonic, breast, head and neck cancer.

Ibudilast (antiasthmatic) (38,39)

Country of Origin:	**Japan**
Originator:	**Kyorin**
First Introduction:	**Japan**
Introduced by:	**Kyorin**
Trade Name:	**Ketas**
CAS Registry No.:	**50847-11-5**



Ibudilast is a leukotriene antagonist and phosphodiesterase inhibitor useful in the treatment of bronchial asthma. It antagonizes leukotriene D_4-induced contractions of guinea pig ileum and tracheal muscles *in vitro*, and inhibits eosinophil accumulation *in vivo*.

γ-Interferon (antiinflammatory) (40-42)

Country of Origin:	**USA**	Introduced by:	**Biogen, Rentschler**
Originator:	**Biogen, Shionogi**	Trade Name:	**Polyferon**
First Introduction:	**W. Germany**		

Recombinant γ-interferon developed by Biogen has been launched for the treatment of rheumatoid arthritis. Other indications under study include nasopharyngeal carcinoma, basal cell carcinoma, chronic hepatitis B and adjunct immunotherapy for cancer.

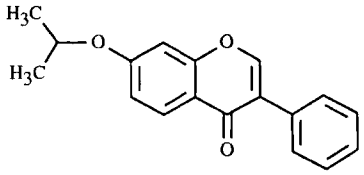
Interleukin-2 (antineoplastic) (43-45)

Country of Origin:	**USA**	Introduced by:	**Farmos**
Originator:	**Cetus**	Trade Name:	**Proleukin**
First Introduction:	**Denmark**		

Cetus has introduced a stable rDNA interleukin-2 (rIL-2) for the treatment of renal cell carcinoma. Recent studies showed that rIL-2 in combination with lymphokine activated killer cells or tumor infiltrating lymphocytes induced regression or partial regression in patients with advanced metastatic cancers.

Ipriflavone (calcium regulator) (46-48)

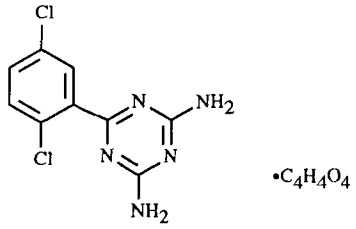
Country of Origin:	**Japan**
Originator:	**Chinoin**
First Introduction:	**Japan**
Introduced by:	**Takeda**
Trade Name:	**Osten**
CAS Registry No.:	**35212-22-7**



Ipriflavone, a derivative of isoflavone, is a calcium metabolism regulator useful in the treatment of primary and secondary osteoporosis, as well as disorders of osteogenesis. It appears to be without significant side-effects.

Irsogladine (antiulcer) (49,50)

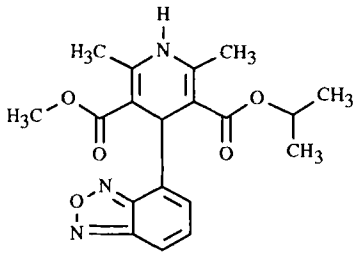
Country of Origin:	**Japan**
Originator:	**Nippon Shinyaku**
First Introduction:	**Japan**
Introduced by:	**Nippon Shinyaku**
Trade Name:	**Gaslon-N**
CAS Registry No.:	**57381-33-6**



Irsogladine is a new once-daily cytoprotective agent useful in the treatment of ulcer. In experimental animals irsogladine is active in a variety of models including stress-, indomethacin-, and histamine-induced ulcers.

Isradipine (antihypertensive) (51-53)

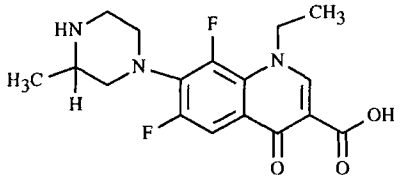
Country of Origin:	**Switzerland**
Originator:	**Sandoz**
First Introduction:	**United Kingdom**
Introduced by:	**Ciba Geigy**
Trade Name:	**Prescal**
CAS Registry No.:	**75695-93-1**



Isradipine is a long-acting calcium blocker effective in the treatment of essential hypertension. The advantages of isradipine as compared with other dihydropyridine calcium antagonists include a "gentle" onset and fewer contraindications. It can be taken by patients with diabetes mellitus, chronic obstructive airway diseases, gout, congestive heart failure or ischemic heart disease.

Lomefloxacin (antibiotic) (54-56)

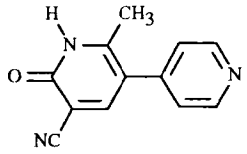
Country of Origin:	**Japan**
Originator:	**Hokuriku Seiyaku**
First Introduction:	**Argentina**
Introduced by:	**Searle (Monsanto)**
Trade Name:	**Uniquin**
CAS Registry No.:	**98079-51-7**



Lomefloxacin is a once-daily, third-generation quinolone antibiotic useful in the treatment of bacterial infections. The new fluorinated quinolone does not interfere with the metabolism of theophylline; it is efficacious against pathogens resistant to cephalosporins, penicillins and aminoglycosides.

Milrinone (cardiostimulant) (57-59)

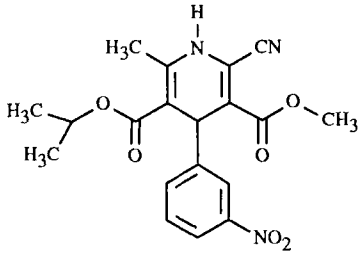
Country of Origin:	**USA**
Originator:	**Eastman Kodak (Sterling)**
First Introduction:	**Netherlands**
Introduced by:	**Eastman Kodak**
Trade Name:	**Corotrope**
CAS Registry No.:	**78415-72-2**



Milrinone, an inhibitor of phosphodiesterase selective for fraction III PDE, exerts a positive inotropic effect on the heart as well as a peripheral vasodilatory effect. Milrinone given intravenously produces significant improvements on cardiac output, pulmonary capillary wedge pressure and vascular resistance without significant effects on the heart rate.

Nilvadipine (antihypertensive) (60-62)

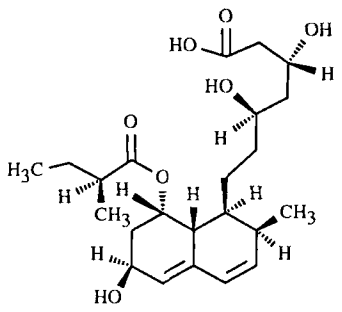
Country of Origin:	**Japan**
Originator:	**Fujisawa**
First Introduction:	**Japan**
Introduced by:	**Fujisawa**
Trade Name:	**Nivadil**
CAS Registry No.:	**75530-68-6**



Nilvadipine is a new, second-generation calcium channel blocker effective in the treatment of hypertension and angina pectoris. Its potent inhibitory effect on the stimulated chemotaxis of smooth muscle cells and protective action against calcium deposition suggest that nilvadipine may be useful for preventing and treating atherosclerosis.

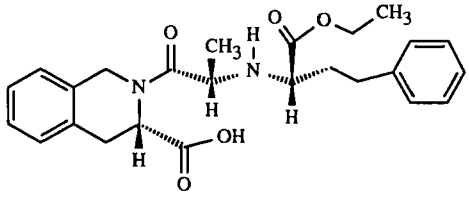
Pravastatin (antilipidemic) (63-66)

Country of Origin:	**Japan**
Originator:	**Sankyo**
First Introduction:	**Japan**
Introduced by:	**Sankyo**
Trade Name:	**Mevalotin**
CAS Registry No.:	**81093-37-0**



Pravastatin is the third HMG-CoA reductase inhibitor introduced for the treatment of atherosclerosis. Compared with lovastatin and simvastatin launched earlier, pravastatin is equipotent as an HMG-CoA reductase inhibitor *in vitro*, yet it is reported to be more tissue-selective.

Quinapril (antihypertensive) (67-69)

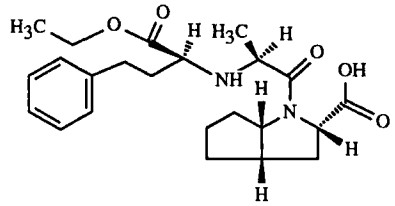


Country of Origin:	**USA**	Introduced by:	**Warner-Lambert**
Originator:	**Warner-Lambert**	Trade Name:	**Accupro**
First Introduction:	**Italy**	CAS Registry No.:	**85441-61-8**

Quinapril is an orally active, non-sulfhydryl ACE inhibitor reportedly useful in the treatment of essential hypertension. The main claimed advantage of quinapril compared with other available ACE inhibitors is its side-effect profile. It is also effective in the management of congestive heart failure.

Ramipril (antihypertensive) (70-72)

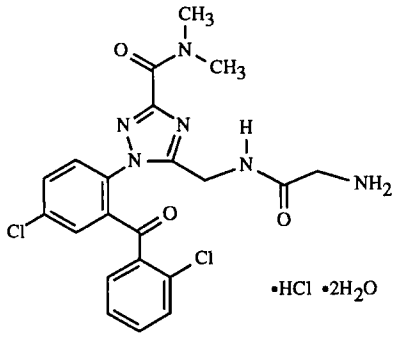
Country of Origin:	**W. Germany**
Originator:	**Hoechst AG**
First Introduction:	**France**
Introduced by:	**Labs Hoechst**
Trade Name:	**Triatec**
CAS Registry No.:	**87333-19-5**



Ramipril, a prodrug of ramiprilat, is a long-acting, tissue-specific and non-sulfhydryl ACE inhibitor useful in the treatment of mild to moderate hypertension. In animal studies ramipril exerted cardioprotective effects resembling those of bradykinin in cardiac reperfusion injuries. Ramipril is also reportedly useful in congestive heart failure.

Rilmazafone (hypnotic) (73,74)

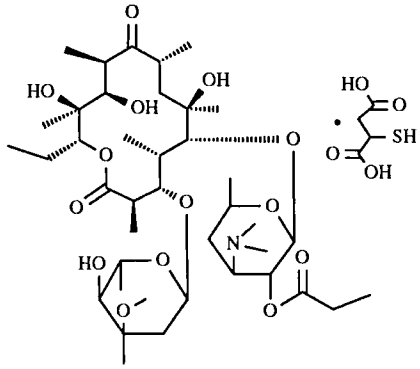
Country of Origin:	**Japan**
Originator:	**Shionogi**
First Introduction:	**Japan**
Introduced by:	**Shionogi**
Trade Name:	**Rhythmy**
CAS Registry No.:	**85815-37-8**



Rilmazafone is a new hypnotic with anxiolytic properties. The major advantages of rilmazafone are claimed to be a significant reduction of motor ataxia and the lack of habituation or hang-over associated with other hypnotics.

RV-11 (antibiotic) (75)

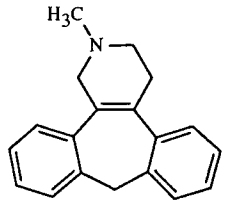
Country of Origin:	**Italy**
Originator:	**Refarmed**
First Introduction:	**Italy**
Introduced by:	**Pierrel**
Trade Name:	**Zalig**
CAS Registry No.:	**84252-06-2**



RV-11 is a derivative of erythromycin useful in the treatment of chronic bronchitis. It is stable in gastric acid, rapidly absorbed and has a high affinity for the respiratory tract. Additional advantages include low toxicity and a moderate mucolytic effect.

Setiptiline (antidepressant) (76,77)

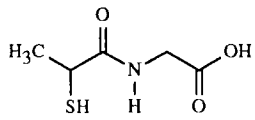
Country of Origin:	**Netherlands**
Originator:	**Organon**
First Introduction:	**Japan**
Introduced by:	**Mochida**
Trade Name:	**Tecipul**
CAS Registry No.:	**57262-94-9**



Setiptiline is a mianserin analog reportedly useful in the treatment of depression with a favorable side-effect profile. It is a potent antagonist of alpha-receptors, with greater affinity at $alpha_2$ than $alpha_1$ sites, as well as an inhibitor of central serotonin receptors.

Tiopronin (urolithiasis) (78-80)

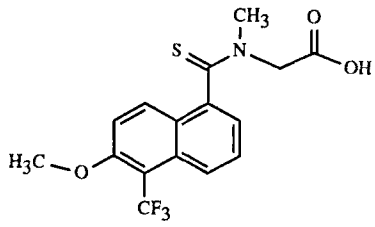
Country of Origin:	**Japan**
Originator:	**Roussel-Uclaf**
First Introduction:	**France**
Introduced by:	**Santeen**
Trade Name:	**Thiola**
CAS Registry No.:	**1953-02-2**



Tiopronin is a sulfhydryl derivative of N-propylglycine useful in the treatment of cystine urolithiasis in children. It is also reportedly effective in the management of rheumatoid polyarthritis, possibly due to its inhibitory effect on the free oxygen radicals produced by inflammatory macrophages and granulocytes.

Tolrestat (antidiabetic) (81-83)

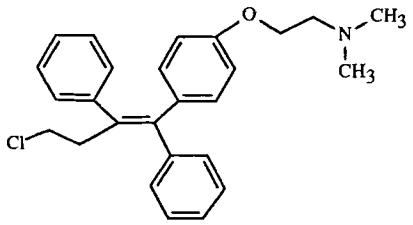
Country of Origin:	**USA**
Originator:	**American Home Products**
First Introduction:	**Ireland**
Introduced by:	**American Home Products**
Trade Name:	**Alredase**
CAS Registry No.:	**82964-04-3**



Tolrestat is a long-acting aldose reductase inhibitor reportedly useful in the prophylaxis of diabetic neuropathy, retinopathy and cataracts.

Toremifene (antineoplastic) (84-87)

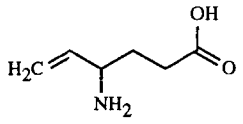
Country of Origin:	**Finland**
Originator:	**Farmos**
First Introduction:	**Finland**
Introduced by:	**Farmos**
Trade Name:	**Fareston**
CAS Registry No.:	**89778-26-7**



Toremifene is an antiestrogenic anticancer agent effective in the treatment of advanced recurrent breast cancer in postmenopausal patients. It is claimed to be more effective than tamoxifen in several rat mammary tumor models. Side-effects are mild, including hot flushes and sweating.

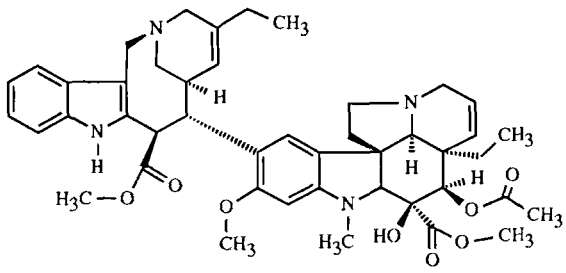
Vigabatrin (anticonvulsant) (88-91)

Country of Origin:	**United Kingdom**
Originator:	**Merrell Dow**
First Introduction:	**United Kingdom**
Introduced by:	**Merrell Dow**
Trade Name:	**Sabril**
CAS Registry No.:	**60643-86-9**



Vigabatrin, the gamma-vinyl derivative of GABA, is a new anticonvulsant reportedly effective in the treatment of intractable seizures unresponsive to currently available therapy. Mechanistically vigabatrin is a potent irreversible GABA aminotransferase inhibitor which modifies the enzyme's active-site by Michael addition. Other potential indications have been suggested for vigabatrin, including depression and schizophrenia.

Vinorelbine (antineoplastic) (92,93)

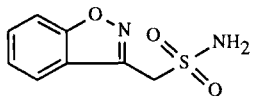


Country of Origin:	**France**	Introduced by:	**Pierre Fabre**
Originator:	**CNRS**	Trade Name:	**Navelbine**
First Introduction:	**France**	CAS Registry No.:	**71486-22-1**

Vinorelbine is a semisynthetic vinca alkaloid differing from vinblastine in the catharantine moiety of the molecule. It is claimed to have a broad spectrum of action both *in vitro* and *in vivo*; clinically it has been found effective in the treatment of non-small cell lung cancer, advanced breast cancer, ovarian cancer and Hodgkins disease.

Zonisamide (anticonvulsant) (94-96)

Country of Origin:	**Japan**
Originator:	**Dainippon**
First Introduction:	**Japan**
Introduced by:	**Dainippon**
Trade Name:	**Exegran**
CAS Registry No.:	**68291-97-4**



Zonisamide is a broad-spectrum antiepileptic effective in the treatment of refractory seizures. In cultured spinal cord neurons, zonisamide blocks the sustained firing of action potentials induced by depolarizing steps of current injected across the membrane.

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Chapter 34 . Significance of Drug Stereochemistry in Modern Pharmaceutical Research and Development

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Introduction - The evolution of the role of stereochemistry in drug development has depended on accumulating knowledge of the synthesis and analysis of chiral molecules and the stereoselective nature of the interaction between drugs and endogenous macromolecules in man. For decades, synthetic drugs and other useful organic molecules have been utilized in enantiomerically pure form as well as in defined racemic mixtures depending on current manufacturing capabilities and other practical considerations. Historically, sponsors have been free to choose between a racemate or enantiomer when identifying a candidate for drug development. Choices have been made on a case-by-case basis considering pharmacologic, toxicologic, metabolic, medical, chemical and economic factors. Limitations in synthetic and separation technologies and the origin (i.e., natural or synthetic) and availability of synthetic intermediates have been the most influential factors. New technologies now allow synthetic obstacles, met in drug development with molecules that contain one or more stereogenic centers, to be more readily negotiated than before.

Advances in chemistry and biology and recognition of the complexities associated with working with racemic drugs, coupled with pressures from regulatory agencies, encourage that more attention be given to the role of stereochemistry in the pharmacology, toxicology, metabolism, safety, effectiveness, manufacture and control of developmental drugs. Uncertainty over the possibility of new regulatory requirements pertaining to drug stereochemistry and the continued acceptability of racemic drugs by worldwide regulatory agencies is causing a reassessment of established development strategies and planning for drugs currently under development. While the regulatory environment is in transition, proceeding with the development of the racemic form of any new drug must be carefully considered. For such drug candidates, time-proven development sequences and strategies may require modification to accommodate the potential for additional regulatory requirements for both racemates and enantiomers. How pharmaceutical manufacturers are adapting to the problem of racemates currently under development and how new drug candidates are chosen today when stereochemistry is an important emerging regulatory issue, is the subject of this perspective. The chapter discusses drug stereochemistry in terms of racemates and single enantiomers. A third category, a mixture of diastereomers, is not specifically discussed. It is recognized that with additional complexity, much of what is discussed about racemates can be extended to mixtures of diastereomers.

Background - The significance of drug stereochemistry in pharmacology was first recognized over 70 years ago by Cushny (1). A decade later this was extended to pharmacokinetics (2) and drug metabolism (3). Despite a long held awareness of the stereoselective nature of drug interaction in animals and man, synthetic drugs that contain one or more stereogenic centers are still developed and marketed as racemates. In earlier decades, emphasis in medicinal chemistry was placed on empirically established structure-activity relationships rather than on drug-macromolecule (i.e., enzyme or receptor) interactions. While it was recognized that certain drugs were acting as inhibitors of enzymatic action, the chemist's ability to assess the importance of drug-macromolecule interactions was only later extended by X-ray crystallography and computer-based 3-dimensional color-graphic molecular modeling. Until synthetic approaches became available to allow the medicinal chemist to realize chiral synthesis and resolution on a commercial scale and until analytical methods became available for the development and control of processes leading to enantiomerically pure chiral drugs, there was little practical reason for the medicinal chemist to be overly concerned with the stereoselective nature of drug action. The established dogma has been, if a drug product is safe, effective, consistently manufactured and adequately labeled, it should be approved and introduced for use regardless of whether it contains a racemate or a pure enantiomer. It has been suggested that, overall, about 25% of the drugs marketed today are racemates (4). Data from 1982 show that while 88% of chiral synthetic drugs were marketed as racemates, 98% of natural or semi-synthetic chiral drugs were marketed as single enantiomers and of chiral drugs introduced between 1983 and 1985, 95% were in the racemic form (5).

An event which raised the prominence of issues of stereochemistry in pharmaceutical research and development was the publication of a manuscript in 1984 entitled, "Stereochemistry, a Basis for Sophisticated Nonsense in Pharmacokinetics and Clinical Pharmacology" by Professor E. J. Ariens (6). This article and the series of articles that followed (4,5,7-14), criticized the practice of conducting pharmacokinetic and pharmacodynamic studies on racemic drugs and ignoring the separate contributions of the individual enantiomers. According to Professor Ariens, almost every conceivable difference in metabolic conversion and/or action or interaction of enantiomers has been observed (5). These papers have served to crystallize some of the important issues surrounding racemic drugs and have stimulated much discussion in industry, academia and government. A recent and comprehensive review, appraising the enantioselective aspects of drug action and disposition, is available (15).

As the result of pharmacokinetic differences and pharmacodynamic interactions between the effects of enantiomeric species and/or their metabolites, the activity and disposition of a racemic drug may be more complex than "the sum of its parts." In a racemate, the inactive isomer and/or its metabolites may agonize or antagonize pharmacologic and/or toxicologic activities. Thus, studies with, and/or development of, a racemic drug can lead to misleading or misinterpreted data and

ultimately impact on approvability and/or labeling. Furthermore, therapeutic drug monitoring of racemic drugs, which disregards the implications of stereochemistry, can be meaningless (16).

Advances in synthetic organic chemistry now make possible, in many cases, the large scale manufacture of enantiomerically pure chiral drugs and advances in asymmetric catalysis and enzymatic kinetic resolution have significantly reduced their cost of manufacture. Catalytic asymmetric induction on an industrial scale and synthetic approaches to the synthesis of enantiomerically pure drugs have been recently reviewed (17-19). Improvements in the separation of enantiomers have been realized through the development of new resolving agents and through the use of enzymes for the kinetic resolution of racemates. Advances in biochemistry and microbiology have improved the efficiency of chiral transformations mediated by enzymes. Preparative HPLC, using columns containing chiral chromatographic substrates or achiral substrates using solvent systems containing chiral solutes, now make possible the production of pilot scale quantities of enantiomerically pure chiral drugs, in sufficient quantities to conduct limited toxicology and even initial clinical studies. Unwanted enantiomers, resulting from chemical or biochemical resolutions, may be recycled using organic chemical or biochemical processes, further improving the economics of manufacture. Since the potency of a drug containing a single enantiomer can be greater than that of a drug that contains the corresponding racemate, reduction of drug substance requirements, combined with efficient synthesis, can produce favorable economics for the manufacture of drugs containing pure enantiomers.

Analytical methods essential for the control of manufacture of enantiomerically pure chiral drugs rely heavily on HPLC. Reviews of the use of enantioselective chromatographic separations for drug development have been recently published (20-22). NMR in the presence of chiral shift reagents is also utilized in the analysis of chiral molecules. Using chiral reagents to produce diastereomeric derivatives, which can be separated and assayed, although an older approach, is still widely used. New chiral derivatizing reagents of high enantiomeric purity continue to be developed.

While the state-of-the-art of synthetic organic chemistry is sufficiently advanced such that the large scale production of enantiomerically pure chiral molecules is feasible and often cost effective, this has not resulted, as yet, in the routine practice of choosing enantiomers over racemates. This is due, in part, to historical precedents, concerns over economics, absence of a synthetic breakthrough at a decision point, need to control isomeric purity, and a multitude of other issues that must be faced during drug development. As synthetic organic and analytical chemistry technologies continue to be refined and practiced, the choice of enantiomers over racemates is becoming more common. However, there will remain cases where a racemate may be the candidate of choice when it exhibits a desirable pharmacologic and/or toxicologic profile.

REGULATORY CONSIDERATIONS

The thalidomide tragedy of the early 1960s resulted in significant regulatory ramifications. At the time of thalidomide's development as a new drug, resolution of racemic mixtures was not routine and the pharmacologic and toxicologic evaluation of the enantiomers of thalidomide was not pursued prospectively. The teratogenicity of thalidomide and the consequences of its approved use in Europe and its limited investigational use in the United States have stimulated research on the pharmacology and toxicology of its enantiomers (23,24). The biological activities of the two enantiomers differ significantly and this has led to speculation that the tragic consequences could have been avoided had the drug been made available in the form of a single enantiomer rather than as a racemate (25).

U.S. Regulatory Environment - The 1962 Kefauver-Harris Amendments to the Food, Drug and Cosmetic Act (F D & C Act), which followed thalidomide, strengthened the Act by tightening safety requirements for investigational drugs and for marketed drugs. The Amendments also added drug efficacy to approval requirements, mandated registration of drug manufacturers and their compliance with good manufacturing practices. The Amendments did not specifically address drug stereochemistry. The F D & C Act is silent on the specific subject of drug stereochemistry, but Section 505(b)(1)(D) requires a full description of the methods used in manufacture and this would include tests for identity, strength, quality and purity. Therefore, molecular structure characterization and, by implication, characterization of drug stereochemistry, is required. Title 21 of the Code of Federal Regulations (CFR) is similarly silent on drug stereochemistry. Currently, the only formal FDA regulatory issuance that specifically addresses drug stereochemistry is, "Guideline for Submitting Supporting Documentation in Drug Applications for the Manufacture of Drug Substances," which is associated with the "NDA Rewrite" (26). In three important contexts, it provides general guidance with regard to chemical and biological characterization of drug substances which contain stereogenic centers. They are as follows:

II.D. **Requirements for a New Drug Application, Manufacture of the Drug Substance:**
"It should be noted that (even in racemates) enantiomers may be considered as impurities."

II.F.2.b. **Requirements for a New Drug Application, Drug Substance Controls, Physical Properties:**
"For a drug substance with chiral centers or other configurational requirements, the specifications and tests should assure that material (whether single enantiomer or isomer, a racemate, or a known ratio of isomers) with the requisite properties for therapeutic activity has been produced."

III.A.1.a. Requirements for an Investigational New Drug (IND), New Chemical Entity, Phases 1 and 2, Physical and Chemical Characteristics:

"When the NDS is asymmetric (e.g. contains one or more chiral centers, or has cis-trans or other types of isomers), the sponsor should ideally (and prior to the submission of an IND) have either separated the various potential stereoisomers of the NDS or synthesized them independently. Physical/chemical information about each stereoisomer should be provided (in detail), or may be requested. Individual stereoisomers may need to be studied for pharmacological and toxicological properties (and/or safety and efficacy)."

The guideline suggests that an "inactive" stereoisomer may be treated as an impurity even if it represents as much as 50% of the drug substance. Thus, with appropriate characterization, control and justification, racemates can be sanctioned under the "NDA Rewrite." However, the possibility of having to provide detailed chemical and biological characterization of the individual enantiomers of a drug substance prior to filing an IND has raised concerns over the premature imposition of stringent chemical and biological characterization requirements for drugs which contain stereogenic centers. This suggestion has prompted inquiries and requests for clarification as sponsors plan the development of new drug candidates.

Insight to current FDA thinking concerning the development of enantiomers and racemates as new drugs can be gained from recent presentations and publications by FDA reviewers who are actively involved in the stereochemistry issue. These should be considered to be "quasi-official" regulatory guidances, but, aside from formal case-by-case discussions with reviewers about specific drug entities, this kind of information is all that may be available until a draft guideline is issued. The importance of stereochemistry in pharmaceutical research and development was appropriately emphasized by positioning an article entitled, "FDA Perspective on the Development of Stereoisomers," as the lead article in the premier issue of a new journal, "Chirality" (27). The article describes the current FDA regulatory environment concerning the approval of drugs containing either racemates or enantiomeric species. A more recent article entitled, "Considerations in the Development of Stereoisomeric Drugs: FDA Viewpoint" describes recent activities at FDA aimed at the development of formal regulatory guidance for the development of drugs which contain stereogenic centers (28). In early 1989, in response to concerns expressed by sponsors of new drugs, the Center for Drug Evaluation and Research (FDA), organized an internal "Stereoisomer Committee." The committee is composed of a cross-section of scientific disciplines (i.e., chemistry, pharmacology-toxicology, biopharmaceutics and medicine) within FDA. The group is responsible for drafting and finalizing guidelines, obtaining outside comments and implementing a scientifically rational approach to the regulation of drugs which

contain stereogenic elements. The results of these efforts are anticipated later this year in the form of an FDA draft guideline.

In response to the aforementioned drug substance NDA guideline and with the intent of providing an industrial perspective to Agency deliberations on the issue of drug stereochemistry, the Pharmaceutical Manufacturers Association (PMA) has published a position paper entitled "Comments on Enantiomerism in the Drug Development Process" (29). The paper recognizes advances in chemistry which make the production and analysis of enantiomerically pure chiral drugs more feasible and suggests that racemates may still be viable candidates for drug development when appropriately justified by their safety and effectiveness and when necessitated by technological, economic and social factors (e.g., a breakthrough AIDS drug). It provides points to consider in the development of drugs which contain stereogenic centers, identifies remaining technological difficulties in the development of enantiomeric drugs, provides examples of where the decision to develop a racemic drug might be justified, and suggests that rational drug development strategies may include switching from the racemic to enantiomeric form of a drug when supported by appropriate pharmacokinetic and metabolic bridging comparisons.

International Regulatory Environments - In the international sphere, the EEC and Japan have expressed views on the subject of drug stereochemistry in drug registrations. An EEC "Notice to Applicants," issued in December, 1988, requires that, if a drug contains a chiral center, the stereochemical form used in human and animal studies as well as the stereochemical form intended for marketing purposes, must be specified in its registration dossier (30). The sponsor must also provide information concerning separation of enantiomers that were used in studies of the drug. The "Expert Report" section of the dossier must address batch-to-batch consistency of the ratio of enantiomers in the drug and contain a discussion of human and animal pharmacologic, toxicologic, and pharmacokinetic and metabolic considerations. It should also include a discussion of clinical considerations with relationship to drug stereochemistry. Preclinical and clinical results should be compared in light of possible differences in the pharmacokinetics and metabolism between animals and man. The Notice also advises that where a pure isomer has been developed from a previously marketed racemate, a complete registration dossier on the enantiomer will be required. In recent meetings with industry, the Japanese Ministry of Health has explained registration guidelines for drugs which contain stereogenic centers (31). For a racemic drug, the sponsor must provide information on the toxicity, pharmacology, and pharmacokinetics and metabolism of each isomer. For a drug containing only one enantiomer, information concerning extent of interconversion of the isomers must be provided and if the "inactive isomer" constitutes a major impurity, then its pharmacology, toxicology and disposition must be described.

Today, when developing worldwide registration strategies for new drugs, sponsors must have an awareness of current regulatory

requirements in all countries where registration is anticipated and it is prudent to plan to satisfy the requirements of the country which has the most stringent requirements. The current state of uncertainty in definitive regulatory requirements, worldwide, for drugs which contain stereogenic centers, requires taking a conservative approach when developing registration strategies. Thus, as a practical matter, enantiomers are being chosen over racemates for drug development.

CURRENT TRENDS

The pharmaceutical industry is acutely aware that issues of drug stereochemistry pervade all aspects of research and development efforts. This feature of organic chemistry is now considered from the screening of new compounds for pharmacologic activity and initial toxicology to the negotiation of licensing agreements between companies. It is recognized that racemic drugs are considerably more complex than drugs that contain only a single enantiomer. Most of the pharmaceutical industry still prefer to approach the issue of drug stereochemistry on a case-by-case basis, but the tendency today is to prefer the development of pure enantiomers unless the racemate exhibits a more favorable biological profile. Some companies have established formal corporate policies requiring, whenever feasible, development of enantiomers. Others are avoiding the issue of stereochemistry and its attendant complications by avoiding, whenever possible, the development of drugs which contain stereogenic centers.

Chemical research activities now tend to focus early on the synthesis or isolation of enantiomeric species in order to make available supplies for initial preclinical pharmacologic, toxicologic, pharmacokinetic and metabolic characterization. These are enantiomers that may show promise by virtue of the activity of a parent racemate or for which the requisite stereochemical configuration is predicted. Racemates or enantiomers may be screened initially but if a racemate is active, many companies follow this with the screening of the individual enantiomers. Once the initial pharmacologic and toxicologic profiles of the enantiomers are established, companies are tending, when practical, to choose the drug development candidate early and these choices are favoring enantiomers. Once a development candidate is well into clinical development or once long-term toxicology studies have been initiated, sponsors may wish to avoid the complications and possible development delays attendant in bridging between racemates and enantiomers. Occasionally, bridging between racemates and an enantiomer is necessitated by the need to proceed with development in the absence of a synthesis or method of isolation for an enantiomer. Here the strategy is to conduct preclinical and possibly clinical development to assess the viability of the racemate in its own right or possibly as a probe for future development of an enantiomer. The initiation of long-term toxicology studies of a racemate early in development may be disadvantageous and is avoided unless the commitment to the racemate is strong. On the other hand, long-term toxicology studies can be delayed while the pilot-scale preparation of a pure enantiomer is being pursued. Such delays can impact other drug development activities.

In drug substance production and drug product manufacture, working with racemates can simplify some issues and make others more complex. The use of enantioselective assays, while as yet not universally practiced in the pharmaceutical industry, is becoming the norm when working with drugs that contain stereogenic centers. These assays are applied to the control of manufacture as well as in preclinical and clinical research as a tool in pharmacokinetic and metabolic studies. Racemization is always a concern when dealing with a single enantiomer and must be assessed at all stages of production and testing.

SUMMARY AND CONCLUSIONS

Considering drug stereochemistry in pharmaceutical research and development is central to rational drug development efforts. Economic and technologic practicalities are largely responsible for the prevalence of racemic drugs in the marketplace. As the result of new synthetic and analytical capabilities, regulatory agencies are now raising more questions about drug stereochemistry and its importance in the various pharmacologic and toxicologic actions of drugs. Pharmaceutical companies are responding by utilizing new technologies and strategies required to support the registration of new drugs in a more demanding worldwide regulatory environment. The additional layer of research and development effort required by the increased attention being given to considerations of stereochemistry is adding cost and time to drug development efforts in many companies. Sponsors with racemic drugs currently in development face special challenges and may need to carefully reassess their commitment to chosen development candidates. Under certain circumstances and with proper justification, racemic drugs are still viable candidates for drug development. Both enantiomeric and racemic drugs are associated with their own special challenges. It can be expected that racemic drugs will continue to be developed, but mainly in exceptional situations.

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Chapter 35. Twenty Five Years of Annual Reports in Medicinal Chemistry

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Introduction - With the publication of the current volume, number 25, of Annual Reports in Medicinal Chemistry (ARMC) we come to the first quarter century mark for this yearly compendium of publications which deal with research activities in the field. We can gain some appreciation for the state of medicinal chemistry in 1965 by considering the drugs then available in major therapeutic categories. Intensive research on steroids had resulted in the availability of a host of adrenocorticoids, most of which are still used today; related research in the field of gonadal steroids had led to the introduction of the anabolic steroids. Hindsight tells us that the most fundamental contribution from this area of research consisted of the estrogen progestin combination oral contraceptives. Antimicrobial agents comprised mainly sulfonamides and modified penicillins from fermentation; research on semisynthetic penicillins was well underway following the isolation of 6-aminopenicillanic acid in 1960. The determination of the structure of cephalosporin at about the same time resulted in research programs based on that nucleus. Treatment of non-insulin dependent diabetes with sulfonylureas was by then well established. Drugs for the treatment of psychosis and depression would differ only slightly for many years since the phenothiazines and tricyclics were being used routinely by 1965; the new anxiolytic agent, diazepam had just been introduced. In contrast, drugs for the treatment of cardiovascular disease were to undergo some major changes in the ensuing 25 years: in 1965 antihypertensive therapy consisted of combinations of a diuretic (the majority of those listed in the PDR today were available by 1965) and reserpine or hydralazine. Digoxin was the venerable standby as a cardiotonic agent.

The undeniable major advances since 1965 should, however, be placed in perspective against events in the 25 years which preceded the publication of Volume 1 in this series. That interval takes us back to 1940, a time when the local anesthetics represented one of the few products from medicinal chemistry available to clinicians; the sulfonamide antibiotics had been only recently introduced and synthetic analgesics (meperidine, methadone) and antimalarials (atabrine) were still largely in early stages of development. This chapter presents a very personal and admittedly subjective view of progress (or lack thereof) over the past 25 years in selected areas of medicinal chemistry as revealed in the pages of ARMC.

Since its very inception, ARMC has been divided into clearly defined sections, one devoted to discussions of specific therapeutic areas and the other to reviews of more general topics related to drug design and development. The four chapters which comprise the initial section of the book are all related to therapeutic agents.

It is reasonable to assume that the therapeutic areas which are included for review are those which are being actively pursued by industry. A review of the changing titles in these sections probably reflects in a very general way the ebb and flow of research targets.

CNS Agents - Volume 1 opens with a chapter on "Antipsychotic and Antianxiety Agents"; the lasting interest in the former is demonstrated by the fact that the first half of the title persists to Volume 25. The changing contents of that chapter provide a particularly apt illustration of progress in the field of CNS pharmacology. The section in Volume 1 devoted to neuroleptic agents deals largely with tricyclics related to phenothiazines and to butyrophenones, while the discussion of pharmacology is restricted to comments on behavioral assays. A perusal of the corresponding chapter in the most recent volume shows the virtual absence of tricyclics or butyrophenones; newer agents consist largely of benzamides, dopamine autoreceptor agonists and agents acting at 5-HT and sigma sites. The discussion of the mode of action of these compounds shows a similar fundamental change, being cast in terms of interaction with subsets of dopamine receptors. In spite of this advanced understanding, only a few new clinical agents such as sulpiride or loxapine have been introduced in the past 25 years. The second part of the title, "...Antianxiety Agents" is represented in the first volume by a group of then new benzodiazepines. The growing research in the field, spurred by the mushrooming sales of anxiolytics, led in 1975 to a separate chapter; this has since appeared on a fairly frequent basis including Volume 24. Until about 1980, this chapter largely chronicled the impressive amount of work devoted to the synthesis of benzodiazepines. The identification of the benzodiazepine receptor in all of its intricacies is duly recorded in these chapters. The clinical efficacy of the piperazine-based compound, buspirone, noted in an isolated comment in Volume 15, led in a few years to a profound change in the contents of the chapter. The availability of this first anxiolytic which operated on a non benzodiazepine site led to new chemistry and recognition of the involvement of 5HT receptors.

The discussion of control of depression in "Antidepressants, Stimulants and Halucinogens" initially dealt preponderantly with the tricyclics and MAO inhibitors; it is of note in passing that the role of neurotransmitter reuptake inhibition, the mechanism of action of the tricyclics, was already recognized in 1965. This chapter, which has appeared almost every year, including 1988, has reflected the significant amount of research aimed at overcoming the known shortcomings of those agents such as slow onset of action and effects on cardiac conduction. Some of the drugs reported in that period, such as the ill fated zimeldine, could be regarded as topological analogues of the tricyclics. The non tricyclic trazodone, reported in the late 1970's, is reportedly free of some of those shortcomings.

A chapter on "Analgetics" has appeared in some form in virtually every volume, including the present one. The persistence of the topic is most easily accounted by the ubiquity of the target, pain, and the seemingly inevitable side effect induced by effective agents, dependence. The discussion at first included both central and peripheral analgetics ("strong" and "weak" in Volume 1). The discussion was dominated over the next few years, as a result of the approval of pentazocine, by the search for mixed agonist-antagonists. Molecular pharmacology

had one of its early successes in this field with the discovery of the opioid receptor in the early 1970's and the endogenous ligands, the enkephalins and endorphins, shortly thereafter. This information, together with the subdivision of the opiate receptor into at least three subtypes, gave new direction to the search for better analgesics. This new knowledge is not yet reflected in compounds introduced during the period; these include the mixed agonist-antagonists butorphanol and alfentanil, a highly potent but classical opioid.

"Anorexigenic Agents" proved of more limited interest, last appearing as such in 1965, and being briefly revived in 1975 as "Agents Affecting Appetite". "Sedatives, Hypnotics, Anticonvulsants, Muscle Relaxants and General Anesthetics" persisted as a discrete title only until 1968; the contents then underwent steady attrition as many of those therapeutic goals were fulfilled by drugs from other classes, most prominently the benzodiazepines.

Cardiovascular and Pulmonary Agents - The majority of chapters in this section, formerly entitled "Pharmacodynamic Agents", persist to the present volume. A chapter on "Antihypertensive Agents", for example, has appeared in every volume to date. The bulk of the discussion in the first Volume is devoted to compounds related to guanethidine, a class of little if any current interest. A portent of things to come is however provided by very brief mention in 1965 of the new ß-blockers, pronethalolol and propranolol. Recognition of the growing importance of this class of drugs is demonstrated by inclusion of a subsection specifically devoted to this class of drugs in Volume 11. The veritable flood of analogues led to the appearance of a parallel chapter devoted specifically to ß-blockers in Volume 14. Prazosin, an important antihypertensive drug, and one which was to play an important role in elucidation of the nature of the α-adrenergic receptor, was described in Volume 9. Captopril, prototype for numerous ACE inhibitors, was fittingly described by its developers in a special chapter "Inhibitors of the Renin Angiotensin System" in Volume 13. The chapter in Volume 25 reflects the importance granted to that system today, with half the pages devoted to renin inhibitors, angiotensin II receptor antagonists and ACE inhibitors.

Antiarrhythmic and antianginal agents have also been reviewed on a fairly continual basis either as separate topics or in a common chapter. This may have been due to the not infrequent occurrence of the syndromes in combination as well as the perceived common therapeutic utility of cardiodepressant drugs. The utility of propranolol and local anesthetics for the former indication was noted in Volume 1; a number of better tolerated compounds which take their point of departure from local anesthetics, such as flecainide and mexiletine, have been introduced in the intervening period. Treatment of angina has taken a direction not anticipated when this series started. The well-established effectiveness of the calcium channel blockers for this indication has spurred a large amount of work on new agents as well as on further elucidation of the role of calcium transport on cell function. It is of passing interest that the antianginal activity of verapamil, which predates the beginning of the series, was originally attributed to adrenergic blocking activity. The first of the dihydropyridines, nifedipine, was described in Volume 8 and diltiazem in Volume 9.

The review on pulmonary and antiallergy agents, which has appeared on an almost constant basis, was for many years devoted to reviews of antihistaminic compounds (H1 in today's parlance) and ß-adrenergic agonist bronchodilators. The arrival of the anti-asthmatic mediator release inhibitor chromolyn sodium, described in Volume 7, led to intensive efforts in many laboratories. The goal of many of these programs was an orally active analogue since chromolyn needs to be administered by insufflation. The model chosen almost universally to assess activity consisted in determining the effect of the test compound on passive cutaneous anaphylaxis (PCA). Significant sections of the chapter for many years have consisted of reviews of compounds designed as orally active chromolyns. Though a significant number of these went to the clinic, most were found wanting when tried on human asthma. Volume 15 includes a chapter on "Slow-Reacting Substances". This chapter which includes the structures of eicosanoids, though without their present names, proved to be a portent of the contents of more recent versions of this chapter which are devoted predominantly to leukotrienes and their antagonists, or synthesis inhibitors.

The chapter dealing with GI disease appeared on an approximately biennial basis until the advent of cimetidine; it then became a yearly feature under the name of "Agents for the Treatment of Peptic Ulcer Disease" starting in 1980. The discussion of antiulcer drugs in the first chapter on this topic consisted of a review of antisecretory anticholinergic agents. It is of note that metoclopramide, which has served as the prototype for antiemetic agents, is described in the same chapter as a "gastric modifier" (quotation marks from source). Speculations on the use of prostaglandins in the treatment of GI disease (see below) appeared quite early in Volume 6. The first specific histamine H2 antagonists, burimamide, appears in this chapter in Volume 8. This comprised the main theme for the chapter in Volume 14 entitled "Histamine Receptors", by one of members of the team responsible for cimetidine.

Research on diuretics reached its pinnacle in the decade preceding ARMC with the introduction of a host of thiazide diuretics. Recognition of the utility of these agents in the treatment of hypertension led to their widespread use. The drawbacks of existing diuretics, which involve mostly their tendency to cause decreases in serum potassium, led to further research in this area. Chapters on this topic persisted into the 1970's. The last of these, in Volume 15, interestingly still deals largely with modifications on the benzenesulfonamide theme.

Chemotherapeutic Agents - The availability of modern antibiotics has been credited with a more important role in prolonging life expectancy than all other medical advances combined. It is thus not surprising to find a discussion of this area of therapeutics in every volume of ARMC to date. What started out as separate chapters on "Antibiotics and Related Substances" and "Synthetic Antibacterial Agents" merged under a single title by 1971.

The original chapter on antibiotics shows that research on semisynthetic penicillins was by then well underway. Three of the amides of 6-APA mentioned, oxacillin, cloxacillin, and dicloxacillin, eventually went on to become entries in the PDR, as did the partly synthetic cephalosporins, cephalothin and cephaloridine. The

companion chapter on synthetic antibacterials interestingly includes a foresighted brief paragraph on a new gram negative agent, nalidixic acid. The recent surge of work on synthetic antibacterials which have grown out of this very early lead resulted eventually in the synthesis of an impressive number of very effective antibacterial agents based on the quinolone nucleus. This intensive work led to the appearance of chapters on "Quinolone Antibacterials" in Volumes 21 through 23. The mode of action of nalidixic acid was attributed in Volume 1 to "...specific inhibition of DNA synthesis...", a not unreasonable consequence of the currently accepted mechanism of action for this class - inhibition of DNA gyrase.

The continuing appearance of new resistant strains of microorganisms has led to ongoing work on new antibiotics intended to overcome that resistance. Appropriate modification of both the penicillin and cephalosporin nucleus, by attachment of ever more complex side chains, has led to antibiotics with very wide spectra of activity. Another rich source of new drugs has come from further examination of natural sources. Imipenem is a semisynthetic derivative of the fermentation product, thienamycin, a beta lactam which contains an unsaturated carbocyclic ring in place of the sulfur containing ring in penicillin. The monobactams, of which azathreonam is an example, consist of beta lactams which entirely dispense with a fused ring. A related theme, of which moxalactam is an approved representative, consists of compounds in which the nucleus itself is modified by partial synthesis. This brief account admittedly omits equally significant work on less familiar classes of antimicrobial agents reported year by year in ARMC.

Mortality and morbidity caused by viral disease approach those due to microbial agents; consequently, a chapter on "Antiviral Agents" has appeared in almost every volume. In considering the relatively slow progress in this area, it must be recalled that the target in this case involves a mammalian cell which differs from a normal one only in that it incorporates a small segment of aberrant DNA. Two of the subheadings in the chapter in Volume 1, Nucleosides, Amantadine, in fact forecast those structural classes which would produce the bulk of the active antiviral drugs. This is closely mirrored by the fact that all but one of the handful of approved antiviral drugs, amantadine, consist of nucleosides or their derivatives. Vidarabine (ara-A) was discussed in Volume 9; the nucleoside with the abbreviated sugar, acyclovir, in Volume 15, and ribavarin in Volume 19. The nucleoside currently in the spotlight as a treatment for AIDS, zidovudine (AZT) ironically does not appear until quite recently in Volume 23. Ironically, because the compound was actually synthesized in the 1960's as part of an antitumor program. The increasing medical and social impact of AIDS is reflected in a chapter devoted to this so far intractable disease in Volume 25.

Cancer has proven an almost equally refractory target for almost the same reasons. All evidence suggests that only very small differences in genetics and possibly biochemistry exist between normal and cancerous cells. Enormous strides have been made in the past twenty five years in the understanding of cancer cells. One can as an example point to the identification of the oncogenes, a topic discussed in separate chapters in Volumes 18 and 21. The level of effort and funding devoted to cancer chemotherapy is reflected in the fact that chapters on

this topic have appeared on an almost yearly basis starting with Volume 2. The handful of antineoplastic drugs available in 1965 has been supplemented by, among others, doxorubicin, cisplatin, etoposide and bleomycin. These drugs, which have significantly added to the oncologist's armamentarium, still lack the specificity obtainable with antimicrobial agents. The difficult accessibility of starting materials for preparing doxorubicin analogues prompted a chapter on anthracycline synthesis in Volume 14. Clinical trials on synthetic estrogen antagonists have shown these compounds, and in particular, tamoxifen, to be effective for adjuvant treatment of breast cancer. Many of the currently known estrogen antagonists were in fact developed in the early 1960's as potential non-steroid antifertility agents; this work was reviewed in Volume 2 in the long since defunct section on "Reproduction".

The irregular coverage of parasitic disease may relate to the fact that this is mainly a Third World problem. Perhaps as a consequence, resources devoted to therapeutic research are not proportional to the magnitude of the problem; malaria for example is still, worldwide, the leading cause of death from infectious disease. An additional reason for the decrease in research in antimalarial research may be attributed to the fact that the problem seemed well on the way to resolution between the synthetic antimalarials and DDT until the more recent development of resistant parasites and mosquitoes. Antifungal therapy has also received somewhat sporadic coverage, probably due to the difficulty of the target (prior to the discovery of the imidazoles such as miconazole) and the relatively low prevalence of infection in developed countries, at least prior to the advent of AIDS and the attendant opportunistic infections.

Metabolic Disorders and Endocrine Function - This section, which started out as "Endocrine and Metabolic Agents", includes a more diverse set of therapeutic targets than do the preceding ones, and as a result fewer topics which persist for the 25 years covered by the series. Atherosclerosis, under that name, or some variant of "disorders of lipid metabolism" regularly occurs as a chapter from volumes 1 through 15 and subsequently on a less regular basis, although the appearance of chapters in Volumes 24 and 25 no doubt reflects the increasing level of research devoted to this disease. The lipid lowering agent clofibrate and the bile acid sequestrant polymer cholestyramine were in advanced stages of development by 1965, though only the latter is referred to in Volume 1; the remaining few drugs in this area, which include probucol, colestipol and gemfibrozil, all appeared in the period of regular coverage. One paragraph in the chapter on "Lipoprotein Research" in Volume 15 refers to a novel competitive inhibitor of HMG CoA reductase, compactin; this was a predecessor of the newest entry in this area, lovastatin. The area of non steroid antiinflammatory agents, which saw significant research investment in the late 1960's, was regularly reviewed in the first decade of this series; this period saw the discovery of most major market entries. As in the case of many other therapeutic fields, research persisted in the hopes of discovering drugs which would be free of the major side effect, in this case a tendency to cause or exacerbate GI ulcers.

Virtually every steroid drug currently available, whether these are corticoids, progestins, androgens or estrogens, traces its origin to the intensive research devoted to this structural class in the 1950's. By the end of that decade it had

become clear to some that further synthesis was unlikely to produce agents with better activity profiles. The diminution of effort is reflected in the gradual attrition in ARMC of chapters by this title. The discovery of the prostaglandins seemed to hold promise of an equally fecund source for new drugs. The massive effort devoted to research on these substances was chronicled on an almost yearly basis in six volumes starting with #3 in 1967. (As a personal note the author can document much earlier work in the area; this consists of an Upjohn research report dated July 1961, describing the start of synthesis work on "prostaglandin" (sic)). The very difficult and elegant chemistry and pharmacology devoted to the area has been subsequently reviewed under somewhat different titles as the field expanded and changed direction; keywords have included prostacyclin, thromboxane, arachidonic acid cascade and leukotrienes. Research on these hormone-like substances has proven very fruitful in new knowledge on molecular pharmacology, providing for example an explanation for the mode of action of the NSAID's as well as a rationale for their deleterious GI effect. The number of drugs which have resulted from this extensive research have, at least to date, been disappointing. Approved drugs include oxytocic agents such as dinoprost, a compound used to regulate fertility in racehorses, fluprostenol and the antiulcer agent misoprostol. This last is not an antiulcer agent in the usual sense, but indicated for prevention of GI ulcers brought on by high dose NSAID's; the agent restores the cytoprotective effect which is inhibited by NSAID triggered diminution of prostaglandin synthesis.

ARMC very occasionally reflects changing societal mores, the first four volumes included chapters on contraceptive agents under the title "Reproduction". This was demoted to an occasional special topic when research on new agents was discontinued. This may well have been due to the then perceived difficulty in having a new agent approved in the face of the availability of the Pill. (The validity of this view is backed by the current furor over mifepristone, perhaps better known as RU 486).

Topics in Biology and Topics in Chemistry and Drug Design - The remaining sections of each book consist of reviews of subjects which are less directly targeted to medicinal chemistry research in specific therapeutic areas. These sections which are somewhat less clearly differentiated than the foregoing, contain a large number of one time articles as for example "Radioimmunoassays" in Volume 11 or "G Proteins" in Volume 23. Other chapters have persisted over the years though not always with the same title. A discussion of Quantitative Structure Activity Relations occurs at the very beginning of the series as part of a chapter of drug-receptor interactions in Volume 1. The first chapter devoted specifically to QSAR then appeared in four consecutive years from Volume 3 on. It is somewhat surprising to note that articles on this subtopic of drug design have appeared on a sporadic basis only five times since Volume 3 and most recently almost 8 years ago.

Receptors were accepted as the target for drug action long before methodology existed for probing their structure, to say nothing of their existence. It is thus not unexpected to find the presence of a chapter which carries the word "receptor" in its title in virtually every volume of the series. Progress in conceptual framework of medicinal chemistry is best visualized by comparing discussions of

receptors in some of the early volumes of the series with the elegant detailed account of those entities presented in "Structure and Function of G-Protein Coupled Receptors" which appeared in Volume 23. The next challenge for the medicinal chemist lies in devising the means for incorporating such detailed knowledge of receptor structure into drug design. The detailed picture of the receptor should help in designing the complementary small compound; it does not however readily provide a method for distinguishing de novo between agonists and antagonists. Nor does it address such factors as specificity, transport to the active site, or delivery to the organ system or a host of related considerations.

Although ARMC is intended as a succinct review of the field rather than an early alert mechanism to significant new findings, the series does often reflect new discoveries by inclusion of specific chapters prompted by recent, significant discoveries. More recently, starting with Volume 19, the ARMC has also incorporated a section devoted specifically to new chemical entities (NCE) which have been introduced as drugs into the marketplace. This chapter, entitled "To Market, to Market" which includes a succinct discussion of pharmacology, allows the reader to appraise progress in a given field at a glance. Successful launches of products which are not strictly medicinal agents can also lead to occasional reviews, such as that on "Non-nutritive Sweeteners" which appeared in Volume 17, not too long after approval of aspartame (Nutrasweet®) or that on "Alopecia" which appeared subsequent to approval of minoxidil, whose antihypertensive activity is recorded in Volume 1, as a hair growth stimulant (Rogaine®)

Authorship - The Editors in Chief and Section Editors of ARMC would seem to have done an exceptionally careful job in avoiding having these reports become a platform for any particular point of view. A perusal of the cumulative author index shows the presence of the respected authorities in various fields as well as less well known medicinal chemists. Equally important is the observation that the index contains in excess of 920 names and that only rarely is an author represented by as many as five entries. The proportion of contributions between industry and academia has remained remarkably constant. Chapters written by industrial authors, not surprisingly, predominate in the first four sections devoted to specific therapeutic areas, while those from academia form a majority of the reviews in the two Topics sections. Comparison of the first few volumes with the most recent confirms a trend to multiple authorship. Volume 1 for example consists of 23 (79%) chapters written by a single author, six by two authors and only one by three authors. The comparable numbers for Volume 25 show a reversal with 35% of the chapters written by a single author and 65% of the chapter multiauthored; seven of the latter list three names at the head of the chapter. This relatively slow trend did not result in full reversal until volume 9, stayed level until volume 17, and then again picked up to assume its present distribution roughly with volume 21.

The Table of Contents in Volume 1 of the series casts an interesting sidelight on transformations which have occurred in the pharmaceutical industry in the past 25 years. Chapters in that book were contributed by authors from an even 12 pharmaceutical laboratories. Fully seven of those companies have undergone mergers or changes in ownership since Volume 1 was published.

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adamantanium bromide	antiseptic	1984	20, 315
adrafinil	psychostimulant	1986	22, 315
AF-2259	antiinflammatory	1987	23, 325
afloqualone	muscle relaxant	1983	19, 313
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amisulpride	antipsychotic	1986	22, 316
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amosulalol	antihypertensive	1988	24, 297
amrinone	cardiotonic	1983	19, 314
amsacrine	antineoplastic	1987	23, 327
APD	calcium regulator	1987	23, 326
apraclonidine HCl	antiglaucoma	1988	24, 297
APSAC	thrombolytic	1987	23, 326
arotinolol HCl	antihypertensive	1986	22, 316
artemisinin	antimalarial	1987	23, 327
aspoxicillin	antibiotic	1987	23, 328
astemizole	antihistamine	1983	19, 314
astromycin sulfate	antibiotic	1985	21, 324
auranofin	chrysotherapeutic	1983	19, 314
azelaic acid	antiacne	1989	25, 310
azelastine HCl	antihistamine	1986	22, 316
azithromycin	antibiotic	1988	24, 298
azosemide	diuretic	1986	22, 316
aztreonam	antibiotic	1984	20, 315
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
benexate HCl	antiulcer	1987	23, 328
betaxolol HCl	antihypertensive	1983	19, 315
bevantolol HCl	antihypertensive	1987	23, 328
bifemelane HCl	nootropic	1987	23, 329
binfonazole	hypnotic	1983	19, 315
binifibrate	hypolipidemic	1986	22, 317
bisoprolol fumarate	antihypertensive	1986	22, 317
bopindolol	antihypertensive	1985	21, 324
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317
bucillamine	immunomodulator	1987	23, 329
bucladesine sodium	cardiostimulant	1984	20, 316
budralazine	antihypertensive	1983	19, 315
bunazosin HCl	antihypertensive	1985	21, 324
bupropion HCl	antidepressant	1989	25, 310
buserelin acetate	hormone	1984	20, 316
buspirone HCl	anxiolytic	1985	21, 324
butoconazole	topical antifungal	1986	22, 318

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butoctamide	hypnotic	1984	20, 316
butyl flufenamate	topical antiinflammatory	1983	19, 316
cadexomer iodine	wound healing agent	1983	19, 316
cadralazine	hypertensive	1988	24, 298
camostat mesylate	antineoplastic	1985	21, 325
carboplatin	antibiotic	1986	22, 318
carumonam	antibiotic	1988	24, 298
cefbuperazone sodium	antibiotic	1985	21, 325
cefixime	antibiotic	1987	23, 329
cefmenoxime HCl	antibiotic	1983	19, 316
cefminox sodium	antibiotic	1987	23, 330
cefonicid sodium	antibiotic	1984	20, 316
ceforanide	antibiotic	1984	20, 317
cefotetan disodium	antibiotic	1984	20, 317
cefpimizole	antibiotic	1987	23, 330
cefpiramide sodium	antibiotic	1985	21, 325
cefpodoxime proxetil	antibiotic	1989	25, 310
ceftazidime	antibiotic	1983	19, 316
cefteram pivoxil	antibiotic	1987	23, 330
cefuroxime axetil	antibiotic	1987	23, 331
cefuzonam sodium	antibiotic	1987	23, 331
celiprolol HCl	antihypertensive	1983	19, 317
cetirizine HCl	antihistamine	1987	23, 331
chenodiol	anticholelithogenic	1983	19, 317
cibenzoline	antiarrhythmic	1985	21, 325
cicletanine	antihypertensive	1988	24, 299
cilostazol	antithrombotic	1988	24, 299
cimetropium bromide	antispasmodic	1985	21, 326
ciprofibrate	hypolipidemic	1985	21, 326
ciprofloxacin	antibacterial	1986	22, 318
cisapride	gastroprokinetic	1988	24, 299
citalopram	antidepressant	1989	25, 311
clobenoside	vasoprotective	1988	24, 300
cloconazole HCl	topical antifungal	1986	22, 318
clodronate disodium	calcium regulator	1986	22, 319
cyclosporine	immunosuppressant	1983	19, 317
dapiprazole HCl	antiglaucoma	1987	23, 332
defibrotide	antithrombotic	1986	22, 319
deflazacort	antiinflammatory	1986	22, 319
delapril	antihypertensive	1989	25, 311
denopamine	cardiostimulant	1988	24, 300
diacerein	antirheumatic	1985	21, 326
dilevalol	antihypertensive	1989	25, 311
disodium pamidronate	calcium regulator	1989	25, 312
divistyramine	hypocholesterolemic	1984	20, 317
dopexamine	cardiostimulant	1989	25, 312
doxazosin mesylate	antihypertensive	1988	24, 300
doxefazepam	hypnotic	1985	21, 326
doxifluridine	antineoplastic	1987	23, 332
doxofylline	bronchodilator	1985	21, 327
dronabinol	antinauseant	1986	22, 319
droxidopa	antiparkinsonian	1989	25, 312
emorfazone	analgesic	1984	20, 317
enalapril maleate	antihypertensive	1984	20, 317
enalaprilat	antihypertensive	1987	23, 332
encainide HCl	antiarrhythmic	1987	23, 333

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enocitabine	antineoplastic	1983	19, 318
enoxacin	antibacterial	1986	22, 320
enoxaparin	antithrombotic	1987	23, 333
enoximone	cardiostimulant	1988	24, 301
enprostil	antiulcer	1985	21, 327
eperisone HCl	muscle relaxant	1983	19, 318
epidermal growth factor	wound healing agent	1987	23, 333
epirubicin HCl	antineoplastic	1984	20, 318
epoprostenol sodium	platelet aggreg. inhib.	1983	19, 318
eptazocine HBr	analgesic	1987	23, 334
erythromycin acistrate	antibiotic	1988	24, 301
erythropoietin	hematopoetic	1988	24, 301
esmolol HCl	antiarrhythmic	1987	23, 334
etizolam	anxiolytic	1984	20, 318
etodolac	antiinflammatory	1985	21, 327
exifone	nootropic	1988	24, 302
famotidine	antiulcer	1985	21, 327
felbinac	topical antiinflammatory	1986	22, 320
felodipine	antihypertensive	1988	24, 302
fenbuprol	choleretic	1983	19, 318
fenticonazole nitrate	antifungal	1987	23, 334
fisalamine	intestinal antiinflammatory	1984	20, 318
flomoxef sodium	antibiotic	1988	24, 302
fluconazole	antifungal	1988	24, 303
flumazenil	benzodiazepine antag.	1987	23, 335
flunoxaprofen	antiinflammatory	1987	23, 335
fluoxetine HCl	antidepressant	1986	22, 320
flupirtine maleate	analgesic	1985	21, 328
flutamide	antineoplastic	1983	19, 318
flutazolam	anxiolytic	1984	20, 318
flutoprazepam	anxiolytic	1986	22, 320
flutropium bromide	antitussive	1988	24, 303
fluvoxamine maleate	antidepressant	1983	19, 319
formoterol fumarate	bronchodilator	1986	22, 321
foscarnet sodium	antiviral	1989	25, 313
fosfosal	analgesic	1984	20, 319
fotemustine	antineoplastic	1989	25, 313
gallopamil HCl	antianginal	1983	19, 319
ganciclovir	antiviral	1988	24, 303
gemeprost	abortifacient	1983	19, 319
gestodene	progestogen	1987	23, 335
gestrinone	antiprogestogen	1986	22, 321
goserelin	hormone	1987	23, 336
guanadrel sulfate	antihypertensive	1983	19, 319
halofantrine	antimalarial	1988	24, 304
halometasone	topical antiinflammatory	1983	19, 320
hydrocortisone aceponate	topical antiinflammatory	1988	24, 304
hydrocortisone butyrate	topical antiinflammatory	1983	19, 320
ibopamine HCl	cardiostimulant	1984	20, 319
ibudilast	antiasthmatic	1989	25, 313
idebenone	nootropic	1986	22, 321
imipenem/cilastatin	antibiotic	1985	21, 328
indalpine	antidepressant	1983	19, 320
indeloxazine HCl	nootropic	1988	24, 304
indobufen	antithrombotic	1984	20, 319
interferon, gamma	antiinflammatory	1989	25, 314

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL., PAGE
interleukin-2	antineoplastic	1989	25, 314
ipriflavone	calcium regulator	1989	25, 314
irsogladine	antiulcer	1989	25, 315
isepamicin	antibiotic	1988	24, 305
isofezolac	antiinflammatory	1984	20, 319
isoxicam	antiinflammatory	1983	19, 320
isradipine	antihypertensive	1989	25, 315
itraconazole	antifungal	1988	24, 305
ivermectin	antiparasitic	1987	23, 336
ketanserin	antihypertensive	1985	21, 328
lenampicillin HCl	antibiotic	1987	23, 336
lentinan	immunostimulant	1986	22, 322
leuprolide acetate	hormone	1984	20, 319
levacecarnine HCl	nootropic	1986	22, 322
levobunolol HCl	antiglaucoma	1985	21, 328
levodropropizine	antitussive	1988	24, 305
lidamidine HCl	antiperistaltic	1984	20, 320
limaprost	antithrombotic	1988	24, 306
lisinopril	antihypertensive	1987	23, 337
lobenzarit sodium	antiinflammatory	1986	22, 322
lomefloxacin	antibiotic	1989	25, 315
lonidamine	antineoplastic	1987	23, 337
loprazolam mesylate	hypnotic	1983	19, 321
loratadine	antihistamine	1988	24, 306
lovastatin	hypocholesterolemic	1987	23, 337
loxoprofen sodium	antiinflammatory	1986	22, 322
mabuterol HCl	bronchodilator	1986	22, 323
malotilate	hepatroprotective	1985	21, 329
medifoxamine fumarate	antidepressant	1986	22, 323
mefloquine HCl	antimalarial	1985	21, 329
meglutol	hypolipidemic	1983	19, 321
melinamide	hypocholesterolemic	1984	20, 320
mepixanox	analeptic	1984	20, 320
meptazinol HCl	analgesic	1983	19, 321
metaclazepam	anxiolytic	1987	23, 338
metapramine	antidepressant	1984	20, 320
mexazolam	anxiolytic	1984	20, 321
mifepristone	abortifacient	1988	24, 306
milrinone	cardiostimulant	1989	25, 316
miokamycin	antibiotic	1985	21, 329
misoprostol	antiulcer	1985	21, 329
mitoxantrone HCl	antineoplastic	1984	20, 321
mizoribine	immunosuppressant	1984	20, 321
mometasone furoate	topical antiinflammatory	1987	23, 338
mupirocin	topical antibiotic	1985	21, 330
muromonab-CD3	immunosuppressant	1986	22, 323
muzolimine	diuretic	1983	19, 321
nabumetone	antiinflammatory	1985	21, 330
nafamostat mesylate	protease inhibitor	1986	22, 323
naftifine HCl	antifungal	1984	20, 321
naltrexone HCl	narcotic antagonist	1984	20, 322
nedocromil sodium	antiallergic	1986	22, 324
nicorandil	coronary vasodilator	1984	20, 322
nilutamide	antineoplastic	1987	23, 338
nilvadipine	antihypertensive	1989	25, 316
nimesulide	antiinflammatory	1985	21, 330

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL., PAGE
nimodipine	cerebral vasodilator	1985	21, 330
nipradilol	antihypertensive	1988	24, 307
nitrefazole	alcohol deterrent	1983	19, 322
nitrendipine	hypertensive	1985	21, 331
nizatidine	antiulcer	1987	23, 339
nizofenzone fumarate	nootropic	1988	24, 307
nomegestrol acetate	progestogen	1986	22, 324
norfloxacin	antibacterial	1983	19, 322
norgestimate	progestogen	1986	22, 324
octreotide	antisecretory	1988	24, 307
ofloxacin	antibacterial	1985	21, 331
omeprazole	antiulcer	1988	24, 308
ornoprostil	antiulcer	1987	23, 339
osalazine sodium	intestinal antinflamm.	1986	22, 324
oxaprozin	antiinflammatory	1983	19, 322
oxiconazole nitrate	antifungal	1983	19, 322
oxiracetam	nootropic	1987	23, 339
oxitropium bromide	bronchodilator	1983	19, 323
ozagrel sodium	antithrombotic	1988	24, 308
pefloxacin mesylate	antibacterial	1985	21, 331
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
picotamide	antithrombotic	1987	23, 340
piketoprofen	topical antiinflammatory	1984	20, 322
pimaprofen	topical antiinflammatory	1984	20, 322
pinacidil	antihypertensive	1987	23, 340
pirarubicin	antineoplastic	1988	24, 309
piroxicam cinnamate	antiinflammatory	1988	24, 309
plaunotol	antiulcer	1987	23, 340
pravastatin	antilipidemic	1989	25, 316
prednicarbate	topical antiinflammatory	1986	22, 325
progabide	anticonvulsant	1985	21, 331
promegestrone	progestogen	1983	19, 323
propacetamol HCl	analgesic	1986	22, 325
propentofylline propionate	cerebral vasodilator	1988	24, 310
propofol	anesthetic	1986	22, 325
quazepam	hypnotic	1985	21, 332
quinapril	antihypertensive	1989	25, 317
quinfamide	amebicide	1984	20, 322
ramipril	antihypertensive	1989	25, 317
ranimustine	antineoplastic	1987	23, 341
repirinast	antiallergic	1987	23, 341
rifapentine	antibacterial	1988	24, 310
rifaximin	antibiotic	1985	21, 332
rifaximin	antibiotic	1987	23, 341
rilmazafone	hypnotic	1989	25, 317
rilmenidine	antihypertensive	1988	24, 310
rimantadine HCl	antiviral	1987	23, 342
rokitamycin	antibiotic	1986	22, 325
ronafibrate	hypolipidemic	1986	22, 326
rosaprostol	antiulcer	1985	21, 332
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antiulcer	1987	23, 342
RV-11	antibiotic	1989	25, 318
schizophyllan	immunostimulant	1985	22, 326

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setastine HCl	antihistamine	1987	23, 342
setiptiline	antidepressant	1989	25, 318
simvastatin	hypocholesterolemic	1988	24, 311
sodium cellulose PO4	hypocalciuric	1983	19, 323
sofalcone	antiulcer	1984	20, 323
somatropin	hormone	1987	23, 343
spizofurone	antiulcer	1987	23, 343
sufentanil	analgesic	1983	19, 323
sulbactam sodium	B-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344
teicoplanin	antibacterial	1988	24, 311
temocillin disodium	antibiotic	1984	20, 323
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terconazole	antifungal	1983	19, 324
tertatolol HCl	antihypertensive	1987	23, 344
thymopentin	immunomodulator	1985	21, 333
tiamenidine HCl	antihypertensive	1988	24, 311
tianeptine sodium	antidepressant	1983	19, 324
tibolone	anabolic	1988	24, 312
timiperone	neuroleptic	1984	20, 323
tinazoline	nasal decongestant	1988	24, 312
tioconazole	antifungal	1983	19, 324
tiopronin	urolithiasis	1989	25, 318
tiquizium bromide	antispasmodic	1984	20, 324
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
toloxatone	antidepressant	1984	20, 324
tolrestat	antidiabetic	1989	25, 319
toremifene	antineoplastic	1989	25, 319
trientine HCl	chelator	1986	22, 327
trimazosin HCl	antihypertensive	1985	21, 333
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
vigabatrin	anticonvulsant	1989	25, 319
vinorelbine	antineoplastic	1989	25, 320
xamoterol fumarate	cardiotonic	1988	24, 312
zidovudine	antiviral	1987	23, 345
zolpidem hemitartrate	hypnotic	1988	24, 313
zonisamide	anticonvulsant	1989	25, 320
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

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gemeprost	ABORTIFACIENT	1983	19, 319
mifepristone		1988	24, 306
nitrefazole	ALCOHOL DETERRENT	1983	19, 322
quinfamide	AMEBICIDE	1984	20, 322
tibolone	ANABOLIC	1988	24, 312
mepixanox	ANALEPTIC	1984	20, 320
alfentanil HCl	ANALGESIC	1983	19, 314
alminoprofen		1983	19, 314
emorfazone		1984	20, 317
eptazocine HBr		1987	23, 334
flupirtine maleate		1985	21, 328
fosfosal		1984	20, 319
meptazinol HCl		1983	19, 321
propacetamol HCl		1986	22, 325
sufentanil		1983	19, 323
suprofen		1983	19, 324
propofol	ANESTHETIC	1986	22, 325
azelaic acid	ANTIACNE	1989	25, 310
nedocromil sodium	ANTIALLERGIC	1986	22, 324
repirinast		1987	23, 341
gallopamil HCl	ANTIANGINAL	1983	19, 319
cibenzoline	ANTIARRHYTHMIC	1985	21, 325
encainide HCl		1987	23, 333
esmolol HCl		1987	23, 334
amlexanox	ANTIASTHMATIC	1987	23, 327
ibudilast		1989	25, 313
ciprofloxacin	ANTIBACTERIAL	1986	22, 318
enoxacin		1986	22, 320
norfloxacin		1983	19, 322
ofloxacin		1985	21, 331
pefloxacin mesylate		1985	21, 331
rifapentine		1988	24, 310
teicoplanin		1988	24, 311
aspoxicillin	ANTIBIOTIC	1987	23, 328
astromycin sulfate		1985	21, 324
azithromycin		1988	24, 298
aztreonam		1984	20, 315
carboplatin		1986	22, 318
carumonam		1988	24, 298
cefbuperazone sodium		1985	21, 325
cefixime		1987	23, 329
cefmenoxime HCl		1983	19, 316

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cefminox sodium		1987	23, 330
cefonicid sodium		1984	20, 316
ceforanide		1984	20, 317
cefotetan disodium		1984	20, 317
cefpimizole		1987	23, 330
cefpiramide sodium		1985	21, 325
cefpodoxime proxetil		1989	25, 310
ceftazidime		1983	19, 316
cefteram pivoxil		1987	23, 330
cefuroxime axetil		1987	23, 331
cefuzonam sodium		1987	23, 331
erythromycin acistrate		1988	24, 301
flomoxef sodium		1988	24, 302
imipenem/cilastatin		1985	21, 328
isepamicin		1988	24, 305
lenampicillin HCl		1987	23, 336
lomefloxacin		1989	25, 315
miokamycin		1985	21, 329
rifaximin		1985	21, 332
rifaximin		1987	23, 341
rokitamycin		1986	22, 325
RV-11		1989	25, 318
sultamycillin tosylate		1987	23, 343
temocillin disodium		1984	20, 323
mupirocin	ANTIBIOTIC, TOPICAL	1985	21, 330
chenodiol	ANTICHOLELITHOGENIC	1983	19, 317
progabide	ANTICONVULSANT	1985	21, 331
vigabatrin		1989	25, 319
zonisamide		1989	25, 320
bupropion HCl	ANTIDEPRESSANT	1989	25, 310
citalopram		1989	25, 311
fluoxetine HCl		1986	22, 320
fluvoxamine maleate		1983	19, 319
indalpine		1983	19, 320
medifoxamine fumarate		1986	22, 323
metapramine		1984	20, 320
setiptiline		1989	25, 318
tianeptine sodium		1983	19, 324
toloxatone		1984	20, 324
tolrestat	ANTIDIABETIC	1989	25, 319
fenticonazole nitrate	ANTIFUNGAL	1987	23, 334
fluconazole		1988	24, 303
itraconazole		1988	24, 305
naftifine HCl		1984	20, 321
oxiconazole nitrate		1983	19, 322
terconazole		1983	19, 324
tioconazole		1983	19, 324
butoconazole	ANTIFUNGAL, TOPICAL	1986	22, 318

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cloconazole HCl		1986	22, 318
sulconizole nitrate		1985	21, 332
apraclonidine HCl	ANTIGLAUCOMA	1988	24, 297
befunolol HCl		1983	19, 315
dapiprazole HCl		1987	23, 332
levobunolol HCl		1985	21, 328
astemizole	ANTIHISTAMINE	1983	19, 314
azelastine HCl		1986	22, 316
cetirizine HCl		1987	23, 331
acrivastine		1988	24, 295
loratadine		1988	24, 306
setastine HCl		1987	23, 342
alacepril	ANTIHYPERTENSIVE	1988	24, 296
alfuzosin HCl		1988	24, 296
amosulalol		1988	24, 297
arotinolol HCl		1986	22, 316
betaxolol HCl		1983	19, 315
bevantolol HCl		1987	23, 328
bisoprolol fumarate		1986	22, 317
bopindolol		1985	21, 324
budralazine		1983	19, 315
bunazosin HCl		1985	21, 324
celiprolol HCl		1983	19, 317
cicletanine		1988	24, 299
delapril		1989	25, 311
dilevalol		1989	25, 311
doxazosin mesylate		1988	24, 300
enalapril maleate		1984	20, 317
enalaprilat		1987	23, 332
felodipine		1988	24, 302
guanadrel sulfate		1983	19, 319
isradipine		1989	25, 315
ketanserin		1985	21, 328
lisinopril		1987	23, 337
nilvadipine		1989	25, 316
nipradilol		1988	24, 307
perindopril		1988	24, 309
pinacidil		1987	23, 340
quinapril		1989	25, 317
ramipril		1989	25, 317
rilmenidine		1988	24, 310
terazosin HCl		1984	20, 323
tertatolol HCl		1987	23, 344
tiamenidine HCl		1988	24, 311
trimazosin HCl		1985	21, 333
AF-2259	ANTIINFLAMMATORY	1987	23, 325
amfenac sodium		1986	22, 315
deflazacort		1986	22, 319
etodolac		1985	21, 327
flunoxaprofen		1987	23, 335
interferon, gamma		1989	25, 314

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isofezolac		1984	20, 319
isoxicam		1983	19, 320
lobenzarit sodium		1986	22, 322
loxoprofen sodium		1986	22, 322
nabumetone		1985	21, 330
nimesulide		1985	21, 330
oxaprozin		1983	19, 322
piroxicam cinnamate		1988	24, 309
tenoxicam		1987	23, 344
fisalamine	ANTIINFLAMMATORY, INTESTINAL	1984	20, 318
osalazine sodium		1986	22, 324
alclometasone dipropionate	ANTIINFLAMMATORY, TOPICAL	1985	21, 323
butyl flufenamate		1983	19, 316
felbinac		1986	22, 320
halometasone		1983	19, 320
hydrocortisone aceponate		1988	24, 304
hydrocortisone butyrate propionate		1983	19, 320
mometasone furoate		1987	23, 338
piketoprofen		1984	20, 322
pimaprofen		1984	20, 322
prednicarbate		1986	22, 325
pravastatin	ANTILIPIDEMIC	1989	25, 316
artemisinin	ANTIMALARIAL	1987	23, 327
halofantrine		1988	24, 304
mefloquine HCl		1985	21, 329
alpiropride	ANTIMIGRAINE	1988	24, 296
dronabinol	ANTINAUSEANT	1986	22, 319
amsacrine	ANTINEOPLASTIC	1987	23, 327
camostat mesylate		1985	21, 325
doxifluridine		1987	23, 332
enocitabine		1983	19, 318
epirubicin HCl		1984	20, 318
flutamide		1983	19, 318
fotemustine		1989	25, 313
interleukin-2		1989	25, 314
lonidamine		1987	23, 337
mitoxantrone HCl		1984	20, 321
nilutamide		1987	23, 338
pirarubicin		1988	24, 309
ranimustine		1987	23, 341
toremifene		1989	25, 319
vinorelbine		1989	25, 320
ivermectin	ANTIPARASITIC	1987	23, 336
droxidopa	ANTIPARKINSONIAN	1989	25, 312
pergolide mesylate		1988	24, 308

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lidamidine HCl	ANTIPERISTALTIC	1984	20, 320
gestrinone	ANTIPROGESTOGEN	1986	22, 321
acitretin	ANTIPSORIATIC	1989	25, 309
amisulpride	ANTIPSYCHOTIC	1986	22, 316
zuclopenthixol acetate		1987	23, 345
diacerein	ANTIRHEUMATIC	1985	21, 326
octreotide	ANTISECRETORY	1988	24, 307
adamantanium bromide	ANTISEPTIC	1984	20, 315
cimetropium bromide	ANTISPASMODIC	1985	21, 326
tiquizium bromide		1984	20, 324
tiropramide HCl		1983	19, 324
defibrotide	ANTITHROMBOTIC	1986	22, 319
enoxaparin		1987	23, 333
cilostazol		1988	24, 299
ozagrel sodium		1988	24, 308
indobufen		1984	20, 319
picotamide		1987	23, 340
limaprost		1988	24, 306
flutropium bromide	ANTITUSSIVE	1988	24, 303
levodropropizine		1988	24, 305
benexate HCl	ANTIULCER	1987	23, 328
enprostil		1985	21, 327
famotidine		1985	21, 327
irsogladine		1989	25, 315
misoprostol		1985	21, 329
nizatidine		1987	23, 339
omeprazole		1988	24, 308
ornoprostil		1987	23, 339
plaunotol		1987	23, 340
rosaprostol		1985	21, 332
roxatidine acetate HCl		1986	22, 326
roxithromycin		1987	23, 342
sofalcone		1984	20, 323
spizofurone		1987	23, 343
teprenone		1984	20, 323
troxipide		1986	22, 327
foscarnet sodium	ANTIVIRAL	1989	25, 313
ganciclovir		1988	24, 303
rimantadine HCl		1987	23, 342
zidovudine		1987	23, 345
buspirone HCl	ANXIOLYTIC	1985	21, 324
etizolam		1984	20, 318
flutazolam		1984	20, 318

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metaclazepam		1987	23, 338
mexazolam		1984	20, 321
flumazenil	BENZODIAZEPINE ANTAG.	1987	23, 335
doxofylline	BRONCHODILATOR	1985	21, 327
formoterol fumarate		1986	22, 321
mabuterol HCl		1986	22, 323
oxitropium bromide		1983	19, 323
APD	CALCIUM REGULATOR	1987	23, 326
clodronate disodium		1986	22, 319
disodium pamidronate		1989	25, 312
ipriflavone		1989	25, 314
bucladesine sodium	CARDIOSTIMULANT	1984	20, 316
denopamine		1988	24, 300
dopexamine		1989	25, 312
enoximone		1988	24, 301
ibopamine HCl		1984	20, 319
milrinone		1989	25, 316
amrinone	CARDIOTONIC	1983	19, 314
xamoterol fumarate		1988	24, 312
brovincamine fumarate	CEREBRAL VASODILATOR	1986	22, 317
nimodipine		1985	21, 330
propentofylline		1988	24, 310
trientine HCl	CHELATOR	1986	22, 327
fenbuprol	CHOLERETIC	1983	19, 318
auranofin	CHRYSOTHERAPEUTIC	1983	19, 314
nicorandil	CORONARY VASODILATOR	1984	20, 322
azosemide	DIURETIC	1986	22, 316
muzolimine		1983	19, 321
cisapride	GASTROPROKINETIC	1988	24, 299
erythropoietin	HEMATOPOETIC	1988	24, 301
malotilate	HEPATROPROTECTIVE	1985	21, 329
buserelin acetate	HORMONE	1984	20, 316
goserelin		1987	23, 336
leuprolide acetate		1984	20, 319
somatropin		1987	23, 343
cadralazine	HYPERTENSIVE	1988	24, 298
nitrendipine		1985	21, 331

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brotizolam		1983	19, 315
butoctamide		1984	20, 316
doxefazepam		1985	21, 326
loprazolam mesylate		1983	19, 321
quazepam		1985	21, 332
rilmazafone		1989	25, 317
zolpidem hemitartrate		1988	24, 313
zopiclone		1986	22, 327
acetohydroxamic acid	HYPOAMMONURIC	1983	19, 313
sodium cellulose PO4	HYPOCALCIURIC	1983	19, 323
divistyramine	HYPOCHOLESTEROLEMIC	1984	20, 317
lovastatin		1987	23, 337
melinamide		1984	20, 320
simvastatin		1988	24, 311
acipimox	HYPOLIPIDEMIC	1985	21, 323
beclobrate		1986	22, 317
binifibrate		1986	22, 317
ciprofibrate		1985	21, 326
meglutol		1983	19, 321
ronafibrate		1986	22, 326
bucillamine	IMMUNOMODULATOR	1987	23, 329
thymopentin		1985	21, 333
lentinan	IMMUNOSTIMULANT	1986	22, 322
schizophyllan		1985	22, 326
ubenimex		1987	23, 345
cyclosporine	IMMUNOSUPPRESSANT	1983	19, 317
mizoribine		1984	20, 321
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sulbactam sodium	β-LACTAMASE INHIBITOR	1986	22, 326
afloqualone	MUSCLE RELAXANT	1983	19, 313
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naltrexone HCl	NARCOTIC ANTAGONIST	1984	20, 322
tinazoline	NASAL DECONGESTANT	1988	24, 312
timiperone	NEUROLEPTIC	1984	20, 323
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oxiracetam		1987	23, 339
epoprostenol sodium	PLATELET AGGREG. INHIBITOR	1983	19, 318
gestodene	PROGESTOGEN	1987	23, 335
nomegestrol acetate		1986	22, 324
norgestimate		1986	22, 324
promegestrone		1983	19, 323
alpha-1 antitrypsin	PROTEASE INHIBITOR	1988	24, 297
nafamostat mesylate		1986	22, 323
adrafinil	PSYCHOSTIMULANT	1986	22, 315
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tiopronin	UROLITHIASIS	1989	25, 318
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cadexomer iodine	WOUND HEALING AGENT	1983	19, 316
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