ANNUAL REPORTS IN MEDICINAL CHEMISTRY Volume 22

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

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

STERLING–WINTHROP RESEARCH INSTITUTE
RENSSELAER, NEW YORK

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ANNUAL REPORTS IN MEDICINAL CHEMISTRY

Volume 22

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PREFACE

Volume 22 of *Annual Reports in Medicinal Chemistry* deviates slightly in format from the previous volumes. The experimental special topic chapter begun last year and the now familiar "To Market, To Market" chapter have been combined in a new section "Special Topics," and the title (and content) of Section IV has been expanded to specifically include topics on immunology. The 32 chapters of this volume are distributed among these two sections and the standard sections: CNS Agents, Pharmacodynamic Agents, Chemotherapeutic Agents, Topics in Biology, and Topics in Chemistry and Drug Design.

In addition to the annual updates on antipsychotics, anxiolytics, sedative-hypnotics, antihypertensives, and pulmonary and antiallergy agents, several chapters are dedicated to less frequently reviewed therapeutic areas. Among these are depression, congestive heart failure, cancer, diabetes, and fungal, viral, dermatological, and peptic ulcer diseases. Juxtaposition of reviews on osteoporosis (Chapter 17) and osteoarthritis (Chapter 18) serves to contrast these disease states and their treatment. The rapidly expanding activities in the area of quinolone antibacterials reviewed in Volume 21 and again this year, promises to be a subject for regular coverage. While a chapter on analgesics is not included in this volume, a detailed review of the mediators of the pain of inflammation can be found in Chapter 24.

Sophisticated molecular approaches to drug design to modulate gene transcription (Chapter 26), neurotransmission (Chapter 28), and protein function (Chapter 29) are reviewed as is the rapidly developing field of computer modeling (Chapter 27). Two important drug delivery modalities, prodrugs and site-specific systems, are detailed in Chapter 30. The special topic of this volume (Chapter 32) discusses and clarifies the complex issues of obtaining patent protection for pharmaceutical entities.

With this volume I conclude my ten-year association with *Annual Reports in Medicinal Chemistry*. During my tenure as Editor-in-Chief we have added modestly to the format and content of the serial and in this, I hope, have added to its utility. I found the experience very stimulating and rewarding and am most grateful to the many contributors for their excellent work and to the section editors for their dedication and support. Special recognition is given to Martha Johnson, whose assistance with *Annual Reports in Medicinal Chemistry* has been invaluable.

Denis M. Bailey
Rensselaer, New York
May 1987

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Section I — CNS Agents

Editor: Barrie Hesp, Stuart Pharmaceuticals
Division of ICI Americas, Wilmington, DE 19897

Chapter 1. Antipsychotic Agents

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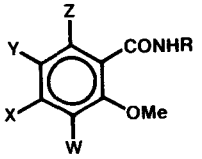
Introduction — Schizophrenia remains a devastating mental disorder and presents an extremely difficult challenge for the discovery of safe and effective therapeutics. Recent studies employing positron emission tomography have provided clear evidence of dopamine (DA) receptor proliferation in untreated schizophrenics (1). This finding may be an important underpinning for the DA hypothesis of schizophrenia, especially in light of the fact that DA antagonists are still the only established pharmacotherapy for psychosis. Nevertheless, antipsychotic medications leave much to be desired, in terms of both efficacy and side effect profile. Consequently, the search continues for DA antagonists with improved therapeutic ratios as well as novel agents that can attenuate dopaminergic neurotransmission without direct interaction at DA receptors.

While neuroleptic drugs are effective against the florid symptoms of psychosis, negative symptoms of schizophrenia, such as social withdrawal and cognitive decline, remain uncontrolled. In this regard, the organic pathology underlying chronic schizophrenia has emerged as a major topic of discussion. Recent studies have provided additional evidence for structural abnormalities in schizophrenic brains which could produce severe functional deficits (2,3). For example, hypofrontality may represent one possible outcome of degenerative change that is expressed as cognitive impairment (4). The etiology of structural abnormalities in schizophrenia is unknown, but genetic (5) and viral (6) hypotheses continue to be debated. Better comprehension of the disease process in schizophrenia holds promise for the development of improved therapeutic strategies.

Substituted Benzamides — The benzamide neuroleptics form a distinct class of drugs on the basis of two attributes — structural commonality and selectivity for D_2 DA receptors (i.e, the virtual absence of other receptor interactions). Substituted benzamides have exhibited varying propensities to induce extrapyramidal side effects (EPS). For example, the well-known anti-emetic agent, metoclopramide (**1**) was found to resemble classical neuroleptics with regard to EPS liability, based on a number of pharmacological properties including the ability to produce catalepsy, inhibit apomorphine (APO)-induced stereotypy and stimulate prolactin release in the rat (7). Early clinical studies with remoxipride (**2**) suggest that this drug is similar in efficacy to classical agents but causes less severe EPS (8-11). It was recently shown to be a very selective, low potency D_2 blocker *in vitro* (12). Remoxipride preferentially inhibits the binding of [^{3}H]spiperone to DA receptors in limbic regions (rat brain). Striatal DA receptors are blocked only to the extent of 60%, even at very high drug doses (13). However, **2** does cause a large increase in DA turnover in the striatum (14).

Raclopride (**3**) possesses very high affinity for D_2 receptors *in vitro* and binds preferentially to the striatum in the rat *in vivo* (15). This finding is most interesting since **3** is 13x more potent in inhibiting APO-induced hyperactivity than in blocking APO-induced stereotypy,

an apparent indication of limbic rather than striatal regional selectivity of action (16). Raclopride was shown to induce catalepsy at an ED_{50} dose approximately 200x higher than the ED_{50} dose for inhibition of APO-induced hyperactivity and is thus claimed to have low cataleptogenic potential (17). The preparation of [^{11}C]raclopride and its use in position emission tomographic (PET) studies in the monkey has been reported. Radioligand was found to accumulate predominantly in the striatum (18). Another new agent, eticlopride (**4**) was found to be approximately 13x more potent than haloperidol in its ability to displace [^{3}H]spiperone from rat brain striatal membranes. Eticlopride is also an extremely potent inhibitor of APO-induced hyperactivity (19). The selective binding of [^{3}H]eticlopride to D_2 receptors has been demonstrated both *in vitro* and *in vivo* and striatal selectivity has been observed (20,21). BRL 20596(**5**), an analog of the gastric motility drug, clebopride, exhibits sulpiride-like potency for D_2 receptors in rat striatum. It has the properties of a central DA antagonist, including the ability to induce catalepsy (22).



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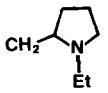
2		Br	H	H	OMe
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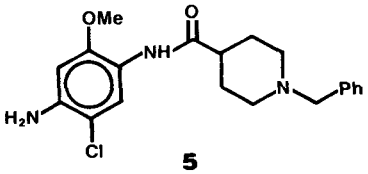

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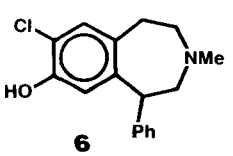
Several structure-activity studies of substituted benzamides were published recently (23,24). It was suggested that a lipophilic substituent *para* to the methoxy group is required for *in vivo* DA antagonist activity; *in vitro* activity is strongly influenced by the ability of the amide group and the *ortho* methoxyl substituent to form a coplanar six-membered pseudo-ring (23). The introduction of a second methoxyl group *ortho* to the carboxamide moiety decreases DA receptor blockade both *in vitro* and *in vivo* (24).

D_1 Antagonists — The selective D_1 antagonist, Sch 23390 (**6**), has been extensively profiled. Tritiated **6** binds with high affinity and specificity to D_1 receptors in mouse brain *in vivo* (25). However, **6** also shows high affinity for 5-HT_2 receptors in rat frontal cortex *in vivo* and *in vitro* and antagonizes the 5-HT agonist-induced syndrome (26). A radio-brominated analog of **6** was used in a PET study in the monkey to show that D_1 receptors are localized in the caudate nucleus (27). Chronic treatment with **6** significantly increased the number of D_1 but not D_2 receptors in rat striatum (28,29). When the binding of [^{3}H]**6** to post-mortem human brain from drug-treated schizophrenic subjects was studied, no increase in the number of D_1 receptors was observed (30). Lastly, the unusually long-lived *in vivo* activity of **6** is believed to be the result of persistent occupation of D_1 receptors (31).



6

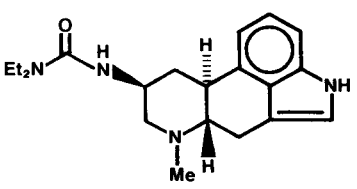
Recent electrophysiological (32,33), behavioral (34-37) and neurochemical (38) studies demonstrated the interdependence between D_1 and D_2 receptors. For example, **6** did not block the ability of APO to inhibit nigral DA cell firing, a D_2 autoreceptor-mediated effect (32). However, **6** partially or fully reversed the effects of APO on globus pallidus and pars reticulata neuronal firing rates. It was suggested that D_1 antagonism attenuates postsynaptic but not presynaptic D_2 agonist effects (32). The cataleptogenic properties of **6** can be inhibited by selective D_2 agonists (34). Conversely, **6** blocks the stereotyped behavior induced by the selective D_2 agonist, RU 24213 (35). Like haloperidol, but in contrast to metergoline or sulpiride, **6** blocked the rewarding effect of intracranial self-stimulation in the rat (37). Since haloperidol blocks both D_1 and D_2 receptors, sulpiride blocks only D_2, and **6** only D_1 receptors, it was postulated that self-stimulation of the prefrontal cortex involves D_1, not D_2 recognition sites (37). Lastly, a comparison of the effects of **6** and haloperidol on the potassium-evoked release of [^{3}H]acetylcholine from rat striatal tissue slices suggested a functional relationship between D_1 and D_2 receptors (38). Low concentrations of haloperidol increased [^{3}H]acetylcholine release, an effect which was antagonized by **6**. Compound **6** also antagonized the effects of haloperidol and spiperone on DA metabolism and plasma prolactin concentration (38). Thus, although D_1 and D_2 antagonists can exhibit similar behavioral profiles, at least certain of their neurochemical properties appear to be in opposition.

DA-Autoreceptor Agonists — DA autoreceptors are located presynaptically on DA-containing neurons of the substantia nigra (A9) and ventral tegmental (A10) areas (39). Since activation of DA autoreceptors inhibits dopaminergic neuronal activity, agonists for these presynaptic sites represent an alternative means for reducing dopaminergic neurotransmission in the forebrain (40,41). However, while DA autoreceptors seem distinct from DA receptors located postsynaptically on non-dopaminergic neurons (42,43), pharmacologically they bear close resemblance to the D_2 class of postsynaptic DA receptors (42,44). Thus, to be effective agents for reducing dopaminergic neurotransmission, DA-autoreceptor agonists must show a high degree of selectivity for autoreceptors without stimulation of postsynaptic D_2 sites, since the latter action is likely to counteract the beneficial effects of the former.

3-(3-Hydroxyphenyl)-N-n-propylpiperidine (3-PPP) is the most-studied DA-autoreceptor agonist. Extensive evidence, recently reviewed (45), indicates that (+)-3-PPP acts as a typical DA agonist with limited autoreceptor selectivity whereas (–)-3-PPP is a weak or partial autoreceptor agonist but may act as an antagonist at postsynaptic D_2 receptors. The apparently selective profile of the racemate, therefore, may result from a complex interplay between the enantiomers, which have negating effects at postsynaptic D_2 sites. This suggestion was confirmed electrophysiologically by examining the action of the racemate and enantiomers on the firing of A9 dopaminergic neurons as well as on the discharge of striatal neurons that receive a dopaminergic innervation (46). It has been claimed that neither racemic 3-PPP nor its enantiomers are effective at inhibiting DA release from striatal slices (47). Since 3-PPP is clearly effective at inhibiting dopaminergic-neuron firing (40), this result may reflect differences between DA autoreceptors on terminals, which control DA release, and those located on soma or dendrites, which influence the firing rate of dopaminergic neurons. Interpretation of the effects of 3-PPP has become more difficult in light of recent work describing high affinity, stereoselective interactions of 3-PPP with the sigma opiate site labelled by SKF-10,047 (48,49). Furthermore, drug discrimination studies have found (+)-3-PPP to either substitute for, or antagonize an SKF-10,047 cue, while (–)-3-PPP had no effect (50,51). Since compounds acting at this sigma site can alter dopaminergic function in the absence of direct interactions with DA receptors (see section on atypical antipsychotics), the action of 3-PPP on dopaminergic systems appears to be multifaceted.

Additional DA-autoreceptor agonists have been identified. Thus, **7** and **8** have been likened to (–)-3-PPP based upon similar biochemical and behavioral effects (45). The latter

compound, however, has been reported to lack DA autoreceptor selectivity (52). CGS-19845A (**9**) reduces *in vivo* dopaminergic activity according to several biochemical indices and is distinguished by good oral potency and a long duration of action (53). A series of 5-substituted-2-aminotetralins (UH-232, **10**, and UH-242, **11**) has been described in which the (–)-enantiomers act as typical DA agonists with some autoreceptor selectivity while the (+)-enantiomers appear to be selective autoreceptor antagonists (54). In contrast, previous work has shown 5-hydroxy substitution on 2-aminotetralins to be detrimental for autoreceptor selectivity, whereas 7-hydroxy-2-di-n-propylaminotetralin possessed maximal agonist potency and selectivity for autoreceptors (55). B-HT 920 (**12**) satisfies biological criteria for a selective DA-autoreceptor agonist but the compound also stimulates α_2-adrenoceptors (56). Interestingly, **12** does induce behavioral signs of postsynaptic DA receptor activation only after these sites have been rendered supersensitive by prior destruction of dopaminergic neurons (56). EMD 23,448 (**13**) continues to be of interest as a selective autoreceptor agonist because of its biochemical, behavioral (57,58) and electrophysiological profile (59).



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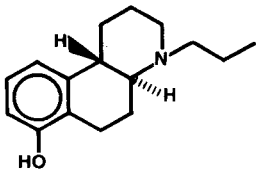

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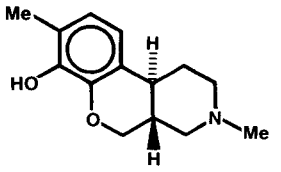

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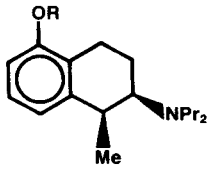

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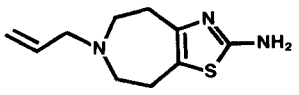

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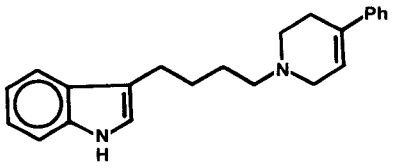

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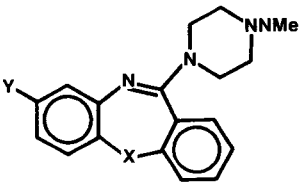
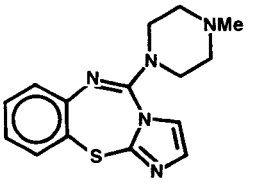
Atypical Agents — Atypical antipsychotic drugs were the subject of a review in last year's issue of this series (60). The adjective, "atypical", is used to describe a drug which is antipsychotic in man without producing acute EPS, regardless of the underlying biochemical mechanism(s). Improved efficacy compared to classical neuroleptics (e.g., amelioration of negative symptomatology) is often considered to be an additional criterion. Since the vast majority of putative atypical agents have not been fully validated in the clinic, it is useful to define an "atypical" drug *pharmacologically* as one which exhibits *in vivo* anti-dopaminergic activity in animals without inducing catalepsy. This definition can be somewhat relaxed to include agents which possess an unusually large separation between their efficacious and cataleptogenic doses (i.e., a high therapeutic ratio). It should be emphasized that the biochemical event, or events, subserving atypical activity remains a topic for speculation (60). A well-substantiated, broadly applicable theoretical explanation has not yet emerged.

Clozapine (**14**), the *bona fide* atypical drug, continues to be the subject of mechanistic investigations. The uptake and binding kinetics of [^{11}C]clozapine in various brain regions of the monkey has been studied using PET (61). It was concluded that **14** binds more transiently to striatal DA receptors than does the classical agent, N-methylspiperone. The short duration of binding of **14** to DA receptors may account for the absence of supersensitivity following chronic administration and may also explain the rapid return of schizophrenic symptoms seen after drug withdrawal (61).



	X	Y
14	NH	Cl
15	CH_2	F

The low affinity and brief timecourse of the binding of fluperlapine (15) to D_2 receptors, rather than a selective action on the limbic system, may underlie the low incidence of EPS reported for this relatively recent successor to clozapine (62). Clinical studies continue to show that 15 is efficacious with only a very low propensity to cause EPS (63-65). Another compound related to clozapine, CGS-10746B (16), decreased neostriatal DA release at behaviorally effective doses without altering DA metabolism or blocking D_2 receptors (66,67). Compound 16 was much more potent in inhibiting APO-induced climbing than stereotypy (66).



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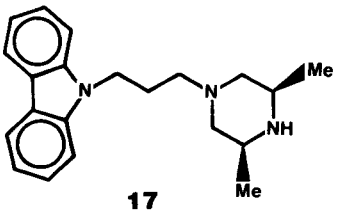
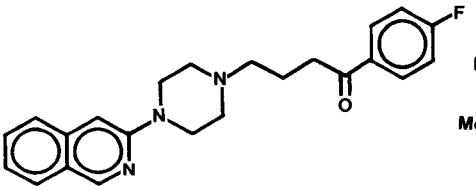


17

Rimcazole (17) is an atypical agent whose mechanism of action appears not to involve direct D_2 receptor antagonism (68). Rather, 17 is a specific, competitive inhibitor of the binding of [^{3}H]SKF-10,047 to sigma opiate sites in rat and guinea pig brain (69). Electrophysiological studies suggest that 17 has an indirect effect on DA neurons and is relatively selective for the limbic DA system (70). Another atypical agent, cinuperone (18), is also a potent ligand at the sigma receptor (71). Compound 19 was found to inhibit spontaneous locomotor activity in mice without causing ataxia and to block conditioned avoidance behavior; it did not displace [^{3}H]haloperidol from rat striatal DA receptors (72). Toxicological problems in animals have precluded clinical evaluation of this compound (72).



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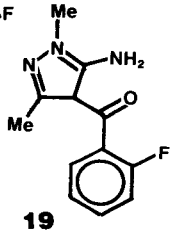

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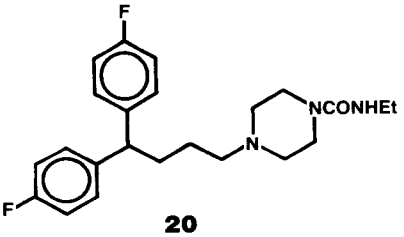
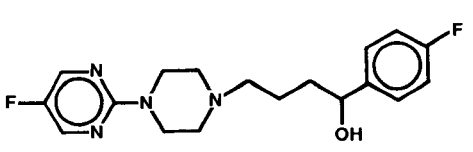


20

Behavioral studies have shown amperozide (20), an agent with extremely low affinity for D_2 receptors, to moderate limbic system dopaminergic function selectively (73). BMY-14802 (21), another potential antipsychotic drug which does not block D_2 receptors, increases DA turnover and the spontaneous firing rate of nigral DA neurons. It also reverses APO-induced depression of neuronal firing rates and the catalepsy induced by haloperidol. An indirect effect on the DA system has been postulated (74).



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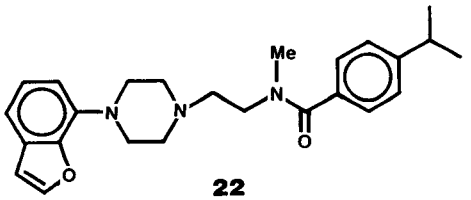
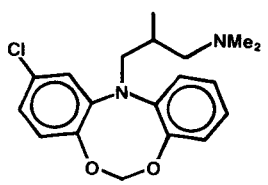


22

Befiperide (**22**) has low affinity for DA receptors *in vitro* but inhibits the conditioned avoidance response and APO-induced stereotypy (75). It does not increase DA turnover. EGYT-2509 (**23**) is a weak inhibitor of [^{3}H]spiperone binding to rat striatal DA receptors but behaves as a DA antagonist as evidenced by its effects on striatal adenylate cyclase, DA release and pituitary prolactin release (76). WY-47,384 (**24**) exhibits moderate affinity for 5-HT_2, and weak affinity for D_2 receptors (77). However, it is active in a number of biochemical and behavioral tests predictive of antipsychotic efficacy. On the basis of its more potent antagonism of APO-induced climbing behavior than stereotypy, **24** may have a low potential for causing EPS (77).



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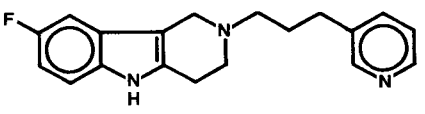

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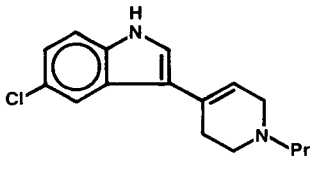

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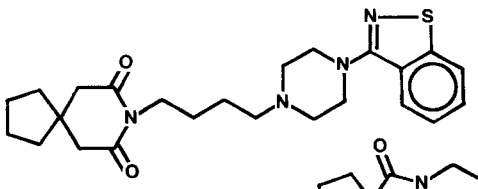

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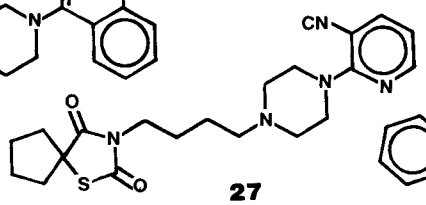

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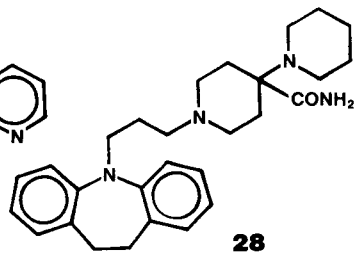
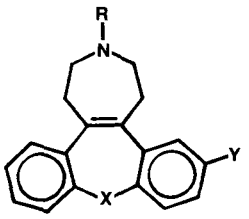


28

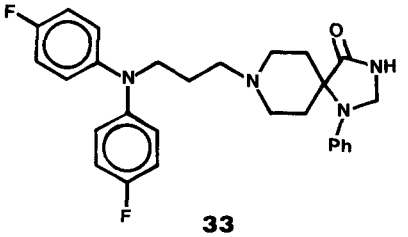
Several potent blockers of DA receptors are also putative atypical antipsychotics. Tepirindole (HR-592, **25**) has a high affinity for DA, 5-HT and NE receptors but has little propensity to cause catalepsy (78). It may have beneficial effects on the negative symptoms of schizophrenia. Tiaspirone (**26**) is a potent D_2 blocker which is active in a variety of standard behavioral tests (79). Compound **26** exhibits a high affinity for both 5-HT_1 and 5-HT_2 receptors and blocks the LSD discriminative cue, an indication of 5-HT antagonism. Although **26** induces catalepsy, it exhibits a relatively high therapeutic ratio. Furthermore, the observed catalepsy may be attributable to sedation, stemming from significant α_1 antagonist activity (79). Acute intravenous administration of **26** appears to exert a greater effect on limbic (A10) than striatal (A9) neurons as assessed electrophysiologically; APO-induced suppression of A10 neuronal firing rates was completely reversed. Chronic administration of **26** resulted in decreased limbic selectivity, i.e., an electrophysiological response similar to a classical antipsychotic was observed (80). When **26** was given to monkeys with persistent tardive dyskinesia (TD), the TD was reduced or abolished and haloperidol-like Parkinsonian symptoms were produced (81). It was noted that **26** might not necessarily elicit acute dystonia and dyskinesia in drug-naive, unsensitized monkeys. Another atypical agent, MJ-13980-1 (**27**), also gave similar results in the same monkey model (81). Compound **27**, however, seems to be more selective than **26** for the limbic system, based on electrophysiological measurements following a chronic treatment regimen (80).

Clocapramine (**28**) is a potent antagonist at DA, α_1 and α_2 receptors, and accelerates both DA and NE turnover (82). The results of a recent Phase I study suggest that **28** may have low EPS liability (83). Maroxepine (**29**), citatepine (**30**), cipazoxapine (**31**) and erespine (**32**) exhibit a limbic (hippocampal) preference for inhibition of [^{3}H]spiperone binding to DA receptors *in vivo* (84). Compounds **29**-**32** antagonize APO-induced stereotypy and climbing. The first three agents elicit catalepsy only at relatively high doses, whereas **32** was non-cataleptogenic at the highest dose tested (84,85).



	X	Y	R
29	O	H	Me
30	S	CN	Me
31	O	CN	CH_2–cyclopentyl
32	NMe	H	Me

Compound **33** is another potent DA receptor blocker which exhibits a large separation between its ED_{50} doses for catalepsy induction and behavioral activity (inhibition of Sidman avoidance behavior and suppression of intracranial self-stimulation) (86). Despite this apparently favorable therapeutic index, **33** elicited EPS from haloperidol-sensitized monkeys at doses similar to those which blocked Sidman avoidance. SAR studies of other compounds from the same series have also been reported (87). Timelotem (**34**) is only marginally cataleptogenic at very high doses. It binds to D_2 but not benzodiazepine receptors and exhibits activity in *in vivo* assays predictive of antipsychotic and anxiolytic properties (88,89). Timelotem also blocks β-receptors and this may contribute to the observed antidopaminergic effects by attentuating DA release (88).



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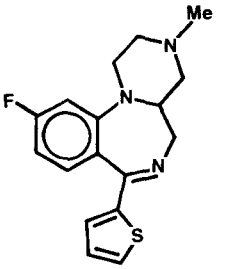
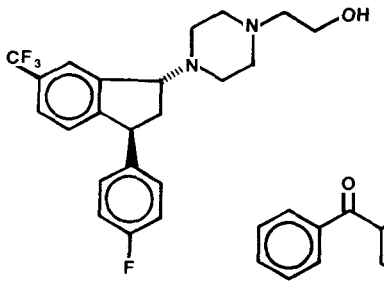


34

Tefludazine (**35**) binds potently to both 5-HT_2 and D_2 receptors and exhibits antiserotonergic and antidopaminergic effects in a variety of tests (90). Although **35** does induce catalepsy at relatively low doses, it is suggested that this agent may possess low EPS liability because of the moderating effect of potent 5-HT antagonism (90). Setoperone (**36**) is also a combined D_2/5-HT_2 antagonist, recently shown to down-regulate 5-HT_2 receptors in rat brain upon chronic administration (91). Ritanserin (**37**) a potent 5-HT_2 antagonist claimed to have clinical antipsychotic efficacy, similarly down-regulated 5-HT_2 sites (91). In an open trial involving twenty schizophrenic patients, **37** reduced existent EPS symptomatology, implying a modulatory effect of 5-HT_2 receptors on the extrapyramidal system (92).



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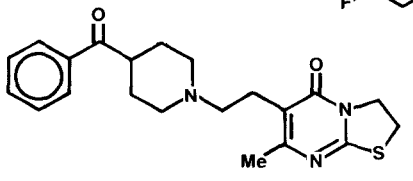

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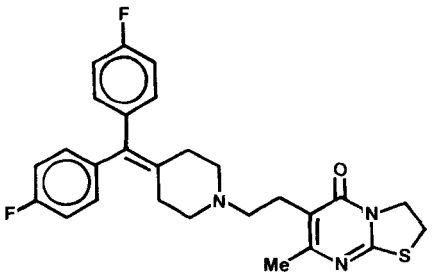

37

Neuropeptides — Interest in neuropeptides continues to grow as basic research provides more evidence for their role as neurotransmitters or neuromodulators in the brain. The discovery that neuropeptides often coexist in the same neuron with classical neurotransmitters has been especially intriguing (93). Identification of cholecystokinin (CCK) (94) and neurotensin (NT) (95) in mesolimbic dopaminergic cells has important implications for schizophrenia research. Drugs which modify the influence of neuropeptides on dopaminergic function offer the prospect of novel antipsychotic agents.

A comprehensive review of neuronal CCK has been published (96). The sulfated, C-terminal octapeptide is the predominant form in brain and is fully active (96). Some investigators consider that CCK acts to potentiate the effects of DA (97-99) while others find that CCK analogs counteract the action of DA (100,101). The existence of multiple CCK receptors in brain may explain these disparate data (99,102). A recent review of electrophysiological experiments focuses on the activation of mesolimbic dopaminergic neurons by CCK-8S, which rapidly leads to depolarization inactivation of these cells (103). Previously, this effect had been observed only after chronic neuroleptic treatment and led to the suggestion that inactivation of DA-containing neurons was essential for achieving antipsychotic efficacy (104). Thus, it follows that CCK agonists might be rapid-acting neuroleptics. On the other hand, if CCK does potentiate the action of DA in the limbic system, then CCK antagonists might prove to be effective antipsychotics. Numerous clinical studies have explored the therapeutic potential of CCK agonists, e.g., CCK octapeptides or the structurally-related decapeptide, ceruletide (caerulein). Generally, the positive findings reported for earlier open trials have not been replicated in more carefully controlled, double blind studies (105-112). To date, the weight of clinical evidence with systemically administered CCK analogs does not suport the contention that they have potential as antipsychotics. However, the therapeutic effect may be severely limited by poor bioavailability and insufficient brain penetration of peptide molecules. Benzotript, proglumide (113) and asperlicin (114) have been reported to be non-peptide antagonists for CCK receptors. A small double blind study found no evidence of antipsychotic efficacy for proglumide (115).

NT is a tridecapeptide located in a variety of areas within mammalian brain (116). The pharmacological effects of NT on central dopaminergic systems have been reviewed in detail and NT appears to oppose the action of DA (117). Accordingly, NT may antagonize the action of DA on neurons in limbic brain regions but it may also enhance DA release by interfering with feedback inhibition at DA autoreceptors. Thus, the antipsychotic potential of NT is difficult to evaluate and will depend on the correct balance of pre- and postsynaptic effects. At present, there are no drugs known to act at NT receptors, nor are there reports of clinical trials investigating the antipsychotic efficacy of NT itself.

The role of endorphins in schizophrenia has been of interest for a number of years and this field was reviewed recently (118). Gamma endorphin analogs have received particular attention. Recent placebo controlled, double blind studies found that 50% of neuroleptic treated patients improved when either (des-tyr)-gamma-endorphin or (des-enkephalin)-gamma-endorphin was added to their therapy, although motor side effect concerns were noted (112).

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Chapter 2. Anxiolytics and Sedative-Hypnotics

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Introduction - As in previous years, the demonstrated safety and efficacy of the benzodiazepines (BZs) causes this group of drugs to dominate the marketplace in the treatment of anxiety and insomnia. Attempts continue to separate the components of the BZs' profile as an approach to more selective drugs. Although the BZ receptor remains a reliable target for anxiolytic drug intervention (1-3), concerns over the side effects and dependence liability have spurred new research into alternate mechanisms of anxiolysis. A number of hypotheses now link central 5-HT neurons with behavioral suppression (4-6), particularly anxiety-related inhibition (7,8). A substantial effort is now involved in this serotonergic approach to novel anxiolytics.

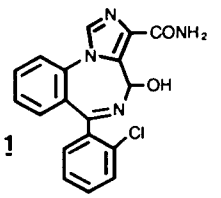
AGENTS ACTING VIA BZ RECEPTORS

There have been continued attempts to produce anxiolytic agents which lack the profound CNS depressant properties and other side effects of the classical BZs (9). However, there is increasing international concern over the potential abuse and dependence liabilities of agents acting through central BZ receptors (10). Others defend the reputation of these drugs, arguing that reports of misuse have been overstated and sensationalized (11). The problem of dependence liability is difficult to evaluate, as clinical accounts of severe symptoms on BZ withdrawal are typically anecdotal, relating isolated and poorly controlled incidents (12,13). Only recently have carefully controlled trials demonstrated that BZ withdrawal symptoms, while clinically significant and relatively common, are generally mild in nature (14).

The agonist/antagonist strategy for improved anxiolytics - There is a wide spectrum of pharmacological profiles theoretically available from BZ receptor ligands, ranging from inverse agonists with anxiogenic potential, through "neutral" antagonists, to full agonists with classical anxiolytic activity (15). It is now widely believed that partial agonists, or mixed agonists/antagonists, would produce anxiolytic effects without sedation or ataxia, regardless of dose (16,17). The classification of compounds as partial agonists or as agonist/antagonists is both confusing and controversial. Neurochemically, such agents may best be described as possessing "intermediate intrinsic efficacy" (18), and available neurochemical methods for their study have been reviewed (19,20). Based on some preliminary supporting evidence (21) and in analogy with opiate receptor ligands (22,23), partial agonists at BZ receptors may possess lessened dependence liability in comparison to full agonists. It remains difficult to demonstrate clearcut BZ dependence in animals, though progress continues in both acute (24) and chronic models of withdrawal (25,26). Reliable neurochemical correlates of BZ dependence appear to be lacking. Agonist activity is generally assessed behaviorally. Apart from classical conflict models, current interest

centers on the development of tests which involve neither food or water deprivation nor behavioral conditioning. Among these are the shock probe conflict procedure (27), the elevated plus maze test (28), quantification of naturalistic behavior (29), and evaluation of nonsocial ambivalent postures in mice (30). There is concern, however, that certain of the newer models may fail to detect some putative anxiolytics, and may also lack specificity (31,32). Antagonist activity is usually evaluated in terms of blockade of one or more of the behavioral deficits induced by BZs, using a battery of neuropharmacological tests (33).

Compounds of interest - Preclinical studies have shown Ro 23-0364 (1) to be a minimally sedative agonist/antagonist (34) while initial clinical investigations demonstrated decreased symptoms of anxiety and some sedation at the higher doses tested (35). Although an early double-blind clinical comparison of the agonist/antagonist Ro 17-1812 (2) and diazepam showed comparable behavioral properties for both compounds, pharmaco-EEG studies indicated that the former may possess a lower dependency risk than the latter (21). Some of the pharmacological activity of the anxiolytic metaclazepam (KC 2547, 3) appears to be the result of the gradual in vivo formation of KC 3756 (4) and KC 3757 (5) as highly active metabolites (36). The pharmacokinetics of bentazepam (QM-6008,



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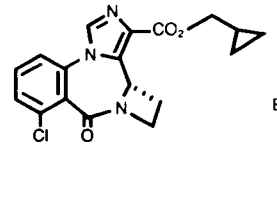

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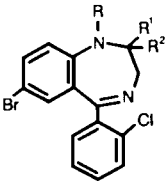
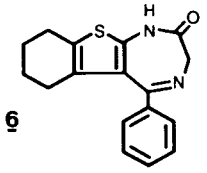


3 R = CH_3, R^1 = CH_2OCH_3, R^2 = H
4 R = CH_3, R^1, R^2 = O
5 R = H, R^1, R^2 = O



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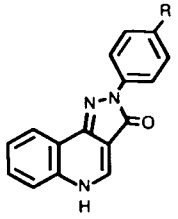
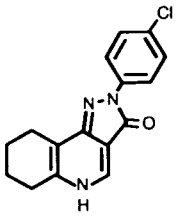


7 R = OCH_3
8 R = Cl
9 R = H



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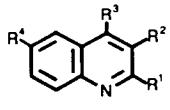
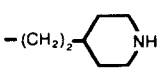
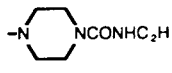
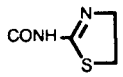
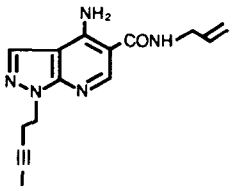
6) have been reported (37). The mixed properties of the partial agonist/antagonist CGS 9895 (7) and agonist/antagonist CGS 9896 (8) have been reaffirmed (38,39). A partially saturated derivative of CGS 9896, CGS 17867A (10) appears to be a non-sedating BZ agonist with no antagonist effects (40). Pipequaline (PK 8165, 11) and other structurally related compounds continue to be of interest as potentially non-sedating anxiolytics (41,42). The quinoline, Ro 23-1590 (12) has a high affin-



	R^1	R^2	R^3	R^4
11	C_6H_5	H		H
12	4-Cl-C_6H_4	H		H
13	H		OH	OCH_3

ity for central BZ receptors and is selective for the Type 1 (cerebellar) subtype (43). This minimally sedative compound has anticonflict and anticonvulsant properties and appears to be a selective agonist (44,45).

Another quinoline, RU 43028 (13) is claimed to be a partial agonist because it antagonizes BZ-induced ataxia while showing anxiolytic activity along with a high affinity for central BZ binding sites (46). The pyrazolopyridine ICI 190,622 (14), an agonist selective for Type I receptors, is a potent anxiolytic agent with anticonvulsant but only weak sedative properties (47). Studies have continued to show that the anxiolytic/anticonvulsant ß-carboline ZK 91296 (15) has anticonflict activity at doses well below those required to produce sedation (48). Early clinical trials indicate that alpidem (SL 800342, 19) is a non-



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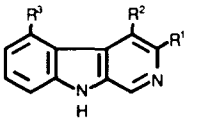
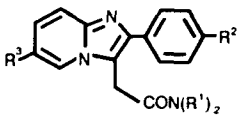
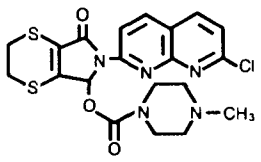


	R¹	R²	R³
15	$CO_2C_2H_5$	CH_2OCH_3	OCH_2Ph
16	CO_2CH_3	H	H
17	$CO_2(CH_2)_3CH_3$	H	H
18	$CO_2C_2H_5$	CH_3	$OCH(CH_3)_2$



	R¹	R²	R³
19	$(CH_2)_2CH_3$	Cl	Cl
20	CH_3	CH_3	CH_3



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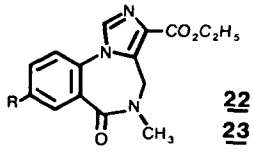
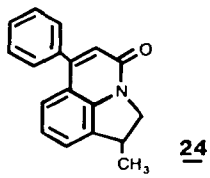
sedative anxiolytic likely to have lower dependence liability in comparison with the BZs (49,50). The side effects of suriclone (21) and diazepam were compared in a double-blind single dose study (51).

BZ antagonists, and especially inverse agonists, are the subject of much speculation over new therapeutic applications, including ethanol antagonism, cognition activation, and appetite suppression. The prototypical antagonist, flumazepil (Ro 15-1788, 22) has proven useful in the reversal of post-operative BZ-induced sedation and anterograde amnesia (52-54). Recent studies in both man (55) and in animals (56) suggest that it may have intrinsic activity, in contrast to its originally postulated "neutral antagonist" profile. A related compound, Ro 15-4513 (23) termed a weak inverse agonist, antagonizes the ethanol-stimulated



22 R = F
23 R = N_3



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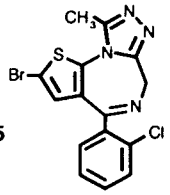
uptake of chloride into brain vesicles by a BZ receptor-mediated mechanism (57). In rats, it blocks the behavioral intoxication and anticonflict effects induced by ethanol, a property not shared by other BZ antagonists or inverse agonists (57-59). There remain serious legal

and ethical questions, however, regarding the development of such an "anti-alcohol" agent (60). That inverse agonists at BZ receptors may serve as cognition activators is supported by the finding that ß-CCM (16) enhances performance in several animal models of learning and memory (61), whereas diazepam impairs such performance (62). Initial clinical studies suggest that ZK 93426 (18), which appears to be an antagonist in animals (63), may possess weak stimulant properties (64). Some inverse agonists, including CGS 8216 (9), induce a dose-dependent suppression of food intake in rats, while the BZ agonist midazolam causes hyperphagia (65). In early human studies, 9 has been shown to reverse the effects of diazepam (66). The indoline AHR-11797 (24) antagonizes the anticonflict and anticonvulsant effects of BZs, but surprisingly, has intrinsic muscle relaxant activity (67). Comprehensive structure-activity relationships for antagonist activity in the BZ (68) and ß-carboline (69) series have been described.

Sedative-hypnotics - Drugs acting at the BZ receptor remain the drugs of choice for the short-term treatment of insomnia (70,71). Animal studies with the clinically effective hypnotic brotizolam (25) indicate the physical dependence liability of this agent may be less than that of tri-



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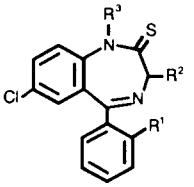
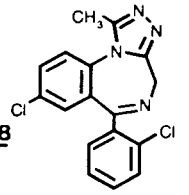
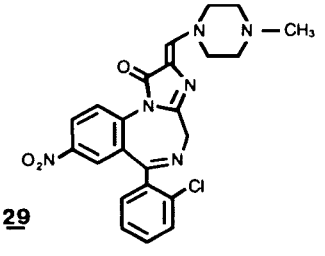




28

	R¹	R²	R³
26	F	H	CH_2CF_3
27	Cl	OH	CH_3

azolam or diazepam (72,73). The clinically effective (74,75) hypnotics quazepam (26) and zolpidem (20) are both selective for the Type 1 BZ receptor (76,77), diminishing credence in an earlier hypothesis correlating Type 1 selectivity with an absence of sedative properties. Although the short acting hypnotic triazolam (28) has been shown to induce a phase-shift in the circadian rhythm in hamsters (78), a similar phenomenon did not appear to be evident in human studies (79). Clinical reports on lormetazepam (27), loprazolam (29) and zopiclone (30) continue to demonstrate the hypnotic efficacy of these agents (80-82). Rilmazafone (31) a hypnotic with anxiolytic activity, appears to exert its effect through metabolic conversion to an active 1,4-benzodiazepine derivative (83).



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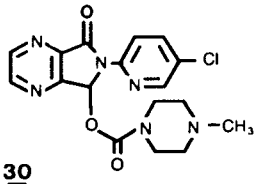

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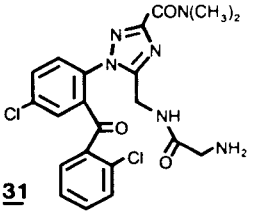

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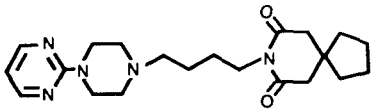
Endogenous ligands - Human diazepam binding inhibitor (hDBI), a polypeptide structurally similar to the previously isolated and partially characterized rat DBI (84), displaces tritiated methyl ß-carboline-3-car-

boxylate (ß-CCM) and is anxiogenic in a rat conflict test (85). Several peptide fragments obtained by trypsin digestion of hDBI are identical to portions of endozepine, a completely sequenced 86-residue polypeptide isolated from human brain which also inhibits the binding of BZs to synaptosomal membranes (86,87). A low molecular weight, heat and protease-stable putative endogenous ligand has been isolated from the plasma of several species, including man. This as yet unidentified substance has an affinity for the BZ receptor comparable to diazepam and may have anxiolytic, rather than anxiogenic activity (88). In an unexpected finding of uncertain biological significance, N-desmethyldiazepam has been isolated from bovine brain (89). The possible role of ß-carboline esters as endogenous ligands for the BZ receptor remains controversial with the suggestion that n-butyl ß-carboline-3-carboxylate (17) is not an artifact of the purification procedure from bovine brain (90).

AGENTS ACTING AT 5-HT RECEPTORS

While some workers have historically maintained that 5-HT plays a key role in anxiety (5,6), it is only recently that pharmacological investigations into the mechanism of action of the novel anxiolytic, bus-



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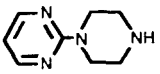

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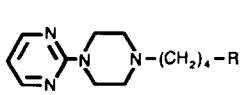
pirone (32) have generated widespread interest in this area. Buspirone lacks affinity for the BZ receptor, but binds avidly to one subclass of serotonin receptors (91), the 5-HT_{1A} subtype (92). It also has weaker interactions with other neurotransmitter systems, showing some dopaminergic (D_2) antagonist (93) and adrenergic (α_1) agonist activities (94), which may contribute to the drug's overall profile. Any mechanistic interpretation of buspirone's clinical efficacy (11,95) is further clouded by the observation that the drug is extensively metabolized to 1-(2-pyrimidinyl)-piperazine (33) a pharmacologically active species (96). Acute and chronic effects must be differentiated, since the peak anxiolytic effects of buspirone are experienced only after several weeks of drug treatment (97). Furthermore, patients with a history of BZ use may prove refractory to the anxiolytic effects of buspirone (98). Nevertheless, buspirone appears to have little sedative (99), abuse, or dependence liabilities (100), and this interesting profile has spawned numerous inquiries into the pharmacological significance of the 5-HT_{1A} receptor.

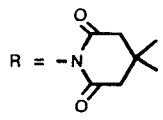
5-HT_{1A} receptors: current understanding - Compounds binding to 5-HT_{1A} receptors are typically detected by the displacement of tritiated 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), which is a highly selective agonist at these sites (101,102). Autoradiographic studies have shown that these receptors are concentrated in the dorsal raphe nucleus, which contains cell bodies and dendrites of serotonergic neurons with projections to numerous areas of the forebrain. The somatodendritic autoreceptors of these cells (but not those on their termini) are believed to be of the 5-HT_{1A} type (103,104). At the molecular level, activation of these receptors by agonists appears to inhibit a forskolin-sensitive adenylate cyclase present in hippocampal membranes (105). The maximal inhibition exhibited by buspirone is less than that of 5-HT itself, and thus buspirone may be acting as a partial agonist in this

system (106). That this negative coupling to adenylate cyclase involves GTP-binding proteins is suggested by the finding that agonists, but not antagonists, have decreased receptor affinities in the presence of added GTP (107). One consequence of agonist binding is a decrease in cell firing, particularly in cells of the dorsal raphe nucleus (108,109). This decrease in cell firing is also produced by the BZs, leading some to suggest a common ultimate pathway for the anxiolytic effects of these two dissimilar classes of drugs (7,95,110).

Animal models for serotonergic anxiolytics - Vigorous attempts are underway to develop specific animal models to detect buspirone-like anxiolytic agents (111), which often produce little or no effect in the classical anticonflict tests used to detect BZ-like activity (112). Nevertheless, an anticonflict model in pigeons has been described, in which buspirone and chlordiazepoxide gave similar and robust response rates (113,114). In rodents, agonists specific for the 5-HT_{1A} receptor, such as 8-OH-DPAT, induce a set of behavioral markers somewhat similar to, but not identical with, the classical 5-HT syndrome. That buspirone has mixed agonist/antagonist activity at 5-HT_{1A} receptors in vivo is demonstrated by its ability to induce certain, but not all, of these behaviors, while retaining the ability to block the others when co-administered with 8-OH-DPAT (115,116). Finally, rodents trained to distinguish the stimulus properties of 5-HT_{1A} agonists from saline have been used to evaluate agonist or antagonist activity at this receptor, and to screen out ligands acting at other serotonin receptors (117-119).

New compounds of interest - Structure-activity relationships detailing the dissociation of 5-HT_{1A} receptor affinity and efficacy among buspirone analogues have been described (120,121). 5-HT agonist activity appears to underlie the anticonflict effects of gepirone (34), a structurally related drug (122). Like buspirone, it inhibits dorsal raphe neuron firing, but differs in that it induces a more pronounced 5-HT behavioral syndrome (123). Ipsapirone (TVX Q 7821, 35) shares many aspects of buspirone's profile, binding to 5-HT_{1A} (but not DA) recep-





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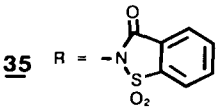
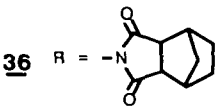
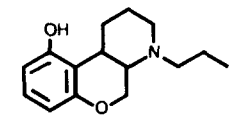




tors (91), and inducing similar behavioral (124,125) and electrophysiological responses in animal studies (126,127). SM-3997 (36) has no CNS depressant properties and is as potent as diazepam in a rat water-lick conflict test (128). CGS 18660A (37) which has considerable structural similarity with 8-OH-DPAT, appears to be a long acting, 5-HT_{1A}-selective agonist (129). LY 165163 (38) is a centrally acting serotonin agonist (130) which appears to be selective for 5-HT_{1A} receptors (131,132), but may have affinity for other serotonin receptors in the periphery (133). Spiroxatrine (40) has been reported to be a selective antagonist at 5-HT_{1A} receptors (134,135), but its in vivo pharmacology is not fully consistent with an antagonist profile (136). MDL 72832 (41) binds with high affinity to 5-HT_{1A} receptors, but also has α_1 activity. Its naphthalene analogue, MDL 72975 (42) is less potent at 5-HT_{1A} sites but is more selective (137). RU 24969 (43)

has been shown to bind to 5-HT_{1B} as well as 5-HT_{1A} receptors, though its behavioral effects are consistent with 5-HT_{1A} agonism (138). In contrast to all of these compounds, CGS 12066B (44) is selective for 5-HT_{1B} receptors (139). That it inhibits firing of substantia nigra DA neurons may have implications for dopaminergic side effects of drugs



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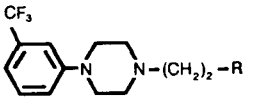
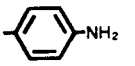
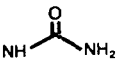
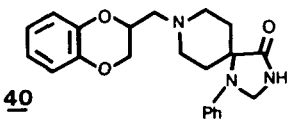


38 R = 

39 R = 



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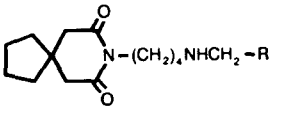
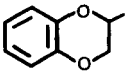
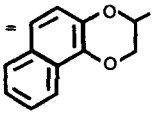
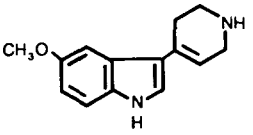


41 R = 

42 R = 

targeted at 5-HT_{1B} receptors, though at this time some doubt the presence of 5-HT_{1B} receptors in human brain (140).



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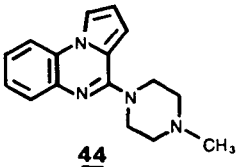

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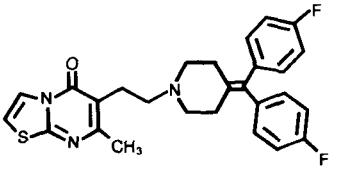
Other serotonergic receptors apart from 5-HT_1 subtypes have been suggested as targets for novel anxiolytics. A large group of phenylpiperazines including fluprazine (39), possess antiagressive properties and have been termed "serenics" (141). A number of structural analogues of these compounds are reported to have 5-HT agonist properties in vivo which may be mediated through effects at 5-HT_2 binding sites (142). Clinical evaluation of the 5-HT_2 antagonist ritanserin (45) continues, after initial reports of clinical efficacy in anxiety (143). The chemical development of this series has been reviewed (144). Finally, an antagonist at 5-HT_3-receptors, GR 38032F (46), may be anxiolytic based on activity in animal models of social interaction (145).



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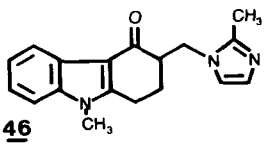

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Chapter 3. Antidepressant Agents

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Introduction - Several reviews of current antidepressant therapy have appeared (1-6). Major concerns are delayed onset of activity, less than ideal efficacy, the importance of proper patient selection, and biochemical predictors of treatment response and side effects. Urinary 3-methoxy-4-hydroxyphenylglycol (MHPG) levels appear not to predict treatment response (7). Platelet serotonin (5-HT) uptake does not show a circadian rhythm in delusional and non-delusional depressed patients unlike healthy subjects (8). Placebo responders are likely to be non-endogenous depressives and have a shorter length of illness with less severe symptomatology (9).

Mechanism of Action - A number of reviews emphasize the chronic effects of antidepressants in animals, which are felt to better model the delayed clinical onset of activity (10-12). Data have been reviewed which suggest that "second generation" antidepressants (e.g. mianserin, nomifensine, trazodone and zimelidine) although differing in their acute actions, have similar effects on noradrenergic function (i.e. normalization) (11).

Receptor modification by antidepressant drugs remains a topical theme for mechanism of action studies. Data pertaining to the effects of depressive illness and treatment with antidepressants on α_2-adrenergic receptors have been critically reviewed (13,14). Platelet α_2-adrenoceptors appeared to be elevated in drug-free depressed patients and antidepressant treatment returned these values to normal (13). These human data concerned peripheral α_2-adrenoceptors, whereas animals studies usually involve central receptors. However, a recent study addressed the effects of antidepressants on peripheral β_1- and α_2-adrenoceptors in rats (15). Interestingly, imipramine (IMI) decreased peripheral β_1- and α_2-adrenoceptor activity, and an effect was noted on α_2-adrenoceptors after 4 days and β_1-adrenoceptors after 11 days of antidepressant administration. β_2-adrenoceptor activity was not affected. Furthermore, as has been demonstrated by others utilizing central receptor binding, coadministration of an α_2-adrenergic antagonist (e.g. yohimbine, idazoxan) and IMI produced a faster decrease in β_1-receptor sensitivity. In other studies, desipramine (DMI) was found to decrease β-adrenergic receptors following chronic exposure to human cells in culture (16).

Recent work has attempted to elucidate the receptor mechanisms subserving the behavioral effects of antidepressants. One approach has been to study the effect of acute and chronic antidepressant administration on β-receptor-mediated potentiation of a 5-hydroxytryptophan (5-HTP)-induced behavioral effect (i.e. head-twitch) (17). Consistent with in vitro studies suggesting β-receptor down regulation, it was observed that chronic, but not acute, antidepressant administration attenuated or blocked the potentiation of 5-HTP-induced head twitch by β-adrenergic agonists. DMI or electroconvulsive shock enhances 5-HT_2 receptor number and 5-HT_2-mediated head-twitch (18). Functional correlates of DMI-induced receptor changes were also investigated by studying the effects of intrahippocampal administration of norepinephrine (NE) (19). Changes in α-adrenergic receptor sensitivity were consistent with in vitro findings. Another

study investigated the combined effects of antidepressants with forced swimming (an antidepressant screening method, vide infra), on β-receptor regulation (20). Forced swim plus IMI produced a decrease in β-receptor binding, but only at doses of IMI which reversed the immobility ("depression") associated with the forced-swim exposure. Comparable results were obtained with pargyline, iprindole and nomifensine. These results imply that the combination of acute antidepressants with forced swim induces adaptive changes similar to those produced by chronic drug administration.

The results from studies with antidepressants on γ-aminobutyric acid (GABA) receptors are not consistent. Thus, in one report chronic antidepressant treatment (e.g. DMI, maprotiline, citalopram, fluoxetine, pargyline, nomifensine) upregulated GABA receptors (21), whereas in another, IMI and nomifensine decreased GABA binding (22) and in a third, DMI and zimelidine did not affect GABA binding (23). The reasons for these differences are not clear, but may be due to differences in species utilized or other methodological considerations. Two of the groups, however, suggested that GABA agonists and antidepressants produce similar effects on GABA binding (21,22). In one study chronic administration of maprotiline did not produce changes in β-adrenergic, 5-HT_2 or DMI receptors; however, a decrease in flunitrazepam binding was observed. This may suggest some interaction of maprotiline with the benzodiazepine/GABA/Cl^- channel complex (24).

IMI binding continues to be of interest. IMI-binding sites are decreased in depressed patients who have not been treated with 5-HT-uptake inhibitors (a potential source of interference with the assay) (25). In healthy volunteers chlorimipramine (but not DMI) rapidly reduces (3 hrs) IMI binding to platelets (26). Although there appears to be a link between the IMI-binding site and the 5-HT-uptake sites in human platelets, this is less clear for rat cortical synaptosomes. Thus, one study reported that the IMI-binding site and the uptake site for 5-HT are not directly linked (27), whereas others have suggested that the sites may be linked (28). Investigations of 5-HT uptake in rat platelets and synaptosomes have suggested some similarities and differences (29). Structure activity relationships were comparable; however, the energy source for transport differs and the two mechanisms respond differently to prolonged drug administration in vivo. IMI binding has been characterized in mouse brain (30); some (e.g. deprenyl), but not all (e.g. DMI), antidepressants decrease the number of IMI binding sites when administered chronically (31). The search continues for the endogenous ligand for the IMI-binding site. One group has suggested that 6-methoxy-1,2,3,4-tetrahydro-β-carboline or a closely related analog, is the putative ligand (32). The effects of coadministration of IMI and chlorpromazine on IMI binding have been investigated following the observation that antidepressants plus neuroleptics may be better than either drug alone for delusional depression (33). The results suggest that coadministration of IMI and chlorpromazine leads to a greater decrease in IMI binding than either alone.

Interest in the role of serotonergic mechanisms in antidepressant action has been recently reviewed (34). Although acute antidepressant treatment has variable effects on serotonergic transmission, chronic administration of most antidepressants appears to enhance serotonergic transmission. The rapid down-regulation of 5-HT_2 receptors observed with mianserin, or yohimbine and DMI in combination, appears not to be dependent on NE or 5-HT for its effect (35).

Receptors are the major focus for mechanism of action studies. This has led to several hypotheses for the discovery/development of new antidepressants (e.g. 36) and for mechanisms involved in the pathogenesis of depression (e.g. 37).

Screening Methods - Eighteen different animal models of depression were critically reviewed and classifed according to predictive validity; predictive and face

validity; and predictive, face and construct validity (38). Behavioral despair, chronic stress, separation and intracranial self-stimulation models were suggested to have the greatest degree of validity.

The forced swim ("behavioral despair") procedure continues to receive attention. Down regulation of β-adrenoceptors may be, in part, responsible for the effects of chronic DMI in this paradigm (39). The rapid down-regulation of β-receptors by antidepressants in combination with forced swim, referred to above, has been suggested as a useful screen for identifying novel antidepressants and eliminating potential false positives (e.g. stimulants) (20). The same researchers conducted studies investigating potential sites of action within the CNS for antidepressants in the forced swim procedure (40). Injection of IMI or pargyline into the central, basolateral and/or lateral nuclear regions of the amygdala antagonized the immobility observed in the forced swim procedure. The novel antidepressant, iprindole, was not active when injected into this region. Interestingly, infusion of IMI into the amygdala did not produce down regulation of cortical β-adrenoceptors in rats exposed to the force swim paradigm, an effect that was noted after systemic administration of IMI and exposure to the forced swim paradigm. We have observed that only serotonin antagonists with concomitant antihistaminic activity are postive in the forced swim procedure (41). Others have reported that the antihistamine-induced reversal of immobility in the forced swim procedure is naloxone sensitive (42).

Purine nucleosides (43) and α_2-adrenergic agonists (44) have been studied in combination with antidepressants in the forced swim paradigm. The purine nucleosides potentiated the immobility, an effect that was reversed by adenosine antagonists, IMI, DMI, tranylcypromine and amphetamine, but not by the selective 5-HT-uptake inhibitor, fluoxetine. The authors suggested that the effect of the purine nucleosides was to inhibit NE outflow. Alpha$_2$-adrenergic agonists were also found to enhance immobility in the behavioral despair test - an effect that could be antagonized by α_2-adrenergic antagonists and antidepressants.

The relationship between brain DMI levels and activity in the forced swim test has been investigated (45). It was concluded that drug accumulation could not account for the enhanced antidepressant effect following repeat dosing.

Paradigms similar to the forced swim procedure have been studied, although not as extensively. Thus, "melancholia" has been induced in rats by initially presenting them with inescapable footshock and then assessing their behavior when the shock is escapable (46). Tricyclic antidepressants, monoamine oxidase inhibitors and other antidepressants (i.e. mianserin and trazodone) were effective in reversing the escape deficit; other psychoactive drugs were not (e.g. benzodiazepines, neuroleptics, sedatives and stimulants). The effects of tricyclic antidepressants in this procedure were antagonized by noradrenergic antagonists (e.g. prazosin) and opioid antagonists (i.e. naloxone), suggesting that activation of both noradrenergic and opioid receptors are an important factor mediating antidepressant effects in animal models (47). In a similar procedure using mice with motor activity used as the endpoint, several antidepressants (e.g IMI, amitriptyline (AMI) and tranylcypromine) were found to reduce the immobility without producing stimulation in shock-naive mice (48). However, mianserin was inactive in this procedure. Another method used to induce antidepressant-sensitive immobility in mice involves suspending them by the tail (49). In addition to antidepressants (including mianserin), amphetamine and atropine were active in this procedure.

Chronic stress decreases basal motor activity - an effect that may be reversed by AMI, chlorimipramine and DMI, but not fluoxetine (50). Similarly, those antidepressants which reverse the decreased activity following stress, also normalize the corticosteroid response. A hamster separation model of

depression has been further characterized (51). Tranylcypromine was found to be effective when administered for 14, but not 7 days.

Olfactory bulbectomy as a model of depression continues to be explored. One review article comparing various animal models of depression to severe depression in humans concluded that the olfactory bulbectomy model holds the most promise (52). The similarities/parallels between olfactory bulbectomy in rats and depression in humans have been studied (53). Thus, bulbectomy alters neuronal function in areas involved in emotional regulation, produces maladaptive behavioral patterns and elevated plasma corticosterone concentrations. Furthermore, chronic antidepressant drug administration reverses the behavioral and endocrine effects for more than five days after drug treatment has been stopped. The effects of 5-HT-uptake inhibitors have also been studied in olfactory bulbectomized rats (54). In some behavioral tests (e.g. passive avoidance) the 5-HT-uptake inhibitors were active, whereas in others they were not (e.g. active avoidance). This observation serves to emphasize the importance of the behavior being measured. Other researchers have found that chlordiazepoxide, as well as AMI can improve discrimination conditioning in olfactory bulbectomized rats (55).

Clinical Studies - Nomifensine was withdrawn worldwide in 1986 because of serious hypersensitivity reactions. Clinical studies with this drug have been reviewed (56). Speed of onset of antidepressants still remains an important area of clinical interest. Studies pertaining to this issue have recently been critically reviewed for amoxapine, maprotiline and trazodone. They appear to have no greater speed of onset of antidepressant activity than IMI (57). In addition, adverse reactions to five of the newer antidepressants (i.e. amoxapine, maprotiline, trazodone, bupropion and nomifensine) have been reviewed (58). Of these, only trazodone was preferred over the tri- and tetracyclic antidepressants with respect to side effects and toxicity. Advantages of 12-week trials compared to the more common 6-week trials for clinical studies have been discussed (59). The 12-week trial appears to be advantageous in non-melancholic depression, a disease characterized by transient mood improvements.

Several uncontrolled trials have concluded that 5-HT-uptake inhibitors are efficacious (60,61), including fluvoxamine (62), Ro-11-2465 (63) and citalopram (64,65). A placebo-controlled trial of sertraline was unable to demonstrate antidepressant efficacy (66). Tomoxetine, a selective NE-uptake inhibitor, was active in an open-label trial (67). Coadministration of haloperidol with maprotiline did not confer any additional benefit, although serum maprotiline concentrations were higher with this regimen (68). Maprotiline was studied in depressives with chronic pain and was found to be efficacious in reducing pain scores (69). AMI was more efficacious than zimelidine in patients with anxiety/depression (70). The antidepressant activities of clovoxamine (an NE/5-HT-uptake inhibitor) and AMI were comparable but the former produced fewer "anticholinergic" complaints and did not produce memory impairment as measured in a signal detection task (71,72). The DA-selective uptake inhibitor, amineptine, was efficacious in open-label and double-blind studies (73). In a pilot study of i.v. clomipramine, utilizing a pulse-loading regimen, patients began to show clinical improvement after achieving transient plasma concentrations that were above the therapeutic plasma concentrations following oral administration (74).

Additional reports on the clinical efficacy of bupropion for depression appeared in the recent literature (75,76). Bupropion also appeared to be efficacious in bipolar or schizoaffective disorder in patients who had been intolerant or showed minimal to moderate improvement on lithium, neuroleptics, antidepressants or a combination of these drugs (77). The monoamine oxidase inhibitor, isocarboxazid, was studied in a double-blind trial in the treatment of atypical depression (78). Isocarboxazid was found to be more efficacious than placebo in this

heterogenous population. Sercloremine (CGP4718A), a reversible MAO-A and 5-HT uptake inhibitor, appeared to be efficacious in an open-labelled exploratory study (79).

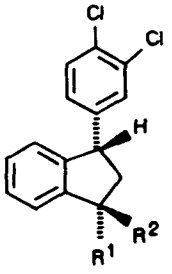
Benzodiazepines (primarily alprazolam) have been receiving attention in the treatment of depression. In two double-blind placebo and antidepressant-controlled studies, alprazolam was more effective than placebo and was comparable to the antidepressants (80,81). One study comparing alprazolam, diazepam, IMI and placebo suggested that all three drugs showed antidepressant effects greater than placebo (82). Adinazolam was also effective in depression (83,84).

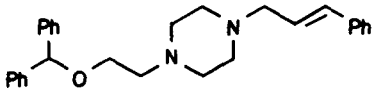
Considering the β-adrenoceptor down-regulation theory of antidepressant activity (vide supra) and the observations that α_2-adrenergic antagonists given with an uptake inhibitor enhance the speed of down-regulation (e.g. 85), the clinical results with a combination of DMI and yohimbine must be considered discouraging (86) However, the patient population was known to be resistant to standard antidepressant therapy. Clinical studies utilizing 5-HT agonists and β-adrenergic agonists as a means of treating depressive disorders via receptor modulation, have also been discussed (87). Preliminary studies with the serotonin agonist, MK-212, or with the β-agonist, salbutamol, did not show striking antidepressant effects.

Potential New Antidepressants

Monoamine-Uptake Inhibitors - The search for monoamine-uptake inhibitors with structures atypical relative to the tricyclic antidepressants continues to be of interest. Presumably, these new structures will not possess the adverse effects associated with the tricyclic antidepressants (e.g., cardiotoxicity, anticholinergic effects and convulsions) (88).

The phenyl-1-indanamines 1 and 2 are monoamine-uptake inhibitors. The trans-isomer 1 (LU 19-005) is a non-selective inhibitor of NE, 5-HT, and dopamine (DA) uptake; while the cis-isomer 2 selectively inhibits 5-HT uptake. LU 19-005 antagonized tetrabenazine-induced ptosis, potentiated 5-HTP-induced behavioral symptomatology, and down-regulated β-adrenergic, 5-HT_2 and D_2 receptors on chronic administration. The (+)-enantiomer of 1 was 5-50 times more potent than the (-)-enantiomer as a monoamine uptake inhibitor in vitro (89,90). GBR 12783 (3) is a selective inhibitor of DA uptake in rodent striatum both in vitro and ex vivo. High-affinity binding of [^{3}H]GBR 12783 to a specific site associated with the neuronal DA-uptake complex in the CNS has been reported. The potencies of various CNS agents for inhibiton of [^{3}H]dopamine uptake and for [^{3}H]GBR 12783 binding correlated very well (91).



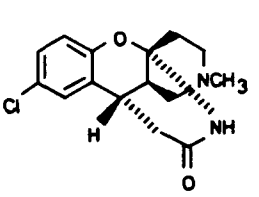


3

1 R^1 = H, R^2 = $NHCH_3$

2 R^1 = $NHCH_3$, R^2 = H

Lortalamine (4) selectively inhibits NE uptake in rat midbrain slices. In the mouse, 4 antagonized reserpine-induced ptosis and hypothermia, and potentiated yohimbine lethality (92). Fezolamine (5) is an inhibitor of NE and 5-HT uptake in vitro and is equipotent with IMI in preventing reserpine-induced ptosis. Compound 5 is not anticholinergic, has a reduced potential for producing antihistamine effects, and does not antagonize the antihypertensive effects of clonidine and guanethidine (93). Fluradoline (HP 494, 6), a centrally-acting antinociceptive agent with antidepressant properties, blocks the reuptake of NE, 5-HT and DA in rat brain synaptosomes (94). Fluradoline was twice as potent as IMI in preventing tetrabenazine-induced ptosis, only partially potentiated yohimbine lethality, and did not potentiate 5-HTP-induced seizures in mice (95). Fluradoline could prove useful in treating pain syndromes with an affective component, such as depression.



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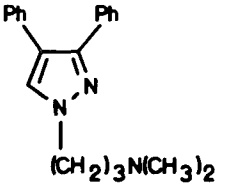

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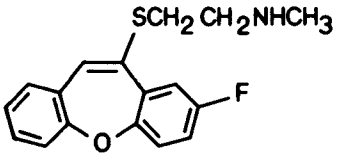
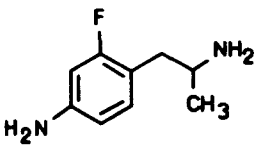


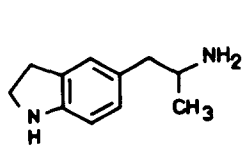
6

Monoamine Oxidase Inhibitors - The utility of selective inhibitors of monoamine oxidase A (MAO-A) or B (MAO-B) in the treatment of depression remains uncertain. Whether selective inhibitors of either enzyme will be devoid of the "cheese effect" still must be demonstrated. This "cheese effect" has been associated with MAO-A inhibition (96). In theory, selective MAO-A inhibition would leave the B form of MAO still active and able to lower tyramine levels by deamination. MAO-A is localized intraneuronally and MAO-B accounts for less than 15% of the total intraneuronal MAO activity in rat cortical synaptosomes (97).



7

The analogs of amiflamine, 7 and 8 are equipotent to amiflamine as selective reversible inhibitors of MAO-A in vitro and ex vivo, but more selective than amiflamine for intraneuronal MAO-A. The compounds 7 and 8 also inhibit monoamine uptake in vitro, produce a 5-HT-like syndrome in reserpinized rats, and reverse reserpine-induced ptosis in rats (98,99). SR-95191 (9), an analog of minaprine, is a selective reversible MAO-A inhibitor in vitro and ex vivo. SR-95191 increases brain 5-HT and DA levels in rodents ex vivo, antagonizes reserpine-induced ptosis, potentiates 5-HTP-induced tremors, and decreases immobility in the behavioral despair test. SR-95191 also possesses DA-stimulant properties since haloperidol and α-methyl-p-tyrosine block SR-95191 induced stereotypies in rats (100,101). The selective MAO-B inhibitor MDL- 72145 (10) is



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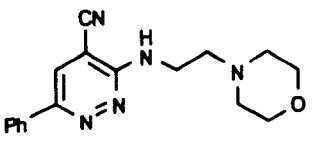

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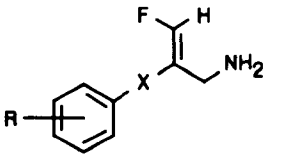
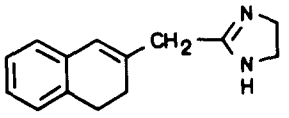


10 X = OCH_2; R = 2,4-Cl_2

11 X = -; R = 3,4-$(OCH_3)_2$

an enzyme-activated irreversible inhibitor whose SAR has been further explored (102). An analog MDL-72638 (11), although structurally related to the selective MAO-A inhibitor, clorgyline, is a selective MAO-B inhibitor (103).

α_2-Adrenergic Antagonists - The role of α_2-adrenergic antagonists as antidepressants and their SAR has been reviewed (104,105). Napamezole (12) is a selective α_2-antagonist which elevates mouse-brain MHPG and increases NE turnover in the rat. Napamezole also inhibited monoamine uptake in rat brain synaptosomes, antagonized tetrabenazine-induced ptosis, blocked parachloroamphetamine-induced depletion of rat forebrain 5-HT, and enhanced 5-HTP-induced decrease in operant responding in the rat (106,107). Napamezole is related to napactadine (13), an inhibitor of monoamine uptake that prevents reserpine-induced ptosis and potentiates yohimbine lethality in mice (108). The (+)-S enantiomer, 14, of the selective α_2-antagonist, RX-821002, is the active enantiomer. The SAR of this series has been described (109).



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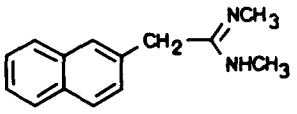

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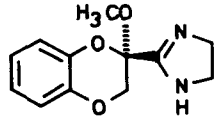
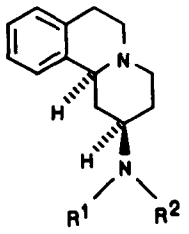


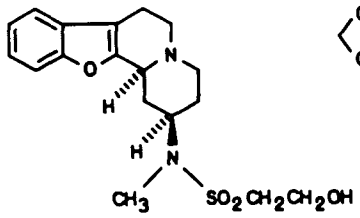
14

The benzoquinolizine Wy-26392 (15) is a selective α_2-adrenergic antagonist in the anaesthetized dog (110). Wy-27127 (16) and idazoxan are equipotent as α_2-antagonists, however, Wy-27127 is 5 times more receptor selective than idazoxan (111). The structurally related L-654,284 (17) is an α_2-antagonist that is more potent than idazoxan with comparable receptor selectivity in vitro. L-654,284 antagonizes clonidine-induced mydriasis and increases central NE turnover in rats (112). CH-38083 (18) is equipotent to idazoxan as an α_2- antagonist, but is 5-14 times more selective than idazoxan for α_2- effects vs. α_1-effects in vitro. CH-38038 enhanced [^{3}H]NE release in the mouse vas deferens and increased NE turnover in rat brain (113).



15 $R^1 = CH_3$, $R^2 = SO_2CH_2CH_2CH_3$

16 $R^1 = SO_2CH_3$, $R^2 = CH_2CH_2NHSO_2CH_3$



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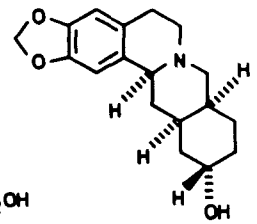
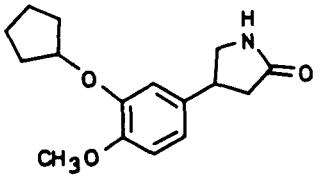


18

Miscellaneous Agents - The phosphodiesterase inhibitor rolipram (19) enhances NE transmission in rats by stimulation of tyrosine hydroxylase and by increasing neural activity (114). The (-)-enantiomer of rolipram inhibited cAMP-phosphodiesterase in vitro and down-regulated β-receptors on chronic administration. The (+)-enantiomer was not active (115). (-)-Rolipram is 20 times more effective than the (+)-enantiomer in displacing (±)-[^{3}H]rolipram from rat forebrain membranes (116). The benzodiazepine 20 possesses antidepressant properties (i.e. inhibits [^{3}H]NE uptake into rat brain synaptosomes and antagonizes tetrabenazine-induced ptosis), as well as moderate anxiolytic properties (i.e. displaces [^{3}H]-

diazepam from rat cerebral cortex homogenates, antagonizes pentylenetetrazol-induced convulsions in mice, and suppresses conflict behavior in rats) (117). A metabolic precursor of NE, L-threo-3,4-dihydroxyphenylserine (L-threo-DOPS) reverses reserpine-induced ptosis after intraperitoneal administration. This effect is potentiated by coadministration with IMI or nialamide. L-threo-DOPS slightly restores the reserpine-induced decrease in mouse brain NE (118). Both L- and D-threo-DOPS are competitive inhibitors of MAO (119).



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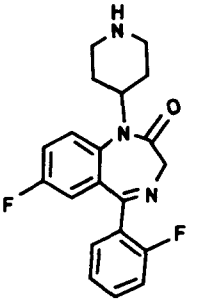

20

Summary - The search has continued to identify new "second generation" monoamine uptake inhibitors and to identify selective inhibitors of monoamine oxidase A or B. The α_2-adrenergic antagonists offer a new therapy for the treatment of depression, although the efficacy of these agents has yet to be demonstrated conclusively in man.

A more rapid onset of antidepressant effect and fewer adverse drug effects than existing therapeutic agents remain as the important hurdles for the newer agents undergoing clinical evaluation.

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Chapter 4.
EXCITATORY AMINO ACIDS AND MAMMALIAN CNS FUNCTION

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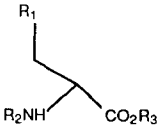
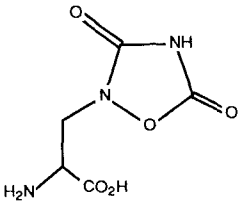
Introduction - The neuroexcitant actions of the amino acids, glutamate (GLU, 1) and aspartate (ASP, 2), so-called excitatory amino acids (EAAs), were first reported more than 25 years ago (1-4). The importance of these EAAs as potential neurotransmitters was slow to evolve however, in part because GLU and ASP play key metabolic roles in both neuronal and non-neuronal tissues. GLU and ASP exist in high concentrations in brain tissue (from 1 - 10 mM intracellularly and 10 - 100 μM extracellularly) with small differences in levels between brain regions (5-6). The possibility that other dibasic acids are the actual neurotransmitters activating EAA receptors (rather than GLU and ASP) cannot be excluded.



	R_1	R_2	R_3
1	CH_2CO_2H	H	H
2	CO_2H	H	H
3	CO_2H	CH_3	H
4	CH_2SO_3H	H	H
5	SSO_3H	H	H
6	$CH_2CO_2C_2H_5$	H	C_2H_5
7	$CH_2CONHCH_2SO_3H$	H	H



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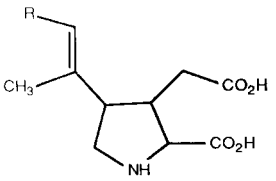
Putative neurotransmitter roles for GLU and ASP were established by the demonstration of specific high affinity, Na^+-dependent neuronal uptake systems (7,8) and depolarization-evoked release (9,10). Based on electrophysiological, biochemical and pharmacological data three major classes of EAA receptor have been described (11-15). These are selectively activated by N-methyl-D-aspartate (NMDA, 3), quisqualate (QUIS, 8) and kainate (KA, 9). A fourth type of EAA receptor, sensitive to the phosphonic acid, AP4 (12) has been reported, and is the subject of controversy (16-19). The nomenclature used to designate these various EAA receptors has been the source of considerable confusion (20,21). Currently accepted convention is to use the NMDA, QUIS, KA and AP4 designations.

NMDA-type Receptors

NMDA-type receptor ligands- NMDA (3), is the prototypical agonist for this EAA receptor type (1-4,22). In vitro, 3 is less active than GLU, the converse being true in intact cell systems where, presumably, active uptake systems exist for GLU (9). Quinolinate (17), an endogenous analog of ASP, is 20 - 40 times less active than NMDA while homoquinolinate (18) is more active than AP4 (26-29). GLU analogs are more active than ASP analogs, suggesting that the endogenous transmitter at NMDA-type receptors is GLU rather than ASP (23-25). Alternatively, the endogenous transmitter

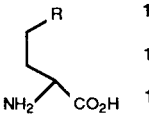
may be another dibasic amino acid, such as homocysteate (4), for which there is substantial supportive evidence (30). Addition of a carboxyl alpha to the basic nitrogen of GABA agonists generally results in an EAA agonist, exemplified by ibotenate (19), which when decarboxylated, yields the GABA-A agonist, muscimol (31).



9 R = H, *alpha* CO_2H

10 R = H, *beta* CO_2H

11 R = $CH{=}CHCH(CH_3)CO_2H$



12 R = PO_3H_2

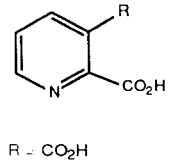
13 R = CH_2CO_2H

14 R = $CH_2PO_3H_2$

15 R = $(CH_2)_3PO_3H_2$

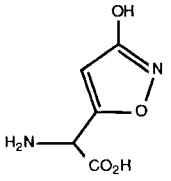
16 R = $(CH_2)_2PO_3H_2$

D-alpha-aminoadipate (13) was the first competitive antagonist described for the NMDA-type receptor (3,4,32). However, the discovery of 2-amino-5-phosphonopentanoate (AP5,14) and subsequently, 2-amino-7-phosphonoheptanoate (AP7, 15), which are metabolically stable, relatively potent, and highly selective antagonists of NMDA receptors, established EAA receptor pharmacology and made NMDA receptors the most studied (33,34). Further exploration of these leads gave CPP (20), CGS 19755 (21) and NPC 451 (22), which are more potent and may have potential as therapeutic agents (vida infra) (23,35-39). Compound 20 was the first radioligand with relatively high affinity for the NMDA-type receptor; 21 is even more potent (38).



17 R = CO_2H

18 R = CH_2CO_2H



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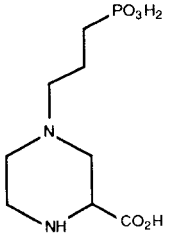

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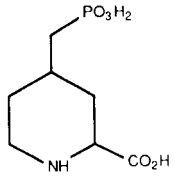

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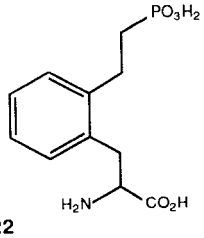
Kynurenic acid (23) while active at NMDA-type receptors also blocks QUIS responses and prevents lesions induced by kainate (40,41). Cis-2,3-piperidine dicarboxylic acid (24) has partial agonist activity at NMDA-type receptors in some systems. The Cis and trans-diastereomers produce excitotoxic lesions, while the trans-diastereomer is a potent agonist (42-44). Phenacylpiperazine derivatives, e.g., BBP (25), and substituted phenylglycines, e.g., 26, show electrophysiological activity at a number of EAA receptors but are generally weak in NMDA receptor binding assays (15).



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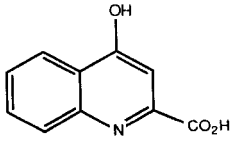

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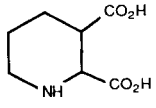

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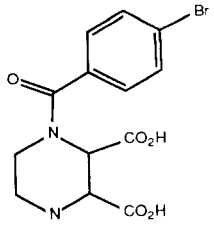

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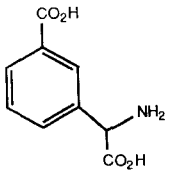
While PK 26124 (27) has been reported to block NMDA-type receptors or associated transduction mechanisms, it fails to inhibit the binding of 20, and its actions are possibly due to intrinsic local anesthetic activity (23,45-47).

NMDA receptor modulators- Dissociative anesthetics such as ketamine (28) phencyclidine (PCP, 29) and dexoxadrol (30) selectively inhibit NMDA-associated excitatory effects, but not those observed with QUIS or KA (48-52). These effects correlate with the ability of such compounds to displace PCP and its thienyl analogue, TCP (31) in receptor binding assays



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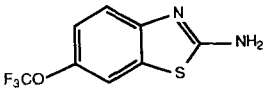

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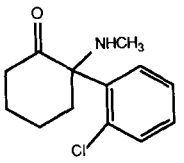

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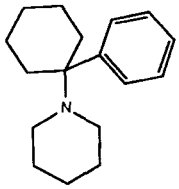

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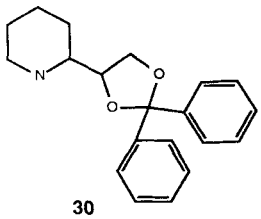
(53,54), leading to speculation that there might be an NMDA-receptor/dissociative-anesthetic receptor complex (50,55) similar conceptually to that



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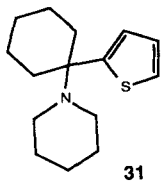

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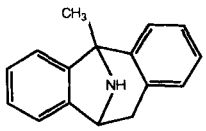

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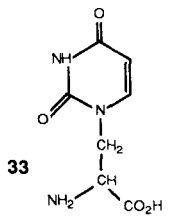

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shown for the inhibitory amino acid, GABA and the anxiolytic, diazepam (56,57). There appear to be unique recognition sites in rat brain for MK801 (32), a novel anticonvulsant (58,59). This compound is also a very potent displacer of PCP binding and selectively inhibits NMDA-induced excitation (60). Binding of TCP and MK 801 is affected similarly by dissociative anesthetics, implying a common site of action. Inhibition of NMDA-induced responses by dissociative-anesthetics is non-competitive (59-61). Furthermore, PCP and related compounds fail to affect the binding of either CPP (20) or GLU to NMDA-type receptors (23,62). However, GLU and Mg^{2+} are effective modulators of both dissociative-anesthetics and NMDA-receptor antagonists at PCP recognition sites (63). Thus binding of radiolabeled TCP is dependent on the presence of GLU (64).

Three models have been proposed for the NMDA-type/dissociative-anesthetic receptor complex. In the first of these (Fig. 1a) the NMDA-type receptor and the dissociative-anesthetic receptor are each linked directly to the same ion channel (65). The second (Fig. 1b) involves allosteric modulation of the NMDA-type receptor/ion channel complex, in direct analogy to the modulation of the GABA-A receptor/chloride ion channel complex by the benzodiazepines (57). The third (Fig. 1c) involves a direct interaction between the dissociative anesthetics and the cation channel associated with the NMDA-type receptor, in direct analogy with the picrotoxinin/GABA-A receptor/chloride ion channel interaction (66).

The observed voltage-dependence of the blockade of NMDA-induced responses, which is similar to that of the blockade produced by magnesium ions, sup-

ports the third model (67,68). Moreover, the blockade of these channels appears to be use-dependent, in that an NMDA-agonist must be present for such effects to be seen (66). Such data are consistent with the potentiation of TCP binding by GLU and Mg^{2+} observed in vitro (64). The stoichiometry of the NMDA-type receptor and the TCP binding site appears constant across brain regions but the actual value remains to be determined rigorously (69,70). The dissociative-anesthetic recognition site associated with the NMDA-type receptor is not a sigma opiate receptor (54). Recently, positive allosteric modulation of the NMDA-type receptor-effector complex by glycine has been reported (71).

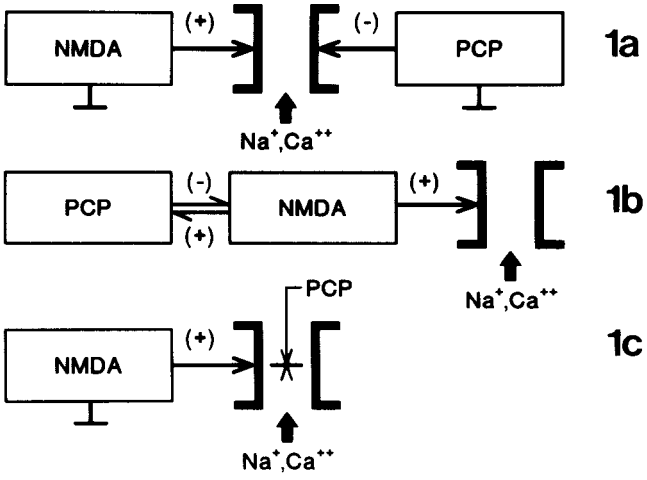
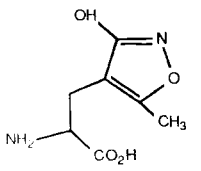


Figure1

NMDA-type receptor mediated processes- In addition to the classical electrophysiological techniques used both to define the NMDA-type receptor hypothesis and to characterize the highly selective phosphonic acid antagonists, 14 and 15, activation of the NMDA-type receptor (e.g. by 21) can selectively inhibit the release of acetylcholine from striatal brain slices (72). Phosphatidyl inositol turnover is stimulated by NMDA-type receptor agonists (73). Changes in cerebellar cyclic GMP in vitro and accumulation in vivo have been associated with actions at NMDA-type receptors, while alterations in EAA turnover are induced by NMDA antagonists (74,75). Behaviorally, NMDA-antagonists can block NMDA-induced convulsions, produce muscle relaxation, can generalize to diazepam, and show activity in anticonflict procedures predictive of anxiolytic activity (37, 76-79). NMDA itself may be used to produce an interoceptive stimulus (80). NMDA-type receptor antagonists block long-term potentiation (an in vitro model for learning and/or plasticity in some but not all species (81,82). Moreover, rats receiving chronic infusion of AP5 into the hippocampus show statistically-significant learning deficits (83).

NMDA-type receptor binding assays - The use of radioligand binding assays to characterize NMDA-type receptors has proven to be more difficult than anticipated for this relatively simple technique. In addition to the low affinities of the available radioligands, the data derived using these have been at variance (15). [^{3}H]NMDA and [^{3}H]AP7 (15) gave inconsistent data while use of [^{3}H]L-GLU and [^{3}H]D-AP5 (14) required laborious membrane preparation procedures (15,25). Even then, specific binding was relatively low and these ligands were only suitable for low-throughput screening. In addition these ligands were capable of labeling uptake sites for EAAs (15). While of relatively low affinity, [^{3}H]CPP (20) was the first NMDA-

receptor ligand to give greater than 70% specific binding in membrane preparations such as are normally used for screening purposes (23). [^{3}H]-CGS 19755 (21) while of higher affinity as a radioligand, still requires centrifugation in the workup procedure (38). The development of compounds with IC_{50} values of less than 10 nM will no doubt allow for the development of more rapid "filtration" binding assays.



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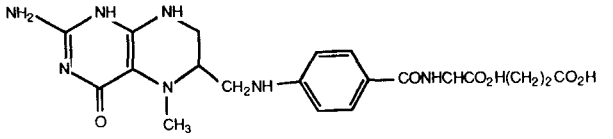

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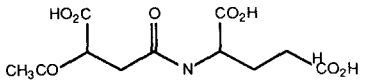

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QUIS-type Receptors

QUIS-type receptor ligands - QUIS and willardiine (33) were the first selective QUIS-receptor agonists reported (86). The isoxazole, AMPA (34), which resembles the NMDA-receptor agonist, 19, was also found to be a selective QUIS-type receptor agonist (87). All three compounds were identified using electrophysiological techniques. Sulfur-containing dibasic amino acids tend to be particularly active at the QUIS-type receptor, with S-sulfo-L-cysteine (5) being the most active (89,90). Putative QUIS-type receptor antagonists include glutamate diethyl ester (GDEE, 6) and gamma-aminomethylsulphonate (GAMS, 7), although the latter also blocks kainate responses (91). Several reports show that pentobarbital and other barbiturates can selectively inhibit QUIS-induced and KA-induced responses (92, 93). However, neither GDEE, GAMS nor the barbiturates inhibit binding of radioligand to QUIS recognition sites (94). Whether this is a reflection of the specificity and/or activity of these compounds or a limitation of the binding assays currently available remains to be seen.

QUIS-type receptor mediated processes - Effector systems linked to QUIS-type receptors have been difficult to characterize, due no doubt to the limited number and diversity of chemical tools available. QUIS agonists can evoke GLU release from cultured cortical neurons (95). While QUIS apparently fails to stimulate cGMP production in the cerebellum *in vitro*, it produces dramatic increases *in vivo* in the cerebellum, even when injected into the cerebellar cortex (53,74). QUIS receptor activation can produce neurotoxic lesions in the immature rat brain, while the compound is essentially devoid of excitotoxicity in the mature rat brain and other excitotoxicity models (96,97). Compound 6 can block alcohol withdrawal-induced seizures and seizures induced by 4 in rats while 7 has been reported to have anticonvulsant activity in the same species (98). QUIS-type receptors in the nucleus tractus solitarius appear to mediate the baroreceptor reflex (99).

QUIS-type receptor binding assays - Both [^{3}H]L-GLU and [^{3}H]AMPA (34) have been used to label QUIS receptors. The use of the former ligand has required the same complex tissue preparation mentioned above and the presence of cold ligands selective for the other EAA sites labeled by [^{3}H]L-GLU (15,25). Reliable assays using [^{3}H]AMPA have been developed (94,100). Binding of [^{3}H]AMPA can be stimulated dramatically by chaotropic agents such as thiocyanate, an effect distinct from that seen with the NMDA-type receptor ligand [^{3}H]CPP. This serves as the basis for a reliable assay useful for screening (94,100). No receptor binding assay has yet been shown to corroborate identification of a QUIS-type antagonist.

KA-type Receptors

KA-type receptor ligands - The prototypic agonist at KA-type receptors is alpha-Kainate (KA, 9). There is a high degree of stereoselectivity, the beta-analog, 10 being inactive in binding (101-103). Domoic acid (11) also has agonist activity at KA-type receptors while QUIS (8) can interact with KA-type receptors at high concentrations (105,106). Tetrahydrofolic acid (35) has been found to be excitotoxic and a mechanism of action via the KA-type receptor has been suggested (107), although this is controversial (106). Antagonists for KA-type receptors have yet to be identified. However, kynurenate (10) can prevent lesions induced by KA (40).

KA-type receptor mediated processes - KA stimulates cGMP formation in slices of adult rat cerebellum and ion fluxes in brain slices. Both effects can be blocked by derivatives of KA (108-110). KA also induces the release of endogenous EAAs from brain slices in vitro (111,112), although the selectivity of this action is controversial (113-116). Compound 10 has anticonvulsant activity (40).

KA-type receptor binding assays - [^{3}H]KA (9) has proven to be an effective and selective radioligand since its introduction over a decade ago (117). The activity of compounds in this assay correlates closely with their excitotoxic actions (118). Unlike binding assays for the other types of EAA receptor discussed, that for KA has a low apparent Bmax. Moreover, in contrast to the low potency of KA in producing lesions inhibiting GLU uptake, or evoking GLU release, [^{3}H]KA binding has high affinity (15).

AP4/NAAG Receptors

AP4/NAAG receptor ligands - AP4 (12) is a potent antagonist of EAA-evoked excitatory responses in the hippocampal dentate gyrus, a locus where NMDA-antagonists are ineffective (19). Compound 16 is however, some 50-fold less active at synapses in the CA3 pyramidal region and on prepyriform pyramidal cell excitatory responses (119,120), an effect ascribed to a blockade of the actions of endogenous N-acetyl-aspartyl-glutamate (NAAG, 36), since in this system the effects of the latter compound are blocked by AP4, while the effects of GLU and ASP are not (120).

Therapeutic Potential of EAA Receptor Modulators - By reducing the actions of excitatory neurotransmission processes in the CNS, EAA antagonists may have actions similar to those of agonists or facilitators of inhibitory amino acid neurotransmission (i.e., GABAmimetics, GABA-transaminase inhibitors, and benzodiazepines) (121). Data related to the therapeutic potential of EAAs have been limited to a large extent to the NMDA receptor; this a reflection of the compounds available.

Anticonvulsant - NMDA antagonists were first recognized as having clinical potential as anticonvulsants (122,123). The electrophysiological characteristics of neurons in epileptic foci (bistable depolarizing shifts) are very similar to the effects produced by continuous ionophoresis of NMDA agonists (124-126).

Anxiolytic - NMDA antagonists such as CPP produce anticonflict behavior in rats, suggestive of anxiolytic potential (79).

Muscle relaxant - Systemic administration of NMDA antagonists produces traction reflex and rotorod deficits that have been ascribed to their central muscle relaxant effects (77,78). When administered intrathecally, NMDA antagonists produce muscle relaxation (37). The increase in muscle tone produced by etorphine may be reversed by systemic administration of TCP (37). In drug discrimination studies NMDA antagonists generalize to diazepam (80). While muscle relaxation may be a useful therapeutic application for NMDA antagonism, it is an undesired side effect when drugs are used as anxiolytics or anticonvulsants.

Central neurodegeneration - Exogenously administered EAAs produce neurodegeneration, a finding that has led to speculation that endogenous EAAs may be involved in various neurodegerative phenomena, including Huntington's chorea, Alzheimer's disease, and stroke (5,97,127,128). When administered intracerebrally or systemically NMDA antagonists can block the neurodegeneration caused by hypoglycemia or seen in animal models of stroke (129-133). Similarly, the non-competitive antagonist, MK 801 (32) is effective in treating cerebral ischemia (134).

Memory and synaptic plasticity - NMDA antagonists can block long-term potentiation (a possible model of learning) in brain slices (81,82). NMDA agonists lacking the ability to produce brain lesions may therefore have potential as nootropic agents.

Future Concerns - Surprisingly, the dissociative anesthetics (the non-competitive NMDA antagonists), have limited therapeutic applications, with the possible exception of muscle relaxation. However, the greatest drawbacks to the NMDA antagonists, which threaten their potential as therapeutic agents, are the side effects they share with the dissociative anesthetics - namely, the production of confusional states, amnesia, and muscle relaxation. Only for stroke, which is the third leading cause of death in the United States and results in 100,000 cases of paralysis per year, would such side effects be acceptable. The concept that NMDA receptor antagonists are equivalent to GABA receptor modulators (i.e. the benzodiazepines), has been validated with available compounds despite their intrinsic limitations. It may be noted, however, that chemical efforts in the EAA area have been somewhat sparse and that increased efforts to discover more novel structures may provide entities that are more specific in their actions and are devoid of some of these side effects. Such compounds would have promise as novel anxiolytics and anticonvulsants in addition to their anti-neurodegenerative properties. These may allow efforts in this area to extend beyond the NMDA receptor based on more recent findings related to the multiplicity of EAA receptor-interactions with associated ion channels (135).

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Chapter 5. Recent Advances in Migraine Research

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Introduction - Headache is a distressing condition which is, as far as we know, restricted to man. The majority of chronic headaches, classified by such terms as classic and common migraine, cluster headache, and muscle contraction or tension headache, are both bilateral and unilateral in nature and idiopathic in origin. Collectively, these conditions are often referred to as vascular headaches. Although numerous studies have sought to define the etiology and pathophysiology of these conditions as separate entities, recent evidence is more supportive of a headache continuum in which the headache types differ only in the severity of symptoms (1,2). The lack of agreement on both the precipitative factors and mechanisms by which headache pain is generated and perceived is evidenced in the numerous treatises on the subject (3-11). Migraine, from the early studies reported by Wolff and others (3), has been assumed to be a vascular phenomenon, however little convincing evidence to support this view has been forthcoming and presently it is held that migraine is more properly considered a neurological disorder (12,13,14).

A result of the complex and poorly understood pathophysiology of migraine is that no specific animal models have been reported. This lack of predictive models has resulted in the identification of effective drugs through an empirical clinical approach. The interpretation of such trials has frequently been hampered by a large placebo effect. Significant progress has been made in understanding the mechanisms behind migraine pain generation and perception. These are briefly reviewed.

Pain Pathways and Mechanisms - Early surgical studies demonstrated that various manipulations of pial blood vessels induced pain which was perceived in the ipsilateral temporal, retro-orbital and frontal facial regions (3). These observations were rationalized by recent histochemical studies in the rat and cat which confirmed that the pial blood vessels receive sensory afferent innervation from the ophthalmic branch of the trigeminal nerve (15). The temporal, retro-orbital and frontal regions of the face, commonly the site of migraine pain, similarly receive innervation from this nerve branch. Using retrograde tracing techniques, it has been further established that although few trigeminal neurons send collaterals to both the middle cerebral artery and the facial regions, separate fibers from these areas do have their central processes converge on common brain-stem trigeminal nucleus cells (16,17). Extracellular recordings from medullary trigeminal neurons confirmed the commonality of response on these relay neurons to both dural artery and infraorbital cutaneous stimulation (18,19). Furthermore, stimulation of the cat periaquaductal grey region suppressed the firing of the trigeminal relay neurons (19). Combined, these observations provide a viable explanation for referral of headache

pain and suggest that one site of convergence of visceral (blood vessel) and somatic information is within the trigeminal nuclear complex.

As for other sensory afferent nerves, the presence in the trigeminal nerve of the pro-inflammatory and pain sensitizing neuropeptide, Substance P is firmly established (20). In addition, two further pro-inflammatory and vasodilatory neuropeptides, Neurokinin A and Calcitonin Gene Related Peptide (CGRP) have also been co-localized with Substance P (21,22). Central or peripheral stimulation of the trigeminal sensory afferents by a variety of humoral or neurogenic stimuli is suggested to modulate or promote the release of these and other co-stored mediators. At the blood vessel wall, direct depolarization or antidromic transmission releases peptide and induces vasodilation and plasma extravasation - two components of the inflammatory response. A variety of neuroactive agents have been shown to stimulate (serotonin, bradykinin, histamine), or inhibit (somatostatin, enkephalins and opiates) the depolarization of sensory fibers and release of Substance P from sensory afferent nerves (15). Interestingly, both dynorphin and serotonin-containing nerve fibers have also been localized to pial blood vessel walls (23,24). Substance P has also been shown to stimulate the release from mast cells of the pro-inflammatory mediator, histamine.

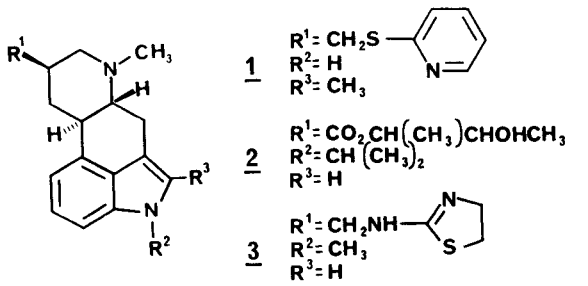
APPROACHES TO MIGRAINE THERAPY

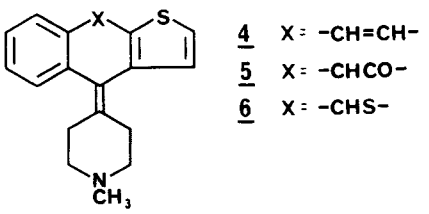
From the foregoing brief discussion, it is apparent that migraine and related headaches are a collection of pain syndromes involving cephalic blood vessels. The sensory nerves which surround these blood vessels serve not only to transmit information about pain to the nervous system, but also promote the development of inflammation within the walls of these same blood vessels. The discussion which follows focuses on drugs which are useful or potentially useful in migraine therapy. Although it is difficult to offer a unifying hypothesis regarding their mechanism(s) of action, many of these agents either modify the release of transmitters or neurmodulators involved in pain transmission (opiates, somatostatin, bradykinin and calcium antagonists), block sensitization of nerve fibers (alpha-adrenergic, bradykinin, serotonin and histamine receptor antagonists, non-steroidal anti-inflammatories (NSAI)), or block inflammatory mechanisms involving the blood vessel wall (serotonin and histamine antagonists, NSAI).

Serotonergic Agents - A central role for serotonin in the mechanism of migraine has developed since the late 50's (25). This close relationship is evidenced by the breadth of serotonergic agents reported to be efficacious in this condition. However, the widely differing receptor subtype specificity, agonist/antagonist profile, and pharmacological action of these agents do not speak to a simple role in migraine. This complication is often compounded by other biochemical actions of these agents. The mixed receptor profile of most antimigraine serotonergic drugs has been reported (26-31). For simplicity, selective antimigraine agents acting at 5-HT_1 or 5-HT_2 receptors will be considered together.

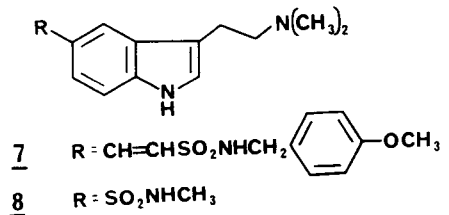
5-HT_1 and 5-HT_2 - Ergotamine and its reduction product dihydroergotamine comprise one of the few abortive therapies for migraine and cluster headache. Ergotamine's effectiveness generally is attributed to a vascular action, and this was reviewed recently (32). However, it has long been recognized that ergotamine could inhibit the serotonin-stimulated release from lung of undefined spasmogens,

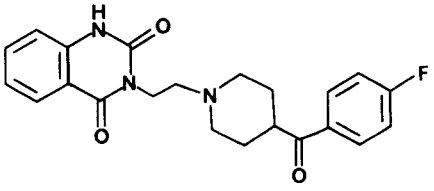
presumably including prostaglandins. Additionally, potent blockade of subdermal serotonin-induced inflammation and release of histamine from mast cells by methysergide, an established prophylactic migraine agent, has also been noted (33,34). A possible role for both ergotamine and methysergide in reducing cerebrovascular inflammation is suggested by the reported inhibition by methysergide and other 5-HT_2 antagonists of the potassium-stimulated release of Substance P in the ventral spinal cord (35). A number of vasoactive lysergic acid derivatives, GYKI-32594 (1), LY 53587 (2), and 3, have been described (36,37,38). LY53587, a selective 5-HT_2 antagonist, is reported to be in phase 2 migraine clinical trials.

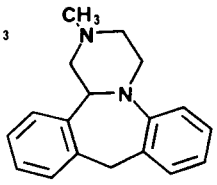




Related to cyproheptadine and pizotifen (4), both established tricyclic antimigraine agents, ketotifen (5) and pipethiadene (6) have also been reported as clinically active (39,40). In addition to a serotonergic action, both compounds are also potent antihistaminergics. Ketotifen is also reported to inhibit the release of histamine and slow reacting substance of anaphylaxis (SRSA) from mast cells.

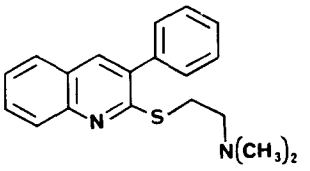






Disclosed as antimigraine agents by virtue of a selective vasoconstrictive action on the dog carotid vascular bed, an extensive series of 3,5-disubstituted indoles has appeared in the patent literature. The most recent reports (41,42), indicate that preferred activity resides in the 5-alkenylsulphonamides 7 and more particularly methylsulphonamide 8. Compounds of this type have been reported to be in phase 2 clinical trials. The clinical efficacy of mianserin (9) in reducing the frequency and intensity of migraine headaches has been related to both its serotonergic and alpha-adrenergic antagonist properties (43,44). Ketanserin (10) was also evaluated in acute and chronic migraine therapy (45,46). The quinolone-based 5-HT_2 antagonist ICI 169369 (11), has been patented for migraine (47). Similarly, iprazochrome (12), a weak serotonergic antagonist with prostaglandin and other antagonist properties, was also reported active in limited

clinical trials (48). Antimigraine activity for AGN-2979 (**13**), through the inhibition of tryptophan hydroxylase, has been claimed (49).



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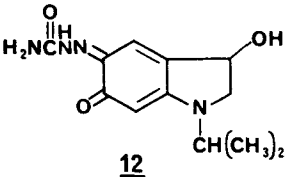

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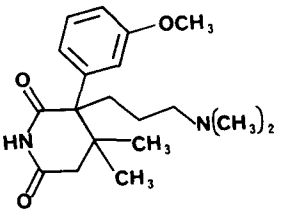

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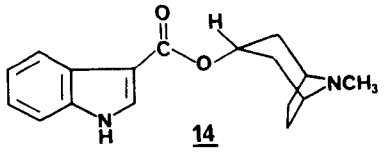
5-HT_3 - Recent studies have demonstrated the existence of an excitatory serotonergic receptor on peripheral sensory afferent nerve terminals (50,51). This has been named the 5-HT_3 receptor and appears related to Gaddum's serotonin M receptor (52). The antagonism of serotonin-induced blister pain and axon reflex by ICS 205-930 (**14**) and MDL 72222 (**15**) confirmed the location and function of the 5-HT_3 receptor (53,54). The probable existence of these receptors on the sensory trigeminal nerve terminals located in the cerebrovascular wall has stimulated the study of new 5-HT_3-receptor antagonists in migraine (55). Significant clinical efficacy in reducing the pain of acute migraine has been reported for MDL 72222 (56). Tropanserin (MDL 72422, **16**), also active against axon reflex (57), is reportedly undergoing clinical evaluation for migraine. Substitution of the ester linkage of MDL 72222 by an amide is also reported to maintain 5-HT_3-antagonist activity (58). Replacing the tropyl group of tropanserin with 1-methylpiperidine as in **17** also maintains 5-HT_3-antagonist activity (59). Similarly **18**, an analogue of ICS 205-930, is also a selective 5-HT_3 antagonist (60). Although substitution of the indole moiety of ICS 205-930 by pyridine, quinoline and related bicyclic rings has been claimed (61), more radical structural alteration, as in **19** and **20**, also affords 5-HT_3 antagonists (62,63).



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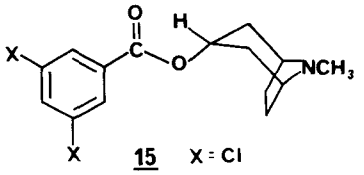
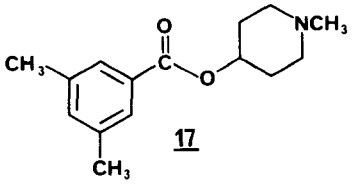


15 X = Cl

16 X = CH_3



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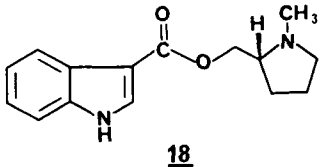

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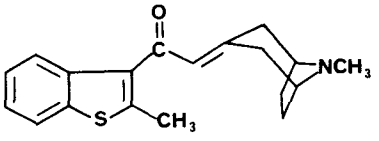

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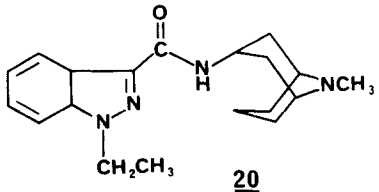

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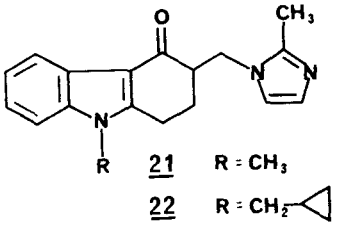
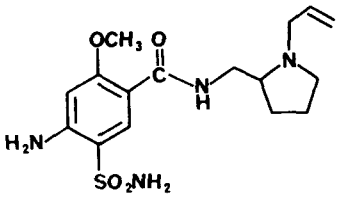
Odanserin (GR 38032F, **21**) represents a new structural class of potent and selective 5-HT_3-receptor antagonists (64). Limited substituent variation on this structure, eg. **22**, has also been reported recently (65). The significance of GR 38032F as a selective 5-HT_3-receptor antagonist is amplified by its reported efficacy and potency in models of emesis, anxiety, and schizophrenia (66). It is apparent from these studies that the 5-HT_3 receptor is not restricted to the peripheral nervous system, and as such, from the perspective of migraine, clinical efficacy of 5-HT_3 antagonists may also result from central mechanisms.



21 R = CH_3

22 R = CH_2-cyclopropyl



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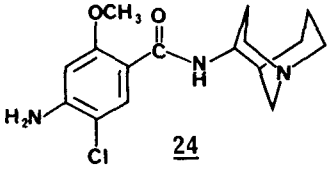

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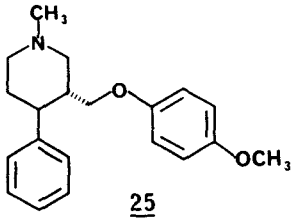
Prior to the discovery of the 5-HT_3 receptor, metoclopramide, a dopaminergic antagonist, had been shown effective in controlling the pain of headache and the associated emesis. However, a 5-HT_3-receptor antagonist profile has recently been established for this compound which may better explain its acute antimigraine action (67). The metoclopramide analogue, alpiropride (RIV 2093, **23**), appears to be superior to pizotifen in the prophylaxis of migraine (68). Another analogue, **24** (BRL-24924), is reported to be a potent inhibitor of the 5-HT_3 receptor-mediated Bezold-Jarish reflex and to be free of dopaminergic antagonist properties (69).

5-HT Uptake Inhibitors - Clinical benefit in migraine has been established for agents which both enhance and deplete the endogenous levels of serotonin. 5-Hydroxytryptophan has been shown recently to be equieffective with methysergide in the treatment of mixed migraine patients (70), although no better than placebo in childhood migraine (71). Amitriptyline, a serotonin reuptake inhibitor with 5-HT_2 antagonist and other biochemical properties, is an established migraine therapy (28). Femoxitine (**25**), a 5-HT reuptake inhibitor, although reportedly under clinical study for migraine, appears not to be superior to placebo (72). An indole derivative **26**, reported to be a 5-HT release stimulant and reuptake inhibitor, has also been claimed for the treatment of migraine (73).



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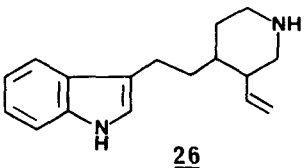

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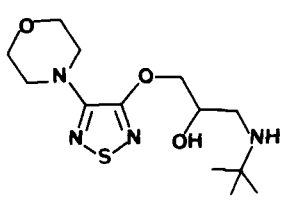
Adrenergic Agents

Alpha-Adrenergic Agents - The vascular actions of ergotamine at

therapeutic doses are predominantly due to its potency as an alpha antagonist (33). A central adrenergic action on endorphinergic pain modulation has also been suggested (74). A predominantly cerebrovascular site of action has been suggested to explain the observed antimigraine activity of indoramine (75). Clonidine was also shown to reduce headache intensity (43). Some support for a therapeutic action of alpha-adrenergic agonists in migraine, other than by a vascular mechanism, has come from the reported noradrenergic inhibition of Substance P release from spinal primary sensory afferent nerve terminals (76).

Beta-Adrenergic Agents - The role of beta-blockers in migraine has been reviewed (77,78). Antimigraine activity is not a pharmacological action shared by all beta-blockers, but has been clinically demonstrated only for propranolol, timolol (**27**), metoprolol (**28**), nadolol (**29**), and atenolol (**30**). In addition to a lack of intrinsic sympathetic activity



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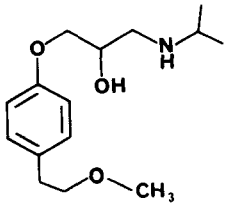

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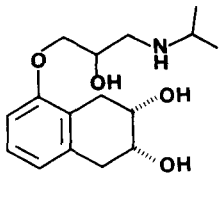

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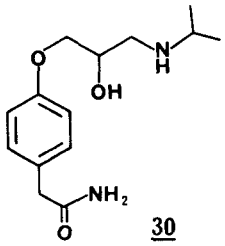

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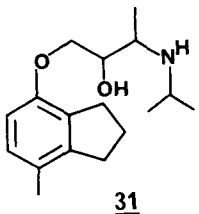

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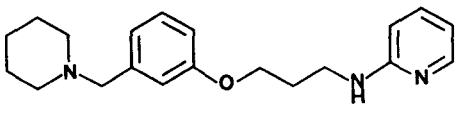
antagonism of $beta_1$ receptors is also a feature common to migraine-active beta-blockers to date. Serotonin antagonism is an action shared by several beta-blockers, although this action is not clearly associated with clinical efficacy (78,79). Although beta receptor-mediated prejunctional inhibition of vascular norepinephrine release has been suggested, a central action, possibly through long term inhibition of the locus coeruleus adrenergic system may better explain the known delayed onset of beta-blocker action in migraine (78). ICI 118551 (**31**), a $beta_2$-selective antagonist (80), is reported to be in phase 2 migraine trials.

Dopaminergic Agents - Hypersensitivity to dopamine agonists among migraine sufferers has been demonstrated and consolidated in the form of a piribedil test (81). However, with the exception of the peripherally-acting antagonist domperidone, the remaining agents effective in the abortive treatment of migraine belong to the metoclopramide family, and as such have been considered under $5\text{-}HT_3$ antagonists.

Histamine - Substance P stimulates the release of histamine from human mast cells (82). In the rat, the release from mast cells of histamine, 5-HT, and acetylcholine has been shown to result from stimulation of the peripheral sensory branch of the trigeminal nerve. The resulting plasma extravasation was antagonized by a cimetidine/mepyramine combination, atropine, and methysergide (83). A cimetidine/mepyramine combination has also been shown effective in treating histamine induced migraine (84). However, cimetidine plus chlorpheniramine was not effective in spontaneous migraine (85). The relevance to migraine therapy of the antihistaminergic and mast cell stabilizing properties of ketotifen and pizotifen is unclear. New histamine (H_2) antagonists, eg **32**, which penetrate into the CNS, have recently been patented for the treatment of vascular headache (86).

Opiates - The role of endogenous opiates in migraine has been reviewed from several perspectives (6). Opiates are effective in the acute treatment of migraine headache. Lofentanil (**33**) has been used to demonstrate a role for opiates in the presynaptic inhibition of Substance P release from both central and peripheral terminals of primary sensory afferent nerve terminals (87). The existence of dynorphin-B-containing nerve fibers surrounding brain blood vessels has recently been established (88). Like enkephalinamide, dynorphin also inhibits the depolarization induced release of Substance P from sensory nerves and reduces neurogenic inflammation (89).



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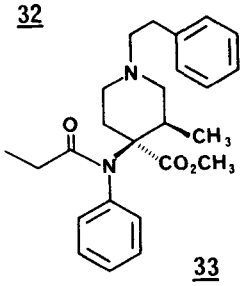

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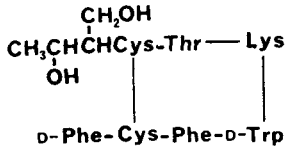

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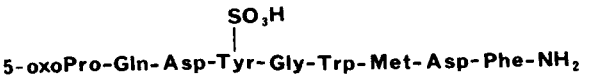

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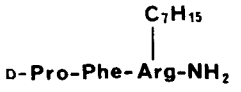
Other Peptides - Somatostatin has been shown to inhibit the release of Substance P from trigeminal nerve endings (90,91). This has led to the successful trial of i.v. somatostatin in the treatment of cluster headache (92). Octreotide (SMS 201-995, **34**), is a synthetic somatostatin analog under clinical investigation for a range of conditions, including cluster headache (93). Other peptides reportedly effective in reducing the pain or frequency of migraine include ceruletid (**35**), a Cholecystokinin (CCK) analog, and salmon calcitonin (94,95). Bradykinin, a nonapeptide, is a major proinflammatory and pain producing substance which is released following acute tissue irritation or damage. A central role for bradykinin in the etiology of migraine has been suggested (96). In addition to a direct vascular action, bradykinin has also been shown to increase vascular permeability and pain perception through the stimulation of Substance P release (97). The tripeptide bradykinin antagonist **36** (S-2441), has been suggested as a potential migraine therapy (98,99).



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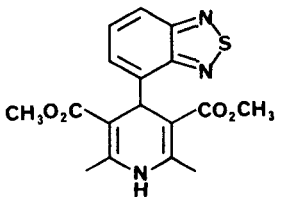
Calcium-Channel Antagonists - The value of calcium-channel antagonists in prophylactic migraine therapy is well established and has been reviewed (100-103). These agents are generally reported to reduce the frequency rather than intensity of attacks. In addition to the clinically recognized antagonists, flunarizine, nimodipine, and verapamil, potent calcium-channel antagonist activity has also been noted for cyproheptadine and pizotifen (104,105). The benzothiadiazole dihydropyridine **37** has been specifically claimed for the treatment of vascular headache (106). The clinical exacerbation of both the intensity and duration of an ongoing migraine



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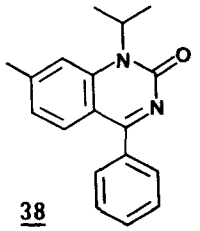
attack by acute nifedipine contrasts with the demonstration of acute efficacy for flunarizine in isosorbide nitrate-induced migraine (107,108). Nonetheless, the well-documented long delay in the onset of prophylactic action of these agents is more consistent with a neuronal than with a vascular mechanism of action. This hypothesis is in accord with the reported inhibition of potassium-stimulated Substance P release from cultured sensory afferents by dihydropyridines and by the potentiation of opiate and non-opiate analgesia by diltiazem and other calcium antagonists (109,110).

Prostaglandins - A role for E-type prostaglandins in the precipitation and evolution of migraine has been suggested (111,112). In addition to a vasoactive role, prostaglandins E_1, E_2 and I_2 have been shown to sensitize pain receptors to the action of bradykinin, or histamine (113,114). A presynaptic action of PGE_1 to enhance the peripheral release of Substance P from trigeminal fibers has also been reported (115). From these and additional observations, it was suggested that E-type prostaglandins exert their hyperalgesic action by lowering the depolarization threshold of primary afferent fibers to noxious stimuli (116). The influence of prostaglandins on pain sensitivity has been reviewed (117). In addition to aspirin, tolfenamic, mefenamic, and diclofenamic acids, and naproxen have been shown effective in acute migraine prophylaxis (118-121). Proquazone (**38**) was also clinically effective (122). Feverfews, an ancient antinflammatory herbal extract comprised of several sesquiterpine lactones eg. **39**, has received significant attention recently as a prophylactic antimigraine agent (123,124). Prostaglandin antagonism, *in vivo* inhibition of phospholipase A_2, a cytotoxic effect on monocyte proliferation, and inhibition of polymorphonuclear leucocyte cell degranulation, all have been suggested as a mechanism of feverfews' action (125-127).



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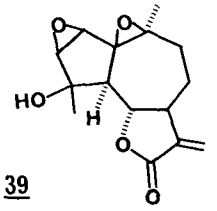

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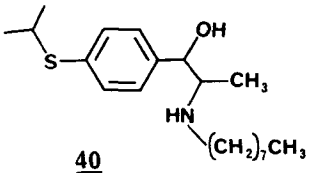

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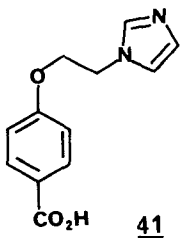
Miscellaneous Other Agents - Although frequently implicated as a precipitating factor in migraine, platelet aggregation and degranulation are now more commonly viewed as an epiphenomenon (128,129). Nonetheless, positive migraine clinical data have been reported for suloctodil (**40**), a platelet aggregation inhibitor, and dazoxiben (**41**), a thromboxane-synthetase inhibitor (130,131). In one patient, transdermal scopolamine has been reported to reduce the frequency and severity of migraine attacks (132). Although known to be effective in the prophylaxis of cluster headache, lithium has been shown to exacerbate migraine headache (133).



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Conclusion - Although past approaches to migraine prophylaxis have been essentially empirical in nature, recent advances in delineating pain-producing mechanisms and pathways offer the future promise of rational and targeted drug therapy of headache.

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Chapter 6. Neuropeptides and Their Processing: Targets for Drug Design

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Introduction* - This chapter updates our previous survey of the formation and degradation of neuropeptides (1), subsequently reviewed by others (2,3) and describes progress in designing drugs which can influence these processes. In principle this includes enzymes, enzyme inhibitors and neuropeptide agonists or antagonists. Although recent developments have made the design of new proteins feasible (4-6) and several enzymes with therapeutic potential exist (7), enzymes as drugs still present serious problems in the context of access to the CNS. Accordingly, this chapter will be limited to enzyme inhibitors and neuropeptides.

THE FORMATION AND DEGRADATION OF NEUROPEPTIDES

Recent Findings - Precursors for neuropeptides processed at paired basic amino acid residues appear to share a common ß-turn secondary structure around the cleavage site (8), while monobasic processing may be directed by adjacent proline residues (9). New endopeptidases include a rat metallopeptidase cleaving NT at the Pro^{10}-Tyr^{11} bond (10) and a bovine enzyme cleaving the OT precursor on the carboxyl side of the Lys-Arg doublet (11). Rat brain may contain a specific TRH-degrading enzyme that is distinct from the more widely distributed pyroglutamyl peptidase (12). A free-radical, alternative mechanism has been suggested for peptide α-amidation on the basis of studies with an abiotic system (13). New exopeptidases include a serine carboxypeptidase of porcine origin which processes human ACTH and ß-LPH (14), and cathepsin B (a preferential peptidyl dipeptidase) which cleaves pro-opioid oligopeptides (15).

Putative neuropeptides, e.g. galanin (16), locust adipokinetic hormone (17) and ODN (18) are widely distributed in rat brain. Galanin acts in human hypothalamus to release growth hormone (19). A DBI-like peptide in human brain may be abnormally high in depression (20). Newly identified precursors include a 255-amino acid protein in rat brain containing 5 copies of the TRH sequence (21), and a 92-amino acid poly-

*The amino acid sequences of most known neuropeptides were given in last year's review (1). Peptide abbreviations used in this chapter are: ACTH, adrenocorticotropic hormone; AMP, 2-aminomethylpyridine; Bua, butyric acid; CCK, cholecystokinin; CRF, corticotropin-releasing factor; DBI, diazepam-binding inhibitor; (DD)AVP, (1-desamino-8-D)arginine vasopressin; Eda, ethylenediamine; GHRH, growth hormone-releasing hormone; Iva, isovaleryl; LH, luteinizing hormone; LHRH, luteinizing hormone-releasing hormone; LPH, lipotropin; MCH, melanin-concentrating hormone; Mcp, ß-mercapto-ß,ß-cyclopentamethylenepropionic acid; MSH, melanocyte-stimulating hormone; Nal(2), 3-(2-naphthyl)alanine; NT, neurotensin; ODN, octadecaneuropeptide fragment of DBI; OT, oxytocin; pAad, L-pyro-2-aminoadipic acid; Pal(3), 3-(3-pyridyl)alanine; POMC, pro-opiomelanocortin; SP, substance P; SS, somatostatin; TRH, thyrotropin-releasing hormone.

peptide in human pituitary containing LHRH and a second distinct sequence capable of stimulating LH release (22). Putative neuropeptides described for the first time in mammalian brain include MCH (1), originally identified in the salmon (23); cerebellin, a 16-amino acid peptide isolated from rat cerebellum (24) and various motilin-like peptides (25) which differ from the previously described porcine intestinal motilins (1). The markedly reduced levels of dynorphins and enkephalins in the basal ganglia of patients with Huntington's chorea imply an important role for neuropeptides in this disease (26). Furthermore, patients with Alzheimer's disease showed an increased CSF level of the 15KD precursor of SS and a lowered level of SS-14 (27), as well as reduced cortical and striatal levels of CRF-like immunoreactivity (28,29).

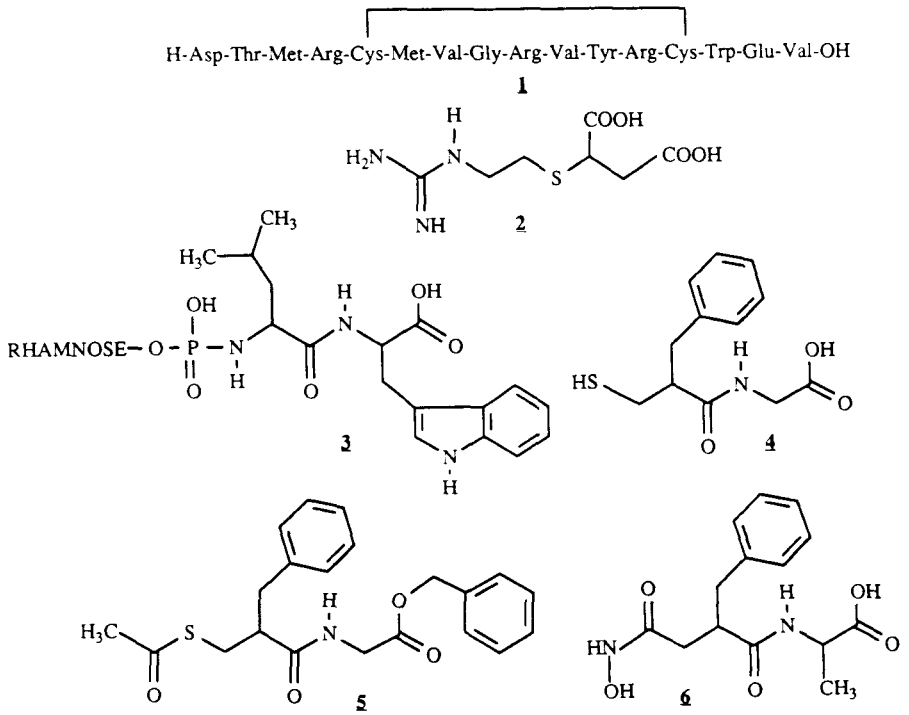
Further information was presented on the role of neuropeptides as co-transmitters in the CNS (30) and their presence in non-mammalian species (31). Neuropeptide processing and levels can be affected by age (32), diet (33), as well as by drugs (1): old rats had significantly altered molecular forms of endorphins and enzymatic α-carboxyamidation is impaired by Vitamin B deficiency. Peptides can be used to inhibit the degradation of endogenous peptides (34). Processing of POMC and pro-enkephalin A and B appear to be modulated by central dopaminergic and serotonergic systems since acute and chronic administration of dopamine antagonists or serotonin agonists raised levels of endorphins, enkephalins and dynorphins in rat brain (35-38) and human plasma (39). Brain levels of SP and neurokinin A are lowered by chronic neuroleptics (38,40,41) and spinal SP is raised by chronic antidepressants (42).

Several general hydrolytic enzymes have been well characterized (e.g. thermolysin, trypsin, chymotrypsin, pepsin and elastase) (43,44), while peptidases of various classes involved in neuropeptide degradation have been obtained in pure form (45,46). Although much less is known about the enzymes involved in the processing of large precursor molecules (i.e. the maturases), or those involved in post-translational modifications, differences in catalytic and physicochemical properties and tissue cellular distribution suggest that each enzyme may act only on its physiological substrate (47). However, others claim that there is a limited number of processing enzymes, which can cleave different prohormones in different tissues at pairs of basic amino acid residues (48).

Enzyme Inhibitors: General Principles - Reversible, irreversible and pseudo-irreversible (cleaved only by chemical means) enzyme inhibitors have been described (49). Reversible inhibitors, typified by the transition-state analogues, can be competitive, mixed or non-competitive in action. The irreversible inhibitors are classified according to their mechanism of action into active site-directed inhibitors (including affinity labels) and mechanism-based inhibitors or suicide substrates (44,49,50).

Strategies for the design of enzyme inhibitors have been reviewed (45,46,49-53). Naturally-occurring inhibitors can be structurally modified to achieve selectivity for a particular enzyme without loss of potency (51,52,54). Proteinase inhibitors for trypsin and chymotrypsin occur in plants (55,56) and animals (54), while several small peptide-like enzyme inhibitors of microbial origin are known, such as pepstatin, bestatin, amastatin, chymostatins, leupeptin, elastinal and phosphoramidon (53,54). The design of inhibitors is often based upon postulated enzyme-reaction intermediates such as tetrahedral transition-state intermediates, collected substrates or bi-products (51,53,57,58). Structural modifications of the peptide backbone and side chains have been amply

employed (59-61). Alternatives for the peptide bond have been utilized to increase resistance to hydrolytic attack while maintaining other binding interactions. Replacements include CO-NAlk, CO-NOH, CO-CH_2, C(OH)-CH_2, CO-C=C, CH_2-NH, CH_2-O, CH_2-S, CH_2-CH_2, CH=CH (E or Z configuration) and CS-NH. Retropeptides (NH-CO), and aza (CO-NH-NR), hydrazino (CO-NH-NH) and intercalated peptides (CH_2-CO-NH, CO-NH-CH_2) are also known. Such changes may not only prolong duration of action but can lead to dissociated or selective biological responses and to altered solubility characteristics (62). Changes at places other than the scissile peptide bond can play an important role through conformational restriction or regiospecific hydrogen bonding (51). Knowledge of the tertiary structures of enzyme and enzyme-inhibitor complexes, obtained by X-ray and other methods, and the application of computer-assisted molecular modeling, has facilitated the design of new inhibitors (5-7).

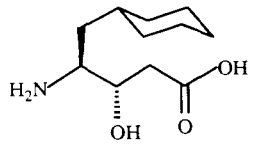


Several specific inhibitors of non-specific proteases have been described (63-67). The highly specific serine protease kallikrein can be inhibited by a blocked hexapeptide obtained *via* the substrate analogue approach (58). Examples of specific inhibitors for CNS enzymes are rare. The best known is guanidinoethylmercaptosuccinic acid (2), which is a potent and selective active site-directed inhibitor of a carboxypeptidase B-like enzyme which removes the Lys or Arg residue after cleavage at a pair of basic amino acids. This enzyme is probably involved in the processing of many peptide hormones (68,69). Pepstatin A effectively inhibits degradation of mouse POMC by the bovine neural lobe prohormone converting enzyme (48). Other examples are *o*-phenanthroline, which specifically inhibits rat brain TRH-degrading enzyme (12), and thiol-group blocking reagents (e.g. bestatin) and metal-ion chelators which inhibit the activity of the major aminopeptidase from human cortex (70).

Opioid Systems - One approach to new centrally-acting analgesics involves inhibiting enzymes which degrade opioid peptides. A thiol protease from

rat brain, which cleaves dynorphin B-29 at a single Arg residue to give the 13-peptide dynorphin B, is inhibited by dynorphin A and its 6-17 fragment but not by the 8-17 fragment. This suggests a role for the Arg^6-Arg^7 pair in the action of this enzyme, which also recognizes opioid peptides of the endorphin and enkephalin families (71). Phosphoramidon (3) inhibits one rat brain synaptosomal endopeptidase but not another, in converting dynorphin A to dynorphin-A(1-8) (72). Studies of enkephalin degradation by enkephalinase (a brain metallopeptidase) suggest that cleavage at Gly-Phe is important. This enzyme, better called endopeptidase 24.11, is not as specific for enkephalins as was once thought (73-76). Several types of inhibitors of this enzyme have been reported and may have potential as analgesics (74-77). Phosphoramidon (3), thiorphan (4), acetorphan (5, a lipophilic pro-drug of 4) and kelatorphan (6) are the most potent (78). Kelatorphan is able to protect enkephalins both *in vitro* and *in vivo* by blocking inactivation by endopeptidase 24.11 and by other enzymes involved in their degradation such as membrane-based aminopeptidases and a dipeptidylaminopeptidase (79). The major cleavage process *in vitro* is the release of the N-terminal Tyr residue, which can be blocked by the non-selective inhibitor bestatin (80,81).

The Renin-Angiotensin System - Both the peripheral and central renin-angiotensin systems, kept independent by the blood brain barrier (BBB), are involved in the control of blood pressure (82). Inhibitors of renin and angiotensin-converting enzyme (ACE, peptidyl dipeptidase A) are used, or are being investigated as antihypertensive drugs (83-85). Renin cleaves human angiotensinogen between Leu^{10}-Val^{11} (Leu^{10}-Leu^{11} in other species) to give angiotensin I. ACE subsequently removes the C-terminal dipeptide (His^9-Leu^{10}) to give the potent pressor substance, angiotensin II (83,84). ACE has additional central roles in hydrolyzing NT, SP and LHRH (1), and may act as a tripeptidylcarboxypeptidase in the brain (86).



7

Iva- His- Pro- Phe- His- ACHPA- Leu- Phe- NH_2

8

Boc-Pro-Phe-MeHis-Leu-Ψ[$CHOHCH_2$]Val-Ile-AMP

9

Early competitive antagonists of renin (87) based on the substrate analogue approach were moderately potent decapeptides (58,83). More potent compounds resulted from SAR and enzyme kinetic studies and molecular modeling (85,88). Incorporation of transition-state dipeptide isosteres [e.g. statine and ACHPA (7)] gave analogues (e.g. 8, 9) with further enhanced potency (85,89-92). Many of the renin inhibitors described in the literature are species specific as well as highly selective for renin among the acid proteases.

Despite its rather low specificity ACE is most closely associated with the formation of angiotensin II (83,93). Based on the success of captopril, extensive SAR studies on N-carboxydipeptides, conformationally restricted inhibitors, phosphorus-based compounds and substances with dipeptide surrogates, as well as computational studies, have resulted in many ACE inhibitors which are now in the final stages of development (93-96). Several of these contain proline replacements and are monoester pro-drugs (83,93). In addition to the peptide inhibitors from snake venom (83) and a recently identified tripeptide in porcine plasma (97), several micro-organisms secrete specific peptide inhibitors with potencies up to one sixth that of captopril (98).

The involvement of ACE in the proteolytic degradation of H-Tyr-Gly-Gly-Phe-Met-Arg-Phe-OH in rodent and primate brain has recently been demonstrated (99-101). Captopril also inhibited the inactivation of LHRH by neuroblastoma cells (102), and after icv administration reduced plasma ACTH and AVP responses (but not that of angiotensin II) to hemorrhagic stress (103). An increased BBB permeability in normotensive rats after icv, intracarotid or topical application of captopril has recently been reported (104). Centrally active and selective ACE inhibitors may be useful in CNS diseases involving memory and cognitive dysfunctions, since captopril and zofenopril delay avoidance extinction in rats (105).

NEUROPEPTIDES

General Considerations - Peptides differ from most other drugs in several important respects which affect the design and biological investigation of structural analogues. Thus, they are relatively large and flexible molecules, are prone to rapid enzymatic degradation, consist of several amino acids each with its own side-chain function, and have more than one effect even in physiological concentrations (106). Attempts to obtain peptides with improved metabolic stability, potency and selectivity of action have generally started with the natural peptide sequence (60,61,106,107). The aim is typically to identify the smallest active fragment through modification of the backbone (at the terminals, the peptide bonds and in the side chains of the amino acid residues) (59-61,106-108). Increases in in vivo potency and/or selectivity may result from enhanced receptor affinity, increased intrinsic activity or stability, improved physico-chemical properties (e.g. lipophilicity), or restriction to a more active conformation. However, a prolonged duration of action may result both from these and additional factors such as increased distribution volume, prolonged half-life and enhanced renal reabsorption (109). Examples are known of α-MSH analogues that combine very prolonged biological activity with high or low potency (108).

Increased selectivity is desirable since peptide hormones generally act on peripheral and central targets. Often the sequence needed for CNS activity is shorter than that required for hormonal effects (110). Another approach is to study the degradation of the neuropeptide with (brain) enzymes and isolate, characterize, and evaluate the resulting fragments (111). Examples include ß-endorphin (1), AVP (112) and OT (113). Typically, SAR studies are conducted on the interesting fragments, or labile bonds in the parent molecule are modified. Analogues with restricted conformational freedom, and cyclic structures in particular, have been studied (5,61,114-116). The application of physical methods to determine conformational aspects of biological activity has been reviewed (117). SAR studies on several intermediate-sized peptide hormones with amphiphilic secondary structures in amphiphilic environments, such as membranes, have identified binding to particular receptors when multiple receptors are known to exist (118). Structural information from the solid state is available only rarely. QSAR studies are also scarce with peptides (60,61). More attention has been paid to application of modern theoretical techniques to the conformationally-based design of peptide analogues (5,117). A computer aided systematic search of peptide conformations from NMR data has recently been reported (119).

Peptide antagonists are very useful for studying the role of peptides in physiological processes, and can also have clinical potential for diagnosis and treatment of hormone excess (120,121). However, effective antagonists do not exist for most of the presently known neuropeptides. Strategies for designing antagonists have been described

(106,115,116,120,121). It is essential to have reliable and reproducible bioassays (108,115,116,121), as well as corroborative receptor binding methods (122), for the meaningful application of SAR studies to antagonists. Speculations on the design of peptidomimetics have been published but have not resulted in many useful examples (123). Among the major obstacles to be overcome if peptides are to be used as CNS drugs are the action of ubiquitous proteolytic enzymes and penetration through the BBB. Several factors can influence the route and method of delivery including molecular size, biological half-life, immunogenicity, conformational stability, dose requirement and rate of administration, and pharmacokinetic-pharmacodynamic considerations (124). Orally administered peptides must be protected against digestion by gastrointestinal (gi) enzymes. Metabolic stability can be enhanced by suitable modifications at potential cleavage sites (125) or the peptide can be directed to parts of the gi tract (e.g. the colon) which lack such enzymes (124,126). However, almost all peptides are administered parenterally, usually as injections of aqueous solutions and sometimes as oily solutions or suspensions. In order to prolong their release and minimize dosing frequency, use has been made of zinc phosphate, liposomes, biodegradable microspheres, implanted osmotic infusion pumps and non-biodegradable tubing systems (124). Non-parenteral routes under investigation include nasal, buccal, rectal, vaginal and transdermal -- bioavailability is better than by the oral route but is still less than after parenteral dosage (124). Several recent studies in man have compared bioavailability from different routes for TRH (127), OT (128), DDAVP (129), LHRH (130) and an agonist (131), glucagon (132), GHRH (133) and CRF (134).

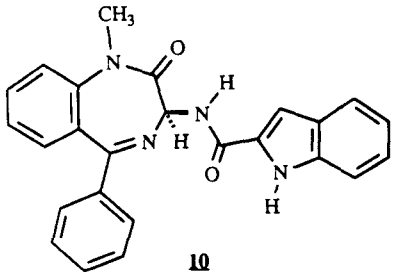
The lipid solubility of peptides is low, and their access to the brain *via* the circumventricular organs, which have no BBB, is slow and limited (135). Evidence has been presented for receptor-mediated peptide transport through the BBB, similar to that which exists for nutrients (136,137). However, peptides may not necessarily have to gain access to the CNS to elicit a response, because peptide receptors do exist on the luminal epithelium of the BBB and such receptors may mediate peptide-induced changes in BBB permeability (135,138). The major route of passage is probably a non-competitive, non-saturable mechanism in which lipophilicity plays the most important role in determining penetration. Opening of the BBB, intrathecal administration and drug latentiation have all been proposed as methods to increase CNS peptide levels (136,137,139).

Peptide pharmacology may be impeded by inconsistent purity of samples and even incorrect primary structures. Considerable variation in the peptide content of commercially available compounds has been reported with several peptides supplied as erroneous sequences and peptide ligands incorrectly radiolabeled (140,141). Purity assessment by HPLC does not eliminate the need for an amino acid analysis, especially for peptides made by solid phase synthesis. Useful additional methods to establish purity include mass spectrometry and capillary isotachophoresis (140,142).

Peptides of Current Interest - Although several peptides are available for therapeutic and diagnostic purposes, current CNS research centers mainly on analogues of natural substances (143,144), examples of which are discussed below.

Gut-brain peptides - SP remains of great interest, in part due to recent information concerning receptor sub-types (145) and descriptions of its preferred conformation in membranes (146-148). Many new agonists and antagonists of SP and its shorter C-terminal hexapeptide have been reported, containing D-amino acids, side-chain replacements and

blocked end groups (149-152). CCK, long known for its effects in the gut, is also wide-spread in the brain, where it acts as a co-transmitter in dopaminergic terminals (153). Neurochemical and pharmacological studies suggest a possible utility as an antipsychotic agent (153-155).



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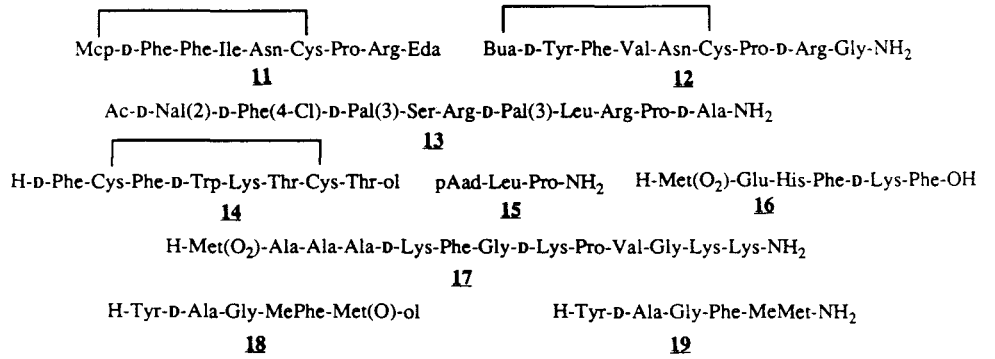
However, clinical trials in schizophrenia of CCK-8S and the related decapeptide ceruletide (caerulein) have given equivocal results (153,156). Conformational analysis of the most studied sulfated C-terminal octapeptide (157) and SAR studies have been described for both endocrine and central activities (153). Screening of fungal fermentation broths resulted in asperlicin, a novel, non-peptide antagonist of CCK (158). Extensive SAR studies on this compound led to L-364,718 (10), a potent orally active peripheral antagonist (159). As yet potent central antagonists remain to be found.

Neurohypophyseal peptides - SAR studies on the neurohypophyseal hormones AVP and OT have yielded agonists and antagonists for endocrine (160) and central systems (110,161), which can be dissociated from each other. The conformational constraint imposed by an N-alkyl residue in position 7 of AVP appears unnecessary for antagonist binding but is important for agonists, suggesting a different mechanism of interaction (162). Compound 11 is one of the most potent antagonists of vasopressin antidiuretic activity known (163). Modification of DDAVP gave 12 which is orally active (164). Highly selective and potent antagonists of OT have been obtained as potential inhibitors of excessive uterine activity (165). The C-terminal tripeptide of OT, H-Pro-Leu-Gly-NH_2, and many of its analogues modulate dopamine receptors (166).

Hypophysiotropic peptides - LHRH agonists containing a hydrophobic D-amino acid residue in position 6, with or without C-terminal modification, appear to be clinically useful as contraceptives or therapeutics for carcinoma (167). In vivo potencies of LHRH agonists and antagonists are greatly influenced by species and animal model, route of administration and biopharmaceutic parameters (168). Recent SAR studies mainly concerned antagonists with increased hydrophobic side-chains in positions 3, 6 and 7 (169-171). Compound 13 is the most potent antagonist yet described (172).

For the most part SS is inhibitory in the CNS, but SS analogues may have therapeutic potential in several areas (173). Classical SAR approaches have led to increased selectivity of action, potency and metabolic stability with oral activity (174-176). Analogue design based on conformational analysis has provided new and highly potent compounds (115,116). Clinical applications of the analogue octreotide (SMS 201-995, 14) in treatment of acromegaly and carcinomas have been reviewed (173,177,178). The involvement of SS in Alzheimer's disease indicates a possible new area for SS analogues (173).

TRH is also widely distributed in the CNS and is involved in many extrathyroidal actions (179). Clinical applications in depression for analogues that are metabolically stabilized by modification of the terminal amino acids have been reviewed (180). Further dissociation of hormonal and CNS effects was observed (e.g. in 15) when the central histidine was replaced by neutral amino acids and the terminal residues by 5- or 6-membered heterocycles (181).



POMC-derived peptides - POMC-derived peptides have received considerable attention. Several fragments and analogues of ACTH have long been available for the stimulation of the adrenal cortex to produce and release steroid hormones (143). Peptides with enhanced behavioral potency and selectivity for CNS receptors have been described (60,182,183). The hexapeptide Org 2766 (16) was orally active in several animal models (182,184), while a 13-peptide analogue (17) was ~ 3 million times as active as the reference compound ACTH-(4-10) in an active avoidance paradigm (60). Comparison of central activities of Org 2766 and ACTH-(4-10) in rats showed different SAR, even resulting in opposite actions (184). Human studies with ACTH derived peptides have been reviewed (185,186). α-MSH, a modified N-terminal 13-peptide fragment of ACTH, is involved in regulating melanin synthesis and melanosome dispersion and in other peripheral and neuroregulatory actions (144,182,187). Recent conformation-activity studies have led to many potent fragment analogues with selective biological activity and which combine high potency with prolonged action in the periphery (108,115,116,188). Different SAR exist for the neurotrophic actions of ACTH analogues (189).

Enkephalins - Since their discovery in 1975, countless analogues of the endogenous opioid peptides Met- and Leu-enkephalin have been synthesized, including orally active compounds (190-193). Some, including FK-33.824 (18) and metkephamid (19), are under clinical investigation as analgesics and for other applications. Conformationally restricted cyclic analogues have been pursued with the aim of increased receptor selectivity (115,116,194).

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Section II - Pharmacodynamic Agents

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Chapter 7. Antihypertensive Agents

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Introduction - Hypertension is a consequence of many diseases: diseases of the components of the central and peripheral nervous systems which regulate blood pressure, diseases of the kidney, vascular disease and abnormalities of the hormonal systems which control blood pressure and fluid volume. Thus, it is not surprising that patients vary in responsiveness to one type or a particular cocktail of medications, presumably based on the etiology of the hypertension. Although the WHO recommends diuretics and beta-blockers as first line therapy (1), recent articles suggest new approaches to treating hypertension, favoring vasodilators (2). The recent use of ACE inhibitors, which are categorized as vasodilators, has resulted in a reported improvement of the quality of life and a feeling of well being among treated patients (3).

Renin Inhibitors - A recent editorial has discussed the history of the development of renin inhibitors (4) and a survey of low-molecular weight inhibitors of renin and pepsin has appeared (5). Substrate analogue inhibitors have been designed employing computer assisted molecular modeling (6). Details concerning the synthesis and renin inhibitory activity have been reported for a series of peptides of general structure A-B-Sta-Ala-Sta where highest potency was observed for compounds in which A=Phe, B=His or Nle and the N-terminal amine was blocked with t-Boc or isovaleryl (7). In this series, pentapeptide 1 showed IC_{50}'s of 28, 600 and 34 nM against human, rat and hog renins, respectively. ES-305 (2) a statine-containing peptide incorporating an achiral, hydrophobic replacement for Phe, inhibited purified human renin in vitro with an IC_{50}= 2.4 nM (8). Structural modification of the unusual amino acid statine has led to a new class of renin inhibitors containing (3S,4S)-3,4-diamino-6-methylheptanoic acid (Asta) as a novel replacement for Leu-10 in angiotensinogen (9). The tetrapeptide, 3, inhibited renin with an IC_{50} of 0.36 mcM compared to 0.19 mcM for the corresponding Sta derivative. In contrast to Sta-containing inhibitors where the (3R)-diastereomer is considerably less potent, both 3-amino isomers in Asta peptides show substantial activity.

Systematic exploration of the size requirements for peptide inhibitors of human renin yielded tetrapeptide 4 which contains a lipophilic P_1 side chain and a reduced amide at the scissile bond (10). This compound inhibited purified human renin in vitro (IC_{50}=7.8 nM) and produced a short-lived hypotensive response in salt-depleted monkeys (0.1 mg/kg, i.v.). Replacement of the Leu-Val scissile bond in angiotensinogen with a stable amino alcohol linkage afforded a new series of renin inhibitors with improved solubility characteristics (11). The most potent congener

in the series, BW 633C (5), competitively inhibited renin (IC_{50}=61 nM) and possessed water solubility to the extent of 0.5 mM. The effect of varying the P_1 side chain has been examined for tetrapeptide analogues containing difluorostatine and difluorostatone (12).

Cmpd. No.	Structure[a]
1	Iva-Phe-Nle-Sta-Ala-Sta-OH
2	BNMA-His-Sta-MBA
3	Boc-Phe-His-Asta-Leu-ABP
4	Boc-Phe-His-Cha$\overset{R}{-}$Val-MBA
5	His-Pro-Phe-His-Leu$\overset{AA}{-}$Val-Ile-Phe-OMe
6	Boc-Pro-Phe-(Me)His-Leu$\overset{OH}{-}$Val-Ile-AMP
7	NPP-His-Nsta-OMe
8	MNP-His-Nsta-OCH$(CH_3)_2$

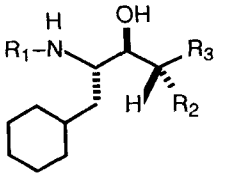
[a]Sta = (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid; BNMA = bis-(1-naphthyl)methylacetyl; MBA = 2-(S)-methylbutylamide; Asta = see text; ABP = 4-amino-1-benzylpiperidine amide; Cha = cyclohexylalanine; R = reduced amide; AA = amino alcohol; OH = hydroxyethylene; AMP = 2-aminomethylpyridine amide; NPP = (+)-2-(1-naphthylmethyl)-3-(phenethylcarbamoyl)propionyl; Nsta = (2RS,3S)-3-amino-2-hydroxy-5-methylhexanoic acid; MNP = (-)-2-(4-morpholinylcarbonylmethyl)-3-(1-naphthyl)propionyl.

U-71038 (6) is a potent inhibitor of human plasma renin (IC_{50}= 0.26 nM) with high metabolic stability (13). Although 6 has limited oral efficacy, this inhibitor displays the best oral activity of any compound reported to date. When administered i.v., it caused a dose-related fall in the blood pressure of salt-depleted monkeys and hog-renin infused, ganglionic-blocked rats (14). KRI-1177 (7) and KRI-1230 (8), relatively small peptides containing a new type of transition state mimic (norstatine), inhibited human plasma renin with IC_{50}'s of 90 and 7.8 nM, respectively (15). Bolus i.v. injection of 7 into salt-depleted, Japanese monkeys (0.1-5 mg/kg) produced a dose-dependent fall in systemic blood pressure accompanied by a corresponding decrease in plasma renin activity (PRA), angiotensin I and II (16). PRA is measured by most investigators; however, it is not clear whether the plasma and/or the tissue renin-angiotensin system (RAS) is responsible for regulating vascular tone (17). Further, PRA may not be predictive of the activity of the vascular tissue derived RAS (18).

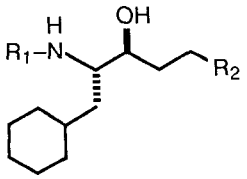
Utilization of (4S,5S)-5-amino-6-cyclohexyl-4-hydroxy-1-hexene-2-carboxylic acid as a new dipeptide analogue (19) provided 9 (IC_{50}=1.5 nM). Thioether 10 (20) inhibited purified human renin with an IC_{50} of 4 nM compared to 10 nM for the corresponding carbon isostere 11 (21). A statine-derived inhibitor containing a lipophilic P_1 side chain and a 2-hydroxyl substituent (12) was equipotent (IC_{50}=4 nM) to the corresponding des-hydroxy congener 13 (22,23). The cyclohexylmethyl derivative of homostatine retro-inverted at the C-terminus and acylated with Boc-Phe-His yielded 14 (24). This compound inhibited renin *in vitro* with an IC_{50} of 15 nM. The hydroxysulfone 15 produced a hypotensive response in salt-depleted monkeys at 0.1 mg/kg, i.v. (25).

In addition to their therapeutic usefulness, renin inhibitors provide an excellent tool for the study of the RAS. Monoclonal antibodies against human renin serve as alternatives to synthetic renin inhibitors to study the effect of renin blockade *in vivo* (26). For example, R-3-36-16 (10 mcg/kg, i.v.) causes a potent and sustained hypotension in salt-

depleted marmosets, which is not enhanced by converting enzyme inhibitors. The production of native human renin in high yield by recombinant DNA techniques has been reported (27). Utilization of transfected Chinese hamster ovary cells followed by tryptic activation and affinity-chromatography gave renin of greater than 95% purity.



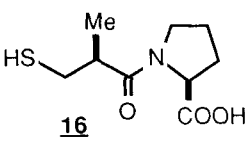
R_1 = Boc-Phe-His



9 $R_2 = H$, $R_3 = C(=CH_2)CONHCH_2CH_2CH(CH_3)_2$
10 $R_2 = H$, $R_3 = SCH(CH_3)_2$
11 $R_2 = H$, $R_3 = CH_2CH(CH_3)_2$
12 $R_2 = OH$, R_3 = CONH-(2S)-methylbutyl
13 $R_2 = H$, R_3 = CONH-(2S)-methylbutyl

14 $R_2 = NHCOCH_2CH_2CH(CH_3)_2$
15 $R_2 = SO_2CH(CH_3)_2$

ACE Inhibitors - The clinical pharmacokinetics of angiotensin converting enzyme inhibitors (ACEI) has been reviewed (28). The chronic treatment of hypertensive patients with ACEI has been evaluated with particular reference to efficacy, metabolic effects and adverse effects (29). Comparison of captopril (16) with SCH 31846 (17) suggests that in dogs and rats the toxicity of ACEI is not dependent upon the presence or absence of a sulfhydryl group (30). The effect of replacing the proline residue in both 16 and enalapril (18) with several conformationally constrained amino acids has recently been described (31); incorporation of a tetrahydroisoquinoline (THIQ)-3-carboxylic acid preserves ACE inhibitory activity for both prototypical structures, whereas the corresponding THIQ-1-carboxylic acid and the homologous isoindoline-1-carboxylic acid do so only for the non-sulfhydryl compounds. CI-925 (19), containing a dimethoxy THIQ-3-carboxylic acid, demonstrated equivalent in vitro and in vivo potency to 18.



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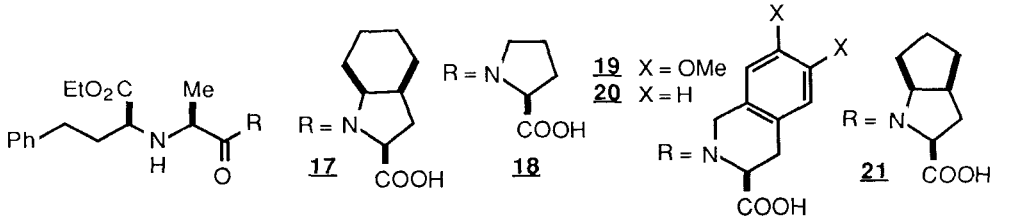
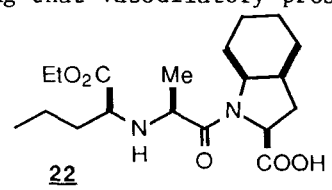


17 **18** **19** X = OMe **20** X = H **21**

Chronic treatment of SHR with quinapril (20) at 20 mg/kg for 8 days caused a marked and progressive reduction in blood pressure, but did not affect plasma kininogen or urinary excretion of kallikrein (32). Urinary excretion of 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 were also unaltered, indicating that vasodilatory prostanoids do not contribute to the hypotensive effect of this compound. Ramipril (21), perindopril (22, both at 1 mg/kg/ day), and 18 (30 mg/kg/day) elicited equal antihypertensive effects in SHR, although their effects on the parameters of the RAS in plasma were different (33).



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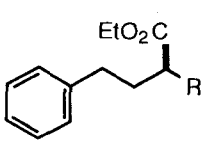
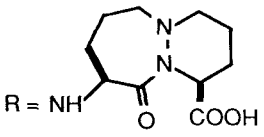
Chronic daily administration of cilazapril (23) at 10 mg/kg/day (4 to 14 weeks) to young spontaneous hypertensive rats (SHR) prevented the

development of hypertension (34). In conscious renal-hypertensive dogs, 23 produced a long-lasting (>24 h) decrease in systolic arterial blood pressure (35). Medium-ring lactams representing conformationally constrained replacements for AlaPro have been incorporated into a new series of ACEI (36). The analogue with an 8-membered ring (24) inhibited ACE with an IC_{50} of 2 nM compared with 1.2 nM for 18.





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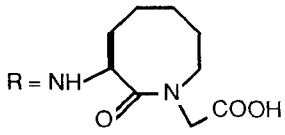

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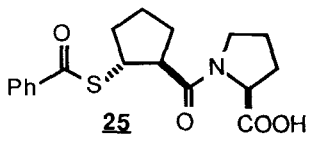
The semirigid compound 25 caused hypotensive effects comparable to 16 in renal hypertensive dogs, but showed considerably lower in vivo ACE inhibition (37). The cardiovascular effects of alacepril (26) in several animal models have been reported (38), along with data suggesting that attenuation of the sympathetic nervous system may also contribute to its hypotensive mechanism (39). A series of thiazepines, thiazines and benzothiazepines have been shown to be potent inhibitors of ACE in vitro and in vivo (40). These heterocycles, which incorporate a thiolactone functionality, are believed to be prodrugs since they generate a free SH in the presence of rat plasma. Benzothiazepine 27 inhibited ACE in conscious, normotensive rats with an ID_{50} of 0.6 mg/kg (40). A marked species difference for the in vitro and in vivo inhibition of ACE by SA 446 (28) has been attributed to differential binding to plasma proteins resulting in variations of the ultrafiltrate drug level (41). Delapril (29) decreased the angiotensin I pressor response in conscious, normotensive dogs with an ED_{50} of 0.75 mg/kg (42).



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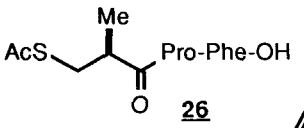

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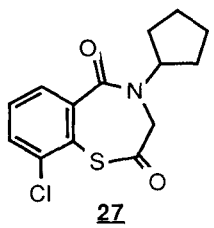

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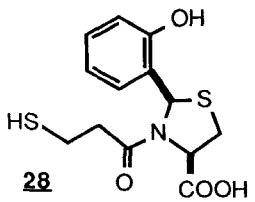

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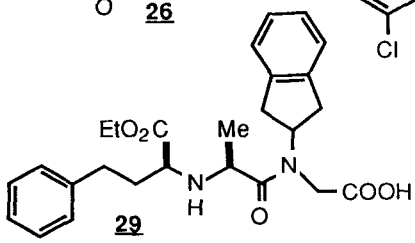

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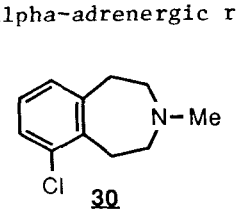
alpha-Adrenoceptor Agonists and Antagonists - The clinical pharmacology and basis for use of $alpha_1$-adrenoceptor antagonists has been delineated (43,44). A summary of the experimental data permitting pharmacological characterization of agents acting at the $alpha_1$-adrenoceptor has been reviewed (45). In a recent survey of agonists and antagonists of $alpha_1$- and $alpha_2$-adrenoceptors, the author suggests that classification of receptors be based upon specificity to ligands and drugs, regardless of location or function (46). A high affinity radioiodinated probe has been used in studies with human platelets and rat hepatic membranes to identify the localization and biochemical characterization of the $alpha_2$-adrenoceptor (47). SKF 86466 (30), a potent and selective $alpha_2$-adrenoceptor antagonist, showed an $alpha_2/alpha_1$ selectivity ratio comparable to yohimbine and demonstrated antihypertensive effects in DOCA-salt rats and SHR (48,49). CI-926 (31) is an orally effective blood pressure lowering agent in renin and non-renin dependent experimental hypertension (50), with a

profile dependent in part on alpha$_1$-adrenoceptor blockade (IC_{50}=82 nM). Compound 32, given orally to normotensive Wistar rats at 10 mg/kg, is more potent than prazosin and is postulated to act by blockade of peripheral alpha-adrenergic receptors (51).



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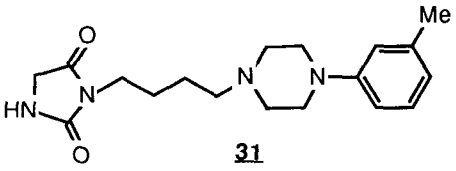

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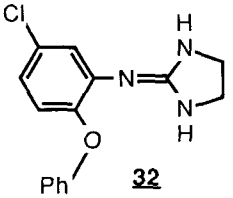
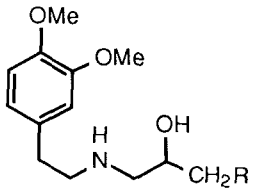
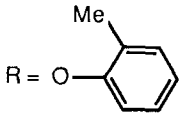


32

beta-Adrenoceptor Blocking Agents - The pharmacologic profile of bevantolol (33), a cardioselective beta-adrenoceptor antagonist devoid of intrinsic sympathomimetic activity, has been reviewed (52). S-51 (34) was shown to be a highly cardioselective beta-blocker and devoid of intrinsic sympathomimetic activity (53). The adrenoceptor blocking properties of the stereoisomers of amosulalol (35) and the corresponding desoxy derivative (36) were examined (54). Both salidiuretic and beta-adrenergic blocking





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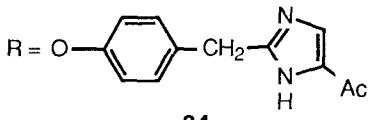

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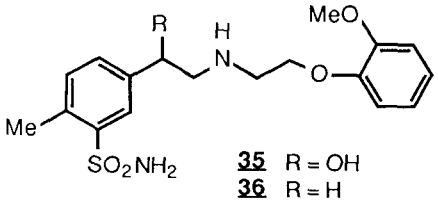
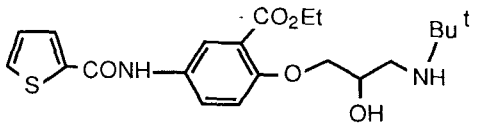
activity were elicited by the thiophene derivative 37 (55). This compound produced a diuresis in water-loaded rats and blocked the contractile force of isoproterenol in mongrel dogs.



35 R = OH
36 R = H



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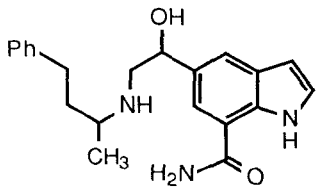

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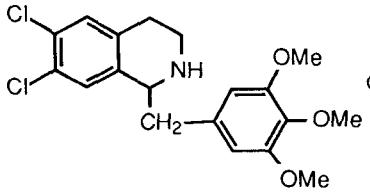

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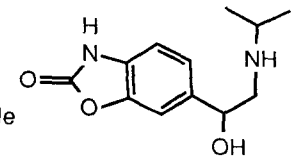

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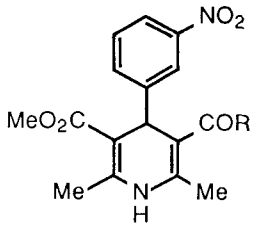
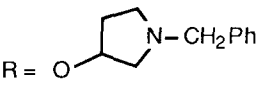
Compound 38, a pyrrolo analogue of labetalol, reduced blood pressure in SHR without inducing bradycardia (56). The tetrahydroisoquinoline 39 demonstrated beta$_1$-adrenoceptor blockade when evaluated in isolated guinea pig atrial pairs (K_B=67 nM). Although less potent than propanolol (K_B= 0.62 nM), this analogue represents a new structural class of beta-adrenergic blocker and is not a partial agonist (57). Utilization of a benzoxazolinone moiety as a bioisosteric (58) replacement for pyrocatechol led to 40, which showed non-selective beta-receptor antagonism in vitro and caused antihypertensive effects in the 2K-2C dog model (59).

Calcium Antagonists - Several pertinent reviews on calcium antagonists have appeared (60,61,62) including a discussion of their mode of action (63,64) and a comparison with other antihypertensive agents (65). The preparation of antibodies with high affinity and specificity for 1,4-dihydropyridine-type calcium antagonists has been described (66). The favorable binding characteristics of these antibodies relative to the corresponding membrane receptor suggests their use for the production of anti-idiotypic antibodies to the dihydropyridine membrane receptor. The (S,S)-diastereomer of YM-09730 (41) showed the highest binding affinity to rat brain cortex membranes (K_i=0.205 nmol/L) and the greatest coronary vasodilator activity (ED_{100}=0.57 mcg i.a.) of the four possible optical isomers (67). The hypotensive and cardiovascular effects of FRC-8411 (42) and several reference calcium antagonists have been reported (68). In dogs, KB-944 (43) was equivalent to diltiazem in decreasing blood pressure and increasing coronary blood flow and showed much less toxicity in mice (69).



R = 

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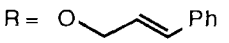
R = 

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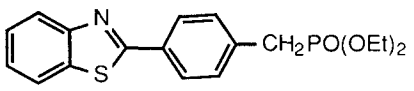

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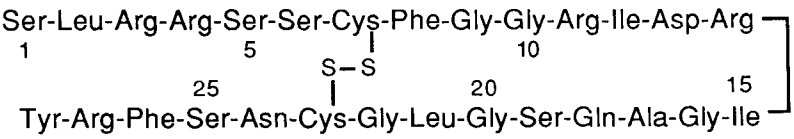
Atrial Natriuretic Factor (ANF) - ANF (44) is a circulating 28 amino acid peptide which causes relaxation of smooth muscle in vitro and a potent natriuresis, usually accompanied by hypotension, in vivo (70,71). Structure-activity studies with ANF analogues must be clearly defined since several classes of ANF receptors exist and the results of binding studies may be dissociated from other biological actions of ANF, e.g., hypotension, inhibition of aldosterone release, increase in intracellular cGMP, vasorelaxation in isolated vascular tissues (72,73,74). Several reports indicate that linear analogues may retain or improve biological activity (74,75). Also, the vasorelaxant activity of cyclic analogues has been reported (74). Deletion of amino acids from the amino or especially the carboxyl termini alters the ability of the analogue to increase cGMP in cultured vascular smooth muscle (72). In this preparation, analogues lacking the carboxyl terminal Phe-26, Arg-27, Tyr-28 were 100-1000-fold less active in promoting intracellular cGMP production (72). However, the sole removal of Tyr-28 does not alter binding or enhancement of cGMP production in renal glomeruli (76). It appears that the disulfide bond stabilizes the biologically active conformation, but is not required for the diuretic activity (76), the ability of ANF to relax pre-contracted isolated smooth muscle, or the binding to or inhibition of aldosterone secretion from bovine adrenal glomerulosa cells (75).

44

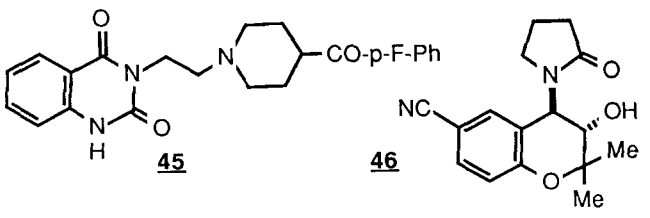


Deletion of the non-functional residues in positions 20-22 disallows the essential interaction of endo-exocyclic residues with their receptor subsites (73) as shown in the aorta relaxation assay (ED_{50}=3380 ± 1400 nM vs. native peptide 13.6±3.9 nM). The parallel (beta'-ANF) and antiparallel dimer (beta-ANF) were equipotent in relaxing isolated rat aorta (medi-

an ED values were 1.7×10^{-8} M and 1.6×10^{-8} M, respectively). Diuretic and natriuretic activity were equally less in magnitude but longer in duration than the native peptide (77). Similar findings were reported elsewhere (78).

Drugs that Interact with Serotonin (5-HT) Receptors - Administration of 5-HT causes a triphasic blood pressure response in anesthetized rats: (a) a short-lasting depressor phase with intense bradycardia, (b) a pressor phase, and (c) a prolonged hypotensive phase. The three phases may be attributed to the activation of 5-HT_M-, 5-HT_2-, and 5-HT_1-receptors, respectively (79). 5-Carboxamidotryptamine (5-CT) is a potent agonist of 5-HT receptors *in vitro* and is believed to reduce blood pressure in conscious DOCA-salt hypertensive rats via direct vasodilation at a "5-HT_1-like" receptor (80). Another approach to the induction of vasodilation by affecting 5-HT receptors is represented by ketanserin (45), a selective antagonist of $5\text{-HT}_2(S_2)$ receptors, which has also been shown to interfere with norepinephrine-induced pressor response in the conscious rabbit (81). This drug has been demonstrated to lower blood pressure in man (82).

Miscellaneous - Several new mechanistic approaches to discovering antihypertensive agents have recently surfaced. BRL 34915 (46) which may act as a direct vasodilator, opens membrane potassium channels, at low concentrations affecting spike repolarization and at high concentrations inducing hyperpolarization and inhibition of membrane excitatory agents (83,84,85). *In vitro* studies of 46 on aorta and portal vein suggest that this agent raises the membrane potential such that it approaches the K_{Eq}. The agent has been shown to be an effective antihypertensive agent in conscious hypertensive animals (86,87).



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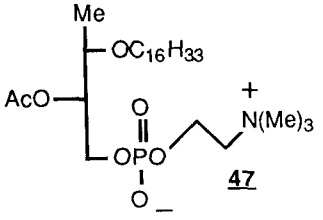

47

Platelet Activating Factor (PAF) is a mixture of homologous alkyl ether phospholipids which possesses a variety of biological activities including a potent effect in lowering blood pressure (88). The report of an attempt to obtain derivatives with specific hypotensive effects has been published (89). Increasing the length of the methylene bridge separating the phosphate and trimethylammonium moieties of PAF decreased both the hypotensive and platelet aggregation responses in SHR and rabbits, respectively (90). Recently, a method for synthesizing chiral cyclic analogues of PAF has been revealed (91). The synthesis of 1-(S)-Me-PAF (47) shows promise as an antihypertensive agent as it causes only weak platelet activation (about 1/115 of C^{16}-PAF), but shows oral antihypertensive activity in SHR which is 200 times greater than PAF (92).

Conclusion - The present review, inclusive of several approaches to antihypertensive therapeutics in 1986, emphasizes the emerging trend to lower blood pressure by inducing peripheral vasodilation. As discussed above, the means by which this goal is achieved are quite varied, but in the final analysis, it is hoped that all vasodilators will promote good tissue perfusion while effectively reducing blood pressure.

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Chapter 8. Pulmonary and Antiallergy Agents

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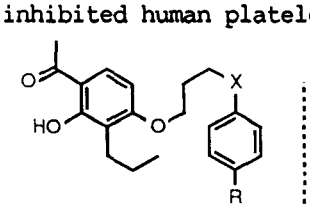
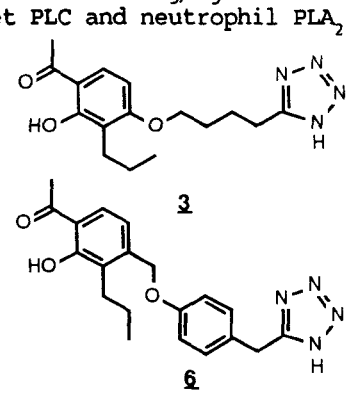
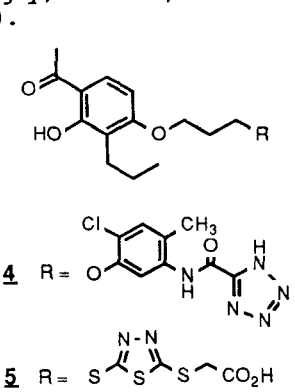
INTRODUCTION - Over the last 7 years there has been an intense search for drugs affecting the leukotriene component of immediate hypersensitivity reactions and in 1986 preliminary clinical results of the first of such drugs, leukotriene receptor antagonists, appeared. A review of the current status of this area has appeared as a National Institutes of Allergy and Infectious Diseases workshop report (1). While medicinal chemistry research in pulmonary and allergic diseases continued to emphasize this area, especially with regard to inhibition of the 5-lipoxygenase (5-LO) pathway of arachidonate metabolism, the focus of new research has shifted toward another mediator, platelet activating factor (PAF). Research also continued into new non-sedating antihistamines, mediator release inhibitors and bronchodilators.

LEUKOTRIENES

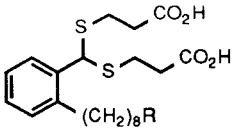
Leukotriene Antagonists: - Reviews on leukotriene receptors (2) and antagonists (3) have appeared. L-649,923 (1) is a selective LTD_4 antagonist in vitro and, unlike the prototype FPL-55712, is active orally in animal models of LTD_4 and antigen-induced bronchospasm (4, 5). The substituents β and γ to the carboxyl group were designed to retard metabolism; the ($\beta R^*, \gamma S^*$) relative configuration had the best pharmacodynamic profile while there was no difference between its enantiomers (5). At a 1 g oral dose, 1 inhibited the bronchial response to inhaled LTD_4 in normal man (6) and in asthmatics caused a small, but significant, attenuation of the early phase bronchospasm with no effect on the late phase (7). The closely related analog L-648,051 (2) is less active orally and is being developed for local administration (8). LY 171,883 (3) at 30 mg/kg p.o. in allergic sheep blocked the late phase bronchospasm and significantly reduced the acute response, but did not affect the antigen-induced fall in tracheal mucous velocity (9). In asthmatics the late phase response to allergen provocation was blocked after treatment with 75-600 mg of LY 171,883 BID for 5 days (10). Several other analogs of this structural type were reported to be orally active in animals including CGP 35,949 (4) (11), YM-16638 (5) (12) and LY 163,443 (6) (13). The latter compound had a duration of at least 8 h at 10 mg/kg. Interestingly, CGP 35,949 also inhibited human platelet PLC and neutrophil PLA_2 (11).





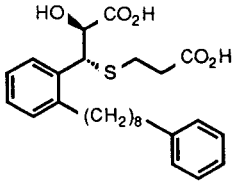


The rationale and SAR leading to the dithioacetals 7 and 8 were reported (14). Both compounds blocked an LTD_4 bronchospasm in guinea pigs at 5 mg/kg i.v. 1 min prior to challenge, but only 8 was effective with a 5 min pretreatment. The longer duration was attributed to blocking of ω-oxidation by the terminal phenyl group. This strategy was also used in a related compound, SKF-104,353 (9) (15) which has the highest receptor binding affinity reported to date (human lung Ki 8 nM). It blocked LTD_4, but not LTC_4, contractions of guinea pig trachea and LTD_4-induced bronchospasm in guinea pigs at 25 mg/kg p.o. The highest affinity and potency resided in the (-) form which corresponds to the natural stereochemistry of the leukotrienes. The LTD_4 analog (10), lacking a C_1-carboxyl group was an LTD_4 antagonist in vitro and in vivo by the aerosol route, but had a short duration of action on i.v. administration (16).



7 R = $(CH_2)_3CH_3$

8 R = C_6H_5



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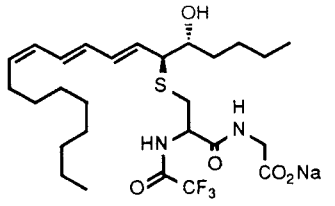

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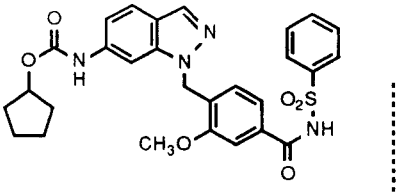
ICI 198,615 (11) is an extremely potent in vitro LTD_4 and LTE_4 antagonist (17). In a conscious guinea pig model of aerosolized LTD_4 induced dyspnea 11 was effective at 0.017 mg/kg i.v. and at 5.7 mg/kg p.o. with half lives of 2.5 h and 16 h, respectively (18). It also reversed antigen-induced allergic bronchospasm in guinea pigs. Both ONO-411 (12) and ONO-347 (13), selective LTD_4 and LTC_4 antagonists in vitro, blocked LTC_4 and LTD_4-induced bronchospasm in guinea pigs on oral and i.v. administration (19). Hydroxamic acid (14) (20) and OT 3473 (15) (21) are dual LTD_4 antagonists and LO inhibitors in vitro. In vivo 14 blocked LTD_4 and antigen-induced, LT-mediated bronchospasm in guinea pigs on intraduodenal administration whereas 15 was not orally active. In general the naphthalene analogs of 14 were 5-LO inhibitors while the quinoline compounds were better LTD_4 antagonists. Analog 16 (22) also exhibited both activities in vivo, but neither 14 nor 16 are being developed because of mutagenic activity in the Ames test (20, 22).



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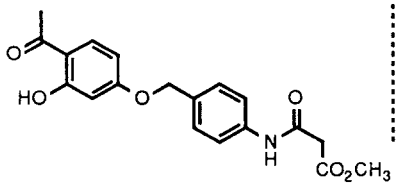

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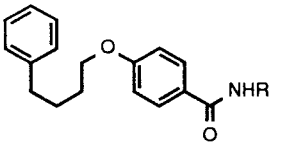

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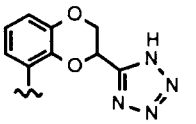

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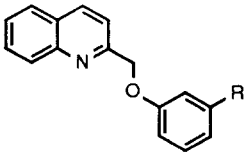

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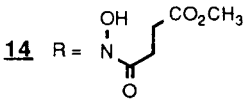

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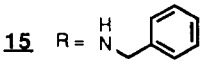
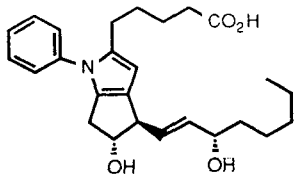


Leukotriene Biosynthesis Inhibitors: - This approach to new agents useful in the treatment of allergic and inflammatory disease was reviewed (23). Piraprost (17) inhibits both 5-LO and glutathione S-transferase of LTC_4 biosynthesis in vitro and is also an LTD_4 antagonist, but it had no effect (1 mg inhaled) on antigen or exercise-induced bronchoconstriction in asthmatic subjects (immediate and late phase) (24). The phenothiazinone L-651,392 (18) selectively blocks 5-LO of both rat and human PMN's (IC_{50} 60-90 nM), but is inactive against 12- or 15-LO at 10 μM. In vivo, 18 prevented antigen-induced bronchial responses in allergic rats (ED_{50} 1.3 mg/kg po) and squirrel monkeys (5 mg/kg po) (25). The antifungal agent ketoconazole is also a selective 5-LO inhibitor and blocked LT-mediated allergic bronchospasm in guinea pigs at 10-40 mg/kg p.o. (26). In a similar assay the potent, specific 5-LO inhibitor TMK-777 (19) was effective at 100 mg/kg p.o. (27). Several 5-LO inhibitors are being targeted for trials in psoriasis since elevated levels of LTs and 12-HETE occur in psoriatic skin. The most advanced compound lonapalene (20), a specific 5-LO inhibitor, was effective in man (28) while L-651,896 (21), a dual LO and CO (cycloxygenase) inhibitor (29), and QA 208-199 (22), which inhibits 5-, 12-, and 15-LO, (30) were evaluated in various models of psoriasis.



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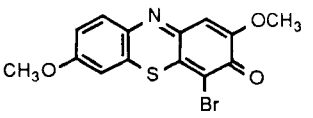

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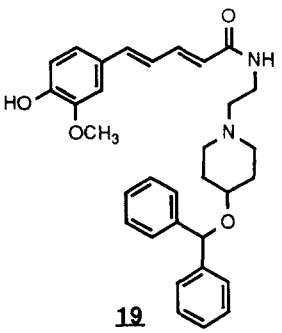

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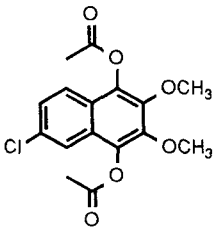

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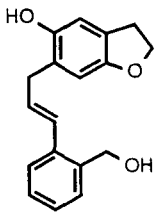

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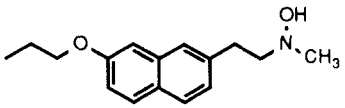
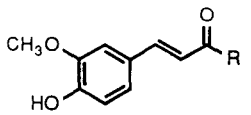
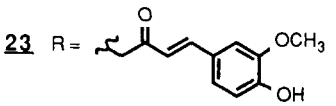


22

Several natural products were reported to be dual inhibitors of 5-LO and CO in vitro. Curcumin (23) and yakuchinone B (24), which inhibited 5-HETE synthesis by human neutrophils, blocked PGE_2 production at 5 fold higher concentrations (31). The related compound gingerdione (25) was equipotent on both pathways (IC_{50} ~ 15 μM) (32). In a homologous series of gingerols (26) relative potencies varied with the length of the alkyl chain with CO inhibition being independent of the chain length n = 4-14, and 5-LO inhibition sharply increasing at n = 8-14 (33). High in vitro 5-LO inhibitory potency is also associated with the catechol unit and several long chain esters of dopamine were potent and specific 5-LO inhibitors (e.g., 27, IC_{50} 2.7 nM) (33). Dual inhibition was also reported for SKF 86002 (28) which was orally active in models of inflammation (34). The quinoline N-oxide (29) is a specific 5-LO inhibitor (35) whereas 3-methoxy tropolone which was also isolated from a fermentation broth selectively inhibits 12-LO (36). 5-LO inhibitory activity was also reported for the pyrroles 30 (R=H, CH_3) (37).





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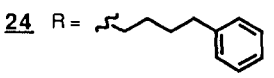

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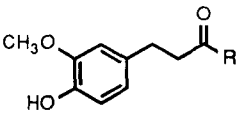

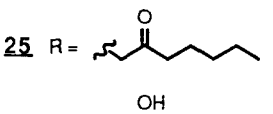

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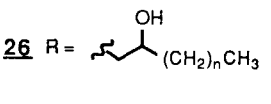

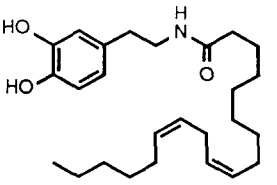

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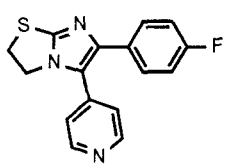

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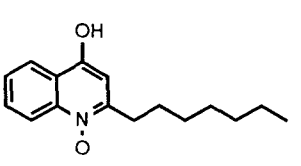

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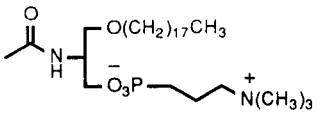
30

PLATELET ACTIVATING FACTOR (PAF)

Biology of PAF: - PAF is a biologically active phospholipid which is implicated in the pathophysiology of various allergic, inflammatory and cardiovascular diseases (38). PAF, originally discovered to be released from antigen challenged rabbit basophils, has subsequently been shown to be produced by many other cells including platelets, macrophages, neutrophils, eosinophils and mast cells (38). However, it is not released extracellularly in all cases (39). The binding of PAF to a specific cell membrane receptor is believed to mediate most of the cellular responses to PAF. The binding characteristics of ^{3}H-PAF to human neutrophil membranes fits a two receptor model (40). Species differences and the existence of classes of PAF receptors is suggested by studies with antagonists (41, 42) while in vitro studies revealed species-dependent effects of PAF on airway smooth muscle (43, 44). Low doses of PAF have also produced transient pulmonary vasoconstriction, edema and acute lung injury in rats (45) and local administration of PAF produced an immediate bronchospasm which was reduced by 5-LO inhibitors (46). PAF is considered a potent inflammatory agent since at doses of 0.1-1 μg it induces a localized inflammatory exudate and cellular infiltrate in the rat pleural cavity (47). Intradermal PAF induced a cutaneous inflammatory response in rats (48), guinea pigs (49), and humans (50). Recently, PAF was shown to be a potent chemotactic and chemokinetic factor for human eosinophils (51). PAF has been implicated as an important mediator of the airway hyperreactivity associated with human asthma. Inhalation of PAF (6-400 μg) produced an immediate bronchospasm, but no late response in human subjects (52). All subjects developed airway hyperreactivity that lasted up to two weeks. Measurements of increased levels of platelet-derived proteins in plasma have suggested the involvement of PAF and platelet activation in patients with bronchitis (53) and exercise-induced asthma (54). However, no evidence for platelet activation was found in allergen-induced human asthma (55, 56).

PAF Antagonists: - Many antagonists were reviewed (57) and a receptor model was proposed (58). The various modifications made on the PAF framework in determining the SAR of PAF were summarized (58). Variations in the 1-O-alkyl chain included unsaturated (59), substituted aromatic (60), and

polyoxygenated analogs (61), which usually produced weak agonists. Likewise, replacement of the 2-O-acetyl moiety with various groups was extensively studied (58). Replacement of the phosphate group with phosphonate (62), sulfone (63), or ether (64) groups produced compounds with little or no activity although the (R) enantiomer of phosphonate 31 was a non-specific antagonist of platelet aggregation with PLA_2 inhibitory activity (65). Structural changes in the alkyl chain of the choline portion of PAF (58, 66, 67) led to some compounds with increased agonist potency and to some with antagonist activity, whereas changes of the glycerol backbone (68, 69) generally led to weaker agonists. However, the 1-(S) methyl analog of PAF 32 demonstrated a much greater hypotensive effect relative to its platelet activity (69). CV 3988 (33) inhibited specific binding of ^{3}H-PAF to washed rabbit, guinea pig and human platelets (70). Even though it may not be as selective as originally reported (71) it was effective against PAF-induced bronchoconstriction (72), hypotension, and endotoxin shock (73). CV 6209 (34) was recently reported to be approximately 80 times more potent than CV 3988 in antagonizing PAF-induced aggregation of rabbit platelets and blocking PAF-induced hypotension in rats (74). ONO-6240 (35) was the most potent inhibitor of PAF-specific binding to human platelets and lung tissue when compared to a variety of other antagonists (41). RO 19-1400 (36), a potent, selective antagonist of PAF-induced aggregation of human platelets, was active intravenously and weakly active orally against PAF-induced bronchospasm in guinea pigs (75). SRI 63-072 (37) given intravenously, is effective against PAF-induced guinea pig bronchoconstriction (76) and rat hypotension (77). RU 45,703 (38) inhibits PAF-induced rabbit platelet aggregation, guinea pig bronchospasm and rat hypotension (78). A number of derivatives possessing a constrained backbone were described (57, 79, 80, 81). In general they were weaker PAF antagonists than the acyclic derivatives.



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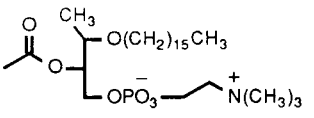

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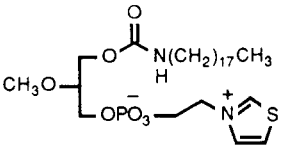

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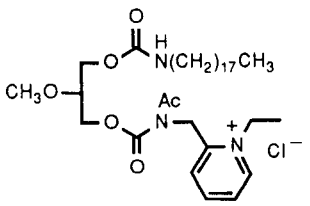

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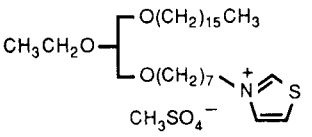

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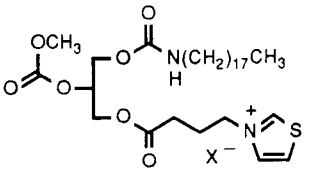

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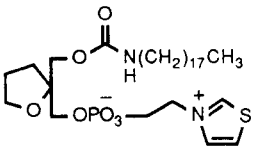

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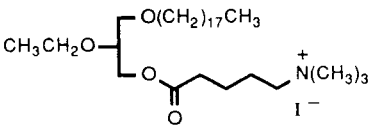

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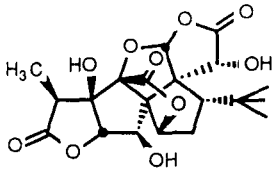
Ginkgolide B (39), which is the most potent of several related compounds (82) was equipotent to CV 3988 in its inhibition of PAF-induced aggregation of washed human platelets, but was about ten-fold more active in platelet-rich plasma (71). In vivo, it was effective orally against bronchospasm induced by PAF, but not by arachidonic acid, acetylcholine, collagen, or serotonin (83). It inhibited endotoxin-induced hypotension

in the guinea pig (84), however, recent reports demonstrate that it has additional actions that contribute to its pharmacological profile including inhibition of the release of histamine (85) and CO metabolites (86) and possible β-sympathomimetic activity (85). Kadsurenone (**40**) is as potent and specific as ginkgolide B in its inhibition of PAF-induced aggregation of washed human platelets (71, 87). It blocked various PAF-induced increases in lysosomal enzymes, hematocrit, and vascular permeability (88) and inhibited endotoxin induced hypotension in the rat (89). By studying the binding of ^{3}H-PAF and ^{3}H-dihydrokadsurenone (**41**) to rabbit platelet membranes, it was shown that these antagonists as well as others probably bind at least partially to the same receptor as PAF (90). The enantiomer of kadsurenone weakly inhibited the binding of ^{3}H-PAF to rabbit platelet membranes at 5 μM (vs. IC_{50} 0.12 μM for **40**) (91). The C-2 and C-3a epimers, as well as the desallyl derivative, were all much weaker than kadsurenone (91). Interestingly, although the activity of the C-3a acetate derivative was very weak, the corresponding benzoate was quite potent (IC_{50} 1 μM) (91). The tetrahydrofuranoid lignan, L 652,731 (**42**), is a specific PAF antagonist that is 15-20 times more potent than either CV 3988 or ginkgolide B in the PAF binding assay (92). The corresponding cis-isomer is about 1000 times less active. L-652,731 was orally active against PAF-induced cutaneous vasopermeability and hypotension in the rat (93). Removing one of the methoxy groups or replacing it with another alkoxy or hydroxyl group significantly reduces the activity (92) as does placement of methyl groups at the C-3 and C-4 positions of the tetrahydrofuran ring (94). The corresponding thiofuran, L-653,150 (**43**) is more efficacious in vivo against PAF-induced vascular permeability and hypotension and is also an inhibitor of 5-LO (95). The two natural products, FR 49175 (**44**) and FR 900452 (**45**), blocked PAF-induced aggregation of rabbit platelets with IC_{50}'s of 8.4 μM and 0.37 μM, respectively (96, 97). This inhibition, however, was not entirely specific at higher doses. They were effective intravenously against PAF-induced bronchospasm in the guinea pig, although only FR 900452 inhibited PAF-induced hypotension (98, 99).



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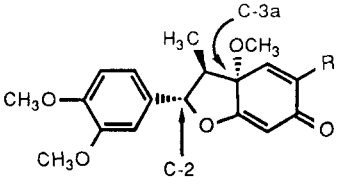
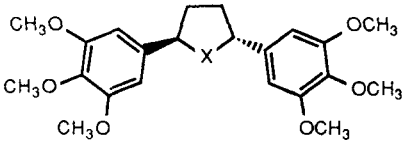


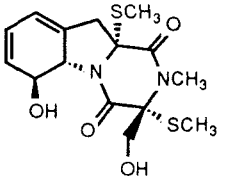
40 R = $CH_2CH{=}CH_2$

41 R = $CH_2CH_2CH_3$



42 X = O

43 X = S



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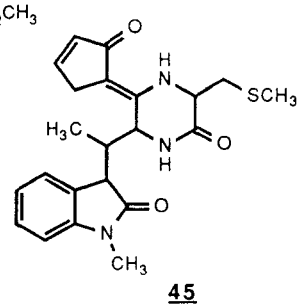

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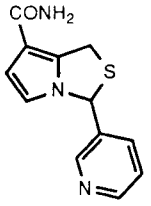
The substituted thiazole 48740 RP (**46**) inhibits PAF-induced platelet aggregation (100), but is not totally specific (57). It effectively competes with the binding of ^{3}H-PAF to rabbit platelet membranes, but is 10-30 times weaker than CV 3988 and 100 times weaker than kadsurenone (90). When given intravenously **46** blocked PAF-induced bronchospasm in the guinea pig and hypotension (ip) in the rat (100). The psychotropic agents,

alprazolam, triazolam, and brotizolam (47) were all specific inhibitors of PAF-induced platelet aggregation (101, 102). Brotizolam, which is the most active of the three, was orally effective in preventing PAF-induced bronchospasm and hypotension in the guinea pig (102). Recently, WEB 2086 (48) was found to be the most potent PAF antagonist in this series (103). Unlike the other triazolobenzodiazepines it is relatively free of CNS activity. In vivo 48 was orally effective at low doses (0.1-2.0 mg/kg) against PAF-induced bronchospasm and hypotension in the guinea pig (103).



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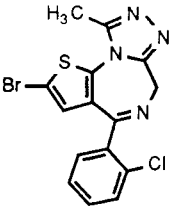

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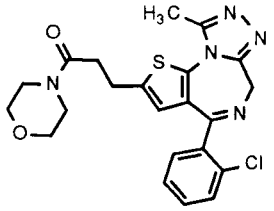
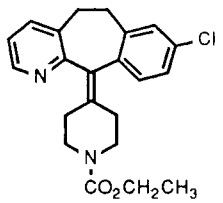


48

ANTIHISTAMINES: - Reports continue to appear indicating that long term treatment with ketotifen was of some benefit in the treatment of asthma (104-106). After 6 months of therapy a decrease in airway hyperactivity was observed using the fog bronchospasm test (106). Terfenadine has received wide acceptance as the first in a new class of non-sedating antihistamines. In conjunction with the H_2-antagonist, cimetidine, terfenadine was effective in the treatment of idiopathic cold urticaria (107). Replacing the N-methyl group of azatadine with various carbamate groups largely eliminated CNS activity (108). Loratadine (49), the most potent in the series, showed no sedating liability in animals (108) or man (109, 110). In vitro radioligand studies indicate that loratadine shows selective binding to peripheral rather than central histamine receptors (111). The animal pharmacology of temelastine (50) was reviewed (112). Antihistamine potency of temelastine is comparable to mepyramine and studies with radiolabelled drug indicate that 50 does not appreciably penetrate the CNS. A complete pharmacological profile was reported for tazifylline (51) (113). It is approximately 10 times more potent than either astemizole or terfenadine in its affinity for H_1-histamine binding sites and appears to be devoid of CNS activity (114). It had a long duration of action in guinea pigs and also had some bronchodilating activity. Cetirizine (52) is a new non-sedating antihistamine which was shown to be about 4-fold more potent than terfenadine in blocking histamine-induced edema in mice (115). Levocabastine (53), an extremely potent antihistamine (116), was 60 times more potent than ketotifen in inhibition of allergen-induced skin reactions in dogs (117). The corresponding diastereomers as well as its enantiomer were much less potent (116). BR 28390 (54) was as potent as mepyramine as an antihistamine (guinea pig ileum), and slightly less potent than cromolyn in its inhib-ition of histamine release in the rat PPA screen (118). Icotidine (55), structurally related to temelastine, lacks CNS activity and is a dual antagonist at both H_1 and H_2-histamine receptors (119).



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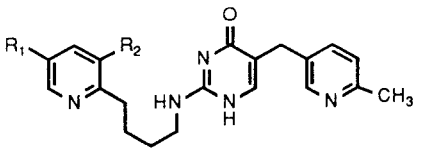
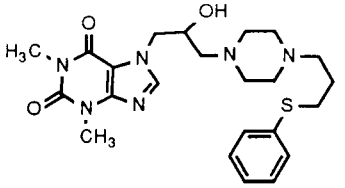


50 $R_1 = Br$, $R_2 = CH_3$

55 $R_1 = H$, $R_2 = OCH_3$



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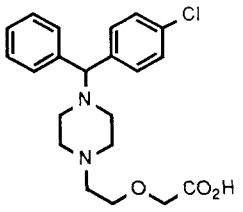

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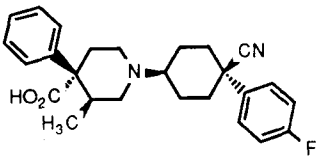

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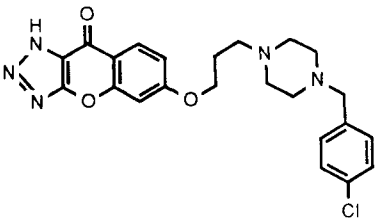

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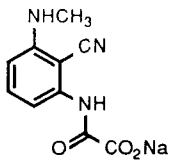
MEDIATOR RELEASE INHIBITORS - Nedocromil was shown to inhibit mediator release from bronchoalveolar lavage cells of allergic monkeys while cromolyn was inactive (120). It is orally active in animal models, but is being used clinically as an inhalation product (121). WY-41,195 (56) is orally active in animal models, but otherwise has a profile similar to cromolyn. It was disappointing clinically at oral doses up to 100 mg despite evidence for its absorption (122). Tiacrilast (RO 22-3747, 57) had protective effects in allergic asthmatics but was less effective than inhaled cromolyn although a pilot study in allergic rhinitis showed promising results (123). Oral activity was also described for MDL-427 (58) (124), quazolast (59) (125), tioxamast (F-1865, 60) (126), eclazolast (RHC-2871, 61) (127) and a series of indolobenzodiazepines (128). Eclazolast is a prodrug which is hydrolysed intracellularly to the active free acid, RHC-3579 (62). Unlike cromolyn, it does not exhibit tachyphylaxis in animal assays (127).



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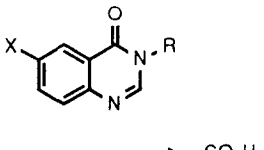
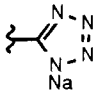
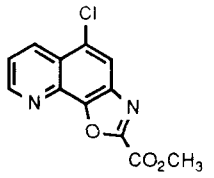


57 X = SCH_3, R =



58 X = H, R =



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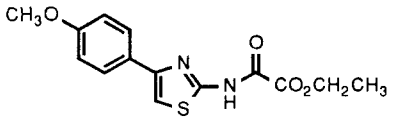

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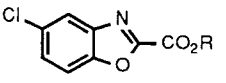
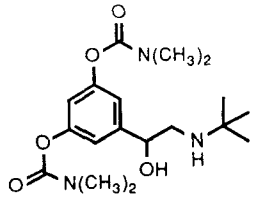


61 R = $CH_2CH_2OCH_2CH_3$

62 R = H

BRONCHODILATORS: - Bambuterol (63), an orally active lipophilic pro-drug diester of terbutaline, produced higher and more stable plasma levels of terbutaline than did oral terbutaline (129). The degree of bronchodilation thus achieved was more prolonged with bambuterol than with terbutaline. The SAR of the potent and highly β_2-selective compound, broxaterol (64), was published (130). LG-30435 (65), a quaternary derivative of the anti-



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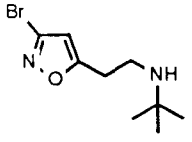

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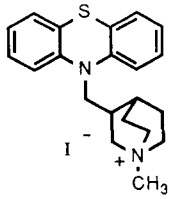

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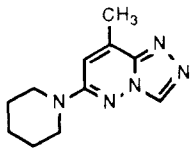

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histamine mequitazine, exhibits bronchodilator activity as an aerosol in animals, but is inactive orally (131). Zindotrine (66), an in vitro PDE inhibitor, was an effective bronchodilator in man at 300 mg po (132). The effect of calcium channel blockers in asthma was reviewed (133) and further demonstrations of the lack of any great beneficial therapeutic effect of such agents in asthma were reported (134, 135).

CONCLUDING REMARKS: - Clinical trials over the next few years should clearly delineate the therapeutic value in asthma and other allergic diseases of selective leukotriene and PAF receptor antagonists and 5-LO inhibitors. Since multiple mediators are likely to be involved in the pathobiology of these diseases the future development of drugs with multiple actions will be a logical extension of this approach. The inflammatory component of asthma is being more widely appreciated and new drug discovery will be directed towards this aspect of the disease rather than bronchodilators. It seems likely that future research will also concentrate on the role of peptide neurotransmitters, airway epithelium and the CNS in the regulation of airway function. Control of allergic diseases by interference with IgE synthesis or action seems to be further in the future.

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Chapter 9. Agents for the Treatment of Congestive Heart Failure

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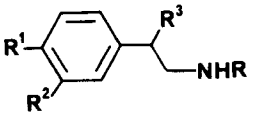
INTRODUCTION - Congestive heart failure (CHF) results when the heart is unable to adequately perfuse the peripheral tissues. While accurate, this definition oversimplifies a complex clinical syndrome (1) which is a common end point for many cardiovascular disorders (2). Insufficient cardiac output initiates compensatory processes involving increased activity of the adrenergic, renin-angiotensin-aldosterone, prostaglandin, bradykinin and vasopressin systems (3). These compensatory mechanisms further decrease peripheral circulation leading to congestive symptoms (2). Estimates of the prevalence of CHF range from 2.3 million people in the US in 1983 (4) to as many as 4 million today (5). Ironically, the incidence of CHF is increasing due in large measure to improved treatment of acute cardiac disease (6). The prognosis for CHF is grim. More than 50% of patients die within five years of diagnosis (6). Even though progress has been made in understanding the disease and developing new therapies, overall mortality has not declined and many unanswered questions remain (7). The etiology, pathophysiology, and drug treatment of CHF have been the subject of a number of recent reviews (1,2,5,8-11).

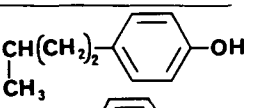
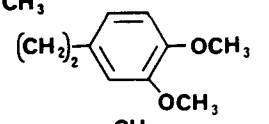
Objectives of Drug Therapy - Contemporary strategies for treatment of heart failure attempt to ameliorate the symptoms of heart failure by directly improving left ventricular (LV) function and/or decreasing peripheral impedence to cardiac output (12). Much of the research in this area is aimed at identifying agents which directly increase cardiac inotropic activity. A number of agents have effects on both cardiac and vascular function. In addition, traditional vasodilators and angiotensin converting enzyme (ACE) inhibitors are the focus of renewed attention. The organization of this chapter reflects these hemodynamic approaches. Cardiac glycosides and diuretics are not discussed since in our view research in these areas appears relatively static. In the case of cardiac glycosides, this may be due to their low therapeutic index (13) and need for drug monitoring (14). As for diuretics, currently available drugs represent a level of efficacy and safety which has limited the incentive for developing improved agents (15).

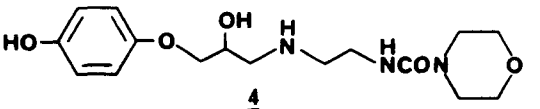
INOTROPIC AGENTS - Recent reviews cover several aspects of inotropic therapy including: rationale for use of inotropic drugs (16), historical (17) and recent (18) inotropic drug discovery, new inotropic drugs in clinical development (19,20), and cardiac inotropic mechanisms (21). Potential negative consequences of chronic inotropic stimulation of the heart have also been outlined (22).

Beta-adrenergic agonists - The autonomic nervous system plays a complex role in the pathophysiology of CHF (23,24). Several studies (25) suggest that long-term benefit may be achieved following short-term, intermittent therapy with the beta-2 receptor agonist dobutamine, 1. However, in severe CHF increased mortality may result with such a regimen (26). The inotropic activities of the stereoisomers of 1 suggest that

alpha-1 and beta-1 receptors are important in the inotropic effect and selectivity of racemic **1** used clinically (27). Denopamine (TA-064), **2**, is an analogue of **1** which is orally active and produces a dose-dependent increase in contractility with little effect on blood pressure in several species. No species differences were noted with regard to either potency or profile (28). Ibopamine, **3**, in clinical studies appeared to prevent deterioration of LV function after 10 weeks of treatment in dilated cardiomyopathy (29), but other studies show lesser efficacy (30-32). Beta receptor partial agonists are purported to improve inotropic activity while protecting against excessive sympathetic stimulation (33). The cardioselective beta-1 partial agonist, xamoterol (ICI 118,587), **4**, has relatively low intrinsic sympathomimetic activity comparable to 400 - 500 pg/mL of plasma norepinephrine. Therefore, **4**, acts as a beta-1 agonist when sympathetic activity is below this level and as an antagonist when it is higher (34). Overall, **4** has a hemodynamic profile similar to dobutamine (35). Patients with mild CHF receive greater clinical benefit from **4** relative to severe CHF where **4** may be harmful (36).



	R^1	R^2	R^3	R
1	OH	OH	H	
2	OH	H	OH	
3	$OCOCH(CH_3)_2$	$OCOCH(CH_3)_2$	H	CH_3

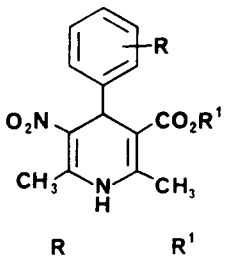


4

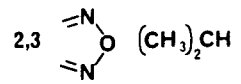
Calcium Channel Activators - Calcium channel activators (37) exert their effects by modulating the gating of the calcium slow channel, stabilizing the channel in an open mode (38). The maximum effect that can be achieved with these agents is less than can be produced by Ca^{2+} alone; therefore, they are calcium partial agonists (39). BAY k 8644, **5**, the prototype calcium channel activator, has a direct effect on regional coronary blood flow and myocardial contractility, *in vivo*, independent of systemic hemodynamic effects (40). CGP-28392, **6**, is a calcium channel activator with effects similar to **5** (41). To date, the limiting factor for use of this class in heart failure is potent vasoconstrictor activity (37) and marked bradycardia (42). In conscious dogs, the vasoconstrictor effects of **5** could be controlled by concomitant administration of nitroprusside (43). No calcium channel activator has been reported to be cardioselective, that is, able to increase myocardial contractility without also increasing vascular resistance and blood pressure.

An important observation is that individual enantiomers of an asymmetric dihydropyridine may have opposite effects on the calcium channel. Examples include the enantiomers of **5** (44) and 202-791, **7**

(45,46), in which one antipode inhibits and the other activates the channel. The racemate, when compared to the individual antipodes, has mixed or biphasic effects. In view of these findings, much of the data on racemic calcium channel activators and blockers should be reevaluated.



	R	R^1
5	2-CF_3	CH_3



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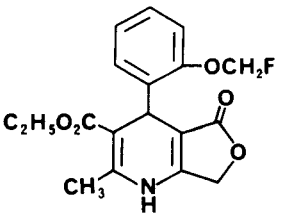
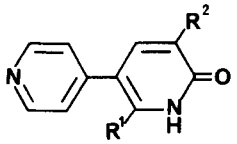


6

AGENTS WITH MIXED INOTROPIC/VASODILATOR ACTIVITY - Phosphodiesterase (PDE) Inhibitors - The prototype agents in this class are amrinone, **8**, and milrinone, **9**. The relative contributions of inotropic and vasodilator activity to the clinical effects of these agents have been controversial. The hemodynamic effects of **8** when compared to those of a pure vasodilator, nitroprusside, suggest that **8** has direct inotropic activity in most individuals studied and this effect contributes to an increase in cardiac output (47). Intracoronary infusion of **9** provided hemodynamic data which also support direct inotropic activity (48). A recent biochemical study suggests that inhibition of the low K_m form of PDE is the prinicipal, if not exclusive, mechanism eliciting the inotropic effect of **9** (49). Inhibition of PDE also was shown to be largely responsible for the positive inotropic activity of **9** in cultured embryonic chick myocardial cells (50). In several reported clinical studies, **9** improved functional status without limiting adverse reactions, however, mortality remained high (51-53).

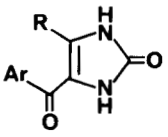


8 R^1 = H; R^2 = NH_2

9 R^1 = CH_3; R^2 = CN

Enoximone, **10**, improves hemodynamic function in CHF patients and acutely increases myocardial performance more than dobutamine (54). Overall, the results of clinical studies are mixed. Side effects, principally gastrointestinal, occurred frequently (55). Despite initial improvement, benefit was not sustained and was associated with high mortality (56). One long-term study showed sustained hemodynamic improvement with a modest increase in exercise capacity (57).

Piroximone, **11**, is the most potent of the related 4-aroyl-1,3-dihydro-2H-imidazol-2-ones (58). Electrophysiologic studies indicate that **11** has no effect on the sodium ion current or sodium-calcium exchange. Inhibition of PDE and stimulation of beta receptors are the principal mechanisms contributing to the positive inotropic activity of **11** (59). In early clinical studies, **11**, exhibited vasodilator and inotropic properties (60,61). Over the long-term, however, the initial hemodynamic improvement waned and mortality was not decreased (62).

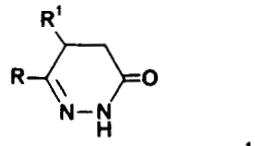


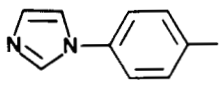
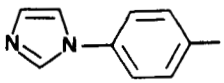
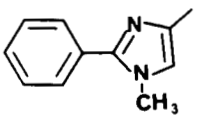
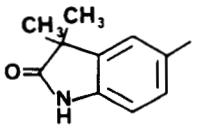
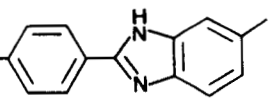
10 R = CH_3; Ar = 4-$C_6H_4SCH_3$

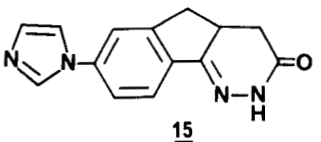
11 R = CH_2CH_3; Ar = 4-C_4H_4N

The first 4,5-dihydro-3(2H)-pyridazinones reported to have inotropic and vasodilator activity are imazodan (CI-914), **12** (63), and its 5-methyl analogue, CI-930, **13** (64). In acute clinical studies, **12**, produced significant increases in cardiac index and contractility (dP/dt) and reduced vascular resistance (65,66). The related 6-[1H-imidazo-4-yl]-3(2H)-pyridazinones, **14**, are also potent inotropic agents while the 5-yl isomers are much less active (67). Rigid analogues of **13**, such as **15**, retain positive inotropic activity with potency comparable to their acyclic analogues (68)

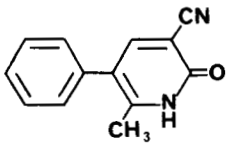
Other dihydropyridazinones which have positive inotropic and vasodilator activity include LY-195115, **16** (69), and pimobendan, **17** (70,71). Differing from other compounds in this class, **17** is reported to enhance calcium sensitivity of myofibrils, in vitro, in addition to inhibiting PDE. Both mechanisms probably contribute to the cardiotonic activity of **17** (72). In anesthetized pigs, **17** increases heart rate and LV contractility, and decreases systemic vascular resistance (72). An analogue of amrinone, APP 201-533, **18**, is reported to also combine PDE inhibition with an enhancement of myofibril calcium sensitivity (73). The pyrazin-2(1H)-one, SK&F 94120, **19**, exhibits a pharmacologic profile similar to the 4,5-dihydropyridazinone cardiotonics (74).



	R	R^1
12		H
13		CH_3
14		H
16		CH_3
17	CH_3O- 	CH_3



15



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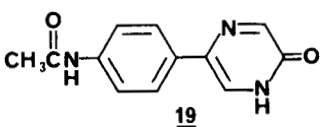
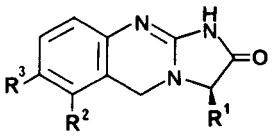


19

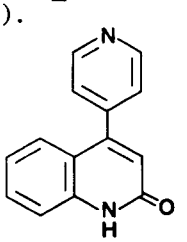
Two anagrelide, **20**, analogues, RO 13-6438 (quazinone), **21** (75,76), and RS-82856, **22** (77,78), have inotropic and vasodilating activity in dogs. In addition to the hemodynamic effects, **22** also inhibits platelet aggregation, ex vivo, in monkeys (77). Evaluation of analogues of **22** reveal significant steric constraints on the heterocycle and sidechain

with respect to inotropic and vasodilator activity (79). In this series, antiplatelet activity is optimal with **22**. More lipophilic analogues have significantly less antiplatelet activity (79).

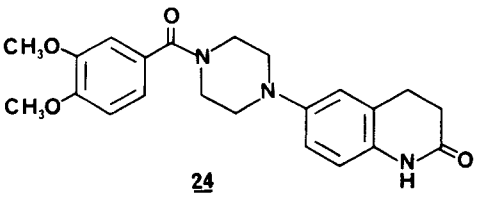
A series of cardiotonic pyridinyl-2(1H)-quinolinones, including **23** (80,81), were designed based on a pharmacophore analysis (82) of several cardiotonic PDE inhibitors including amrinone, milrinone, piroximone, imazodan, anagrelide, and sulmazole. Another 2(1H)-quinolone, OPC-8212, **24**, also shows positive inotropic activity (83).



	R^1	R^2	R^3
20	H	Cl	Cl
21	CH_3	Cl	H
22	H	H	$-NCO(CH_2)_3O-CH_3$



23

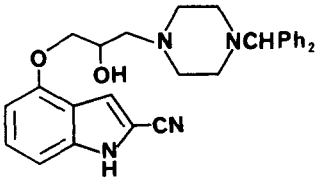


24

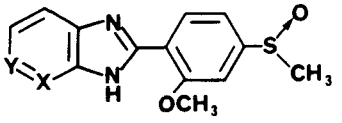
In CHF patients, the inotropic activity of PDE inhibitor cardiotonics may be less than predicted by preclinical studies. In isolated myocardial tissue from CHF patients, compounds acting through a cAMP mechanism produce a lower maximal increase in contractility than non-cAMP mediated mechanisms (84). Not only does the diseased myocardium have a lower baseline inotropic activity, the maximum response is attenuated perhaps due to a diminished ability to produce c-AMP (85). These results may explain the controversy over the relative contributions of inotropic and vascular activity to the clinical effects of these agents (47,48).

Other Mechanisms. DPI 201-106, **25**, is a novel 2-cyanoindole with potent positive inotropic and vasodilator activity. It is bradycardic and prolongs the action-potential (86). The bradycardic effect is particularly noteworthy since many inotropic agents increase heart rate. The electrophysiologic and inotropic effects of **25** are stereospecific, while the vasodilator action is not, suggesting that **25** may act through multiple mechanisms (86). Patch clamp experiments indicate the inotropic activity is due to activation of the cardiac sodium channel (87). The involvement of the sodium channel also is implicated by the antagonism of the positive inotropic effect by the sodium channel blocker tetrodotoxin and comparable effects on the action potential by **25** and the sodium channel activator ATX II (88). In addition, **25** increases the sensitivity of myofibrils to calcium (86). Mechanism studies in anesthetized dogs and pithed open-chest cats pretreated with propranolol and reserpine show that the inotropic activity is not due to beta-receptor stimulation or release of catecholamines (89). The compound has local anesthetic effects and may be antiarrhythmic (90). The enantiomers of **25** exert different effects on the sodium channel. R-**25** blocks the channel, while S-**25** increases the sodium current by slowing the kinetics of sodium channel inactivation (91).

Stimulation of sodium-calcium exchange may be the prinicipal mechanism of the positive inotropic activity of sulmazole, **26**. PDE inhibition and calcium sensitization of the contractile apparatus, which had been implicated previously, may not contribute significantly to the activity of **26** (92). Isomazole (LY175326), **27**, is the most potent of a series of imidazopyridines related to **26**. This agent is reported to have potent inotropic activity combined with vasodilation and little chronotropic activity (93). Examination of the SAR reveals that while 1H-imidazo[4,5-c]pyridine derivatives are generally active, only **26** is active among the isomeric 3H-imidazo[4,5-b]pyridine series (94). Various napthyl and benzothienyl analogues in these series generally retain activity and follow the [4,5-c] > [4,5-b] relationship (95).



25

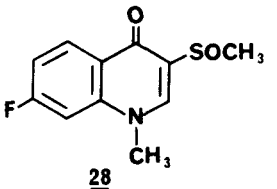


	X	Y
26	N	CH
27	CH	N

VASODILATORS - The use of "traditional" vasodilators has been reviewed including the concept of afterload and preload reduction (96). The efficacy of vasodilator therapy in chronic CHF also has been reviewed (97).

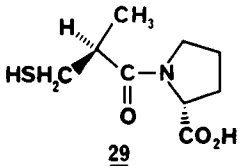
Traditional Vasodilators - The Veterans Administration Cooperative Study on Vasodilator Therapy in Heart Failure (V-HeFT) is the first controlled study to show statistically significant improvement in mortality in CHF with drug therapy (98). A therapeutic regimen consisting of hydralazine and isosorbide dinitrate reduced two-year mortality by 25%. The difference in mortality relative to placebo declined with time, with a 7% difference observed at four years. These results support the use of vasodilator therapy for CHF (99).

A new, orally active, direct-acting vasodilator, flosequinan (BTS 49465), **28**, produces comparable effects on both arterial and venous circulation (afterload and preload). In patients with severe CHF, these effects were sustained for at least 24 hours after a single oral dose (100).



28

ACE Inhibitors - In CHF patients, ACE inhibitors enhance diuresis and reduce afterload (101). In clinical trials, both captopril, **29** (102,103), and enalapril, **30** (104,105), produce sustained improvement in hemodynamic function and exercise capacity in CHF patients. The lysine analogue of enalaprilat (MK-422), **31**, lisinopril (MK-521), **32**, produces hemodynamic effects similar to **31**, but with a much longer duration of action, exceeding 24 hours (106). Another non-sulfhydryl ACE inhibitor, quinapril, **33**, also produces significant hemodynamic improvement in CHF patients (107). The efficacy of ACE inhibitors in heart failure may not be simply a result of their ability to decrease vascular resistance. By interrupting the renin-angiotensin system, which is activated in heart failure, these agents may disrupt a self-perpetuating cycle contributing to the progression of the disease (101).



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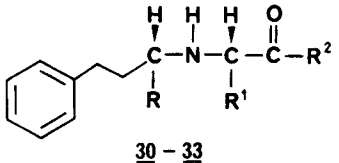
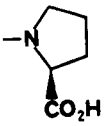
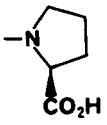
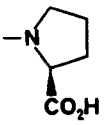
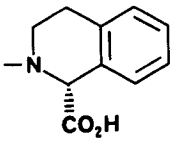
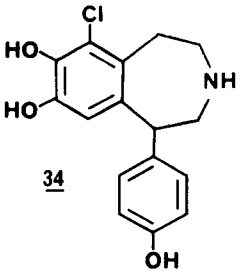


30 - 33

	R	R^1	R^2
30	···$CO_2C_2H_5$	···CH_3	
31	—CO_2H	—CH_3	
32	—CO_2H	—$(CH_2)_4NH_2$	
33	—$CO_2C_2H_5$	—CH_3	

Dopaminergic Agents - Peripheral dopaminergic agonists reduce afterload and selectively dilate the renal vasculature, a beneficial combination in CHF (108). Fenoldopam (SK&F 82526), 34, is a potent, short-acting dopamine (DA) agonist selective for peripheral DA receptors. Alpha adrenoceptors are also blocked by 34 (109). Its polarity prevents entry into the brain and activation of central receptors (110). In CHF patients, 34 improves cardiac index and reduces systemic vascular resistance (111). Dopexamine, 35, combines peripheral DA agonist activity with stimulation of beta-2 receptors to increase myocardial contractility. Furthermore, unlike DA, 35 does not produce arrhythmias in the isolated guinea pig heart (112).



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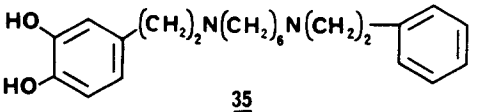
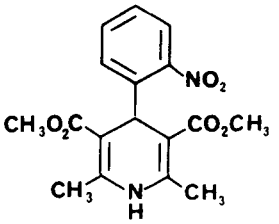


35

Other Mechanisms - Calcium channel blockers, such as nifedipine, 36, and verapamil, 37, have had an intriguing but limited role in the treatment of heart failure (113,114). These agents may be more attractive than other vasodilator agents because of their potential anti-ischemic effects (115). They effectively reduce vascular resistance, however myocardial contractility may be adversely affected (116). Cardiodepression has been observed in some studies (115,116) and thus calcium channel blockers must be used with caution in severe CHF (115,117).

Atrial natriuretic factor (ANF) levels (118) have been found to increase markedly in CHF patients and correlate inversely with cardiac output (119). Infusion of ANF promotes diruesis, natriuresis, and kaluresis. It also inhibits aldosterone and cortisone secretion (119).

Infusions of the more stable ANF analogue, Ileu-ANF, produced substantial drops in blood pressure and increased cardiac output, however diuresis and natriuresis were not increased (120). The precise role of ANF in heart failure and its potential as a therapeutic agent remain uncertain.



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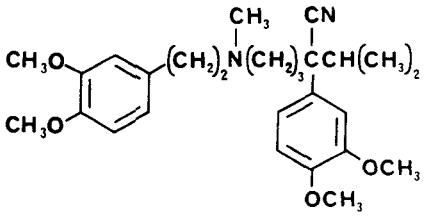

37

CONCLUSIONS - Clearly, the number and diversity of new agents and mechanisms now available or under development will impact favorably on future therapy for CHF. The increase in survival demonstrated in the V-HeFT study is a milestone and other agents, including ACE inhibitors, are undergoing similar trials. The potential role of the PDE inhibitor cardiotonics will be defined in the near future following publication of multicenter trials involving milrinone and enoximone. As a result of this progress, CHF may be better controlled and overall survival may improve. Nonetheless there remains a great challenge to discover drug therapies which not only ameliorate symptoms but have a substantive effect on the disease process and progression.

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Chapter 10. Thromboxane Synthetase Inhibitors and Antagonists

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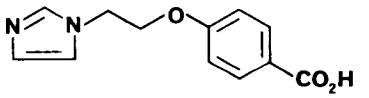
Introduction - Thromboxane A_2 (TxA_2) is an exceptionally potent pro-aggregatory and vasoconstrictor substance produced by the metabolism of arachidonic acid (AA) in blood platelets and other tissues. Together with the potent anti-aggregatory and vasodilator prostacyclin (PGI_2) it is thought to play a role in the maintenance of vascular homeostasis, and may contribute to the pathogenesis of a variety of vascular disorders. Approaches towards limiting the effect of TxA_2 have focussed on either inhibiting its synthesis, or blocking its action at its receptor sites by means of an antagonist. TxA_2 is formed from the prostaglandin endoperoxide PGH_2, and inhibition of the synthetase enzyme may lead to an accumulation of endoperoxide. This is potentially beneficial since the accumulated PGH_2 is available to be converted into PGI_2, although PGH_2 does have similar properties to TxA_2. On the other hand, TxA_2 antagonists also block the actions of PGH_2 but do not have the potential to elevate PGI_2 levels. The two approaches were last reviewed in this series in 1982 (1).

Thromboxane Synthetase Inhibitors

Mechanism and Structure Activity Relationships - TxA_2 synthetase from human platelets has been purified and found to be a cytochrome P-450 enzyme (2). The first step in the isomerization of PGH_2 to TxA_2 is probably interaction of the C-9 oxygen with the heme iron of the enzyme (2, 3). Most TxA_2 synthetase inhibitors are imidazole or pyridine derivatives and several, including dazoxiben, 1, and OKY-1581, 2, produce characteristic optical difference spectra with the enzyme, indicating coordination of the basic nitrogen with the heme iron (2, 3). Most inhibitors also contain a carboxylic acid side chain, and the importance of the distance between the basic nitrogen and the carboxyl group for optimal potency has been noted (3-8). This distance is close to the C-9 oxygen to carboxyl distance in PGH_2, and it seems likely that the substrate and inhibitor carboxyl group interact with the same site on the enzyme (3, 4). The presence of a carboxyl group also has the advantage of reducing activity against liver and adrenal cytochrome P-450 enzymes, thereby reducing the potential for side effects (4, 7, 9, 10).



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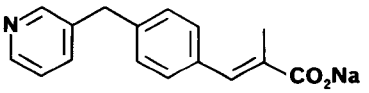
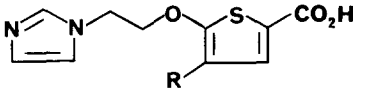


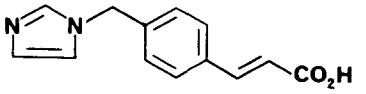
2

Structure activity relationships for 1 and analogues have been discussed (7). Two thenoic acid analogues, LG 82-4-00, 3, and LG 82-4-01, 4, have similar potency to 1 *in vitro* (11). The pharmacological properties of OKY-046, 5, have been reported (12).

Structure-activity relationships for dazmegrel (UK-38,485, 6) have been discussed (9). 6 is more potent and has a longer duration of action than 1 in conscious dogs (9). A series of imidazole derivatives incorporating bicyclic heterocyclic carboxylic acid substituents has been reported (10). In conscious dogs, 80% inhibition of TxA_2 synthesis was observed 24 hours after 0.5 mg/kg p.o. of the benzo[b]thiophenes 7 and 8. Y-20,811, 9, also had a long duration of action in rabbits and dogs (13). CBS-645, 10, (14) and amides related to 11 (15) are examples of imidazole inhibitors that lack a carboxylic acid side chain.



3, R = H
4, R = Cl



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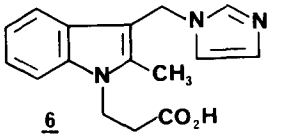

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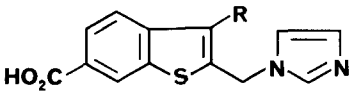
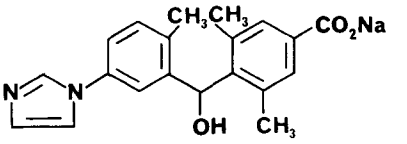


7, R = CH_3
8, R = Cl



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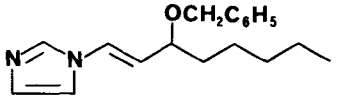

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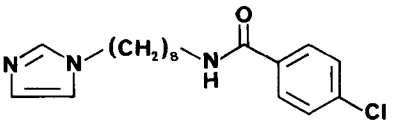
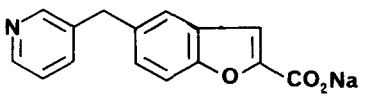


11

Structure-activity relationships have been reported for furegrelate (U-63,557A, 12) and related pyridyl-substituted benzofurans (8). CV-4151, 13, was the most potent of a series of pyridinealkenoic acid analogues (6). Both 12 and 13 produce characteristic optical difference spectra with the purified enzyme (3). The pyridylindole CGS-12,970, 14, has been reported to have a long duration of action in rabbits (16). CGS-14,854, 15, and CGS-15,435A, 16, protected rabbits against AA-induced death when administered 24 hours before AA (17, 18). The pyridine 17 and analogues selectively inhibit TxA_2 synthesis in human platelets (19). Pirmagrel (CGS-13,080), 18, is the most potent member of a series of imidazopyridines (15).



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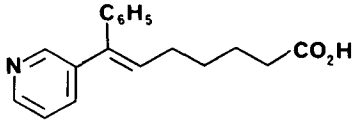

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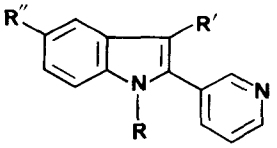
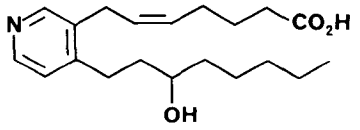


14, R = $(CH_2)_7CO_2H$, R′ = CH_3, R″ = H
15, R = $(CH_2)_5CO_2H$, R′ = CH_3, R″ = Cl
16, R = CH_3, R′ = $(CH_2)_5CO_2H$, R″ = Cl



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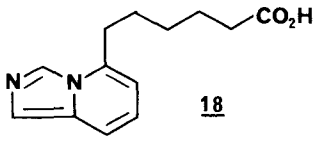


18

Effects on Platelet Aggregation in vitro - Although TxA_2 is a potent platelet aggregating agent, TxA_2 synthetase inhibitors are less effective than aspirin in inhibiting platelet aggregation due to AA or a low concentration of collagen, which mobilises intracellular AA (20). This is probably due, at least in part, to the pro-aggregatory effect of PGH_2. Studies with human platelet-rich plasma (PRP) have shown that **1** will inhibit aggregation due to AA (21, 22) or collagen (23) in plasma of some individuals (responders) but not from others (non-responders). Stimulation of aggregation of human PRP in the presence of **1** results in diversion of PGH_2 to PGD_2, PGE_2 and $PGF_{2\alpha}$ (21-23). PGD_2 is anti-aggregatory while PGE_2 potentiates aggregation. The balance between pro- and anti-aggregatory prostaglandins may govern whether an individual is a responder or a non-responder (20).

Animal Studies with TxA_2 Synthetase Inhibitors - The antithrombotic activity of TxA_2 synthetase inhibitors in vivo has been assessed by examining effects on prosthetic graft patency. Compound **1** failed to decrease platelet consumption in a chronic polyurethane arteriovenous shunt in baboons (24). A combination of **6** and aspirin at a dose that did not abolish PGI_2 synthesis maintained patency in carotid prostheses in dogs (25). Compound **12** increased the patency of canine carotid prosthetic grafts when they were previously seeded with endothelial cells (26), and improved early patency of autologous canine femoral vein grafts, although there was no reduction in graft platelet deposition (27). Both **2** (28) and **18** (29) prevented thrombotic occlusion of coronary arteries of dogs caused by prolonged electrical stimulation, but **1** and **12** were ineffective (29).

TxA_2 synthetase inhibitors have been widely studied for effects on myocardial damage and arrhythmias caused by coronary occlusion. Treatment of anaesthetised rats with **12** or **14** before ligation of the left coronary artery reduced myocardial creatine kinase and amino nitrogen loss up to 24 hr after ligation (30, 31). Both **1** and **6** reduced the incidence of early (up to 30 min) ventricular arrhythmias after coronary ligation in anaesthetized rats, but **6** only affected late (up to 4 hr) ventricular ectopics in combination with metoprolol (32). Combination of **6** with metoprolol was also necessary to reduce ventricular fibrillation (VF) after coronary artery ligation in conscious rats, indicating a role for catecholamines as well as TxA_2 (33). Administration of **18** prior to coronary occlusion in anaesthetized cats attenuated the decrease in ventricular fibrillation threshold (VFT) and reduced the incidence of spontaneous ventricular arrhythmias in the first 30 min after occlusion (34). Similar results were obtained acutely with **12** but there was no effect on VFT 2 weeks after occlusion, suggesting that TxA_2 may only have a role in causing arrhythmias in acute infarction (35).

Reperfusion after coronary occlusion in anaesthetized dogs results in VF in up to 90% of animals (36). Administration of **1** (36), **5** (37), **6** (36) or **13** (38) prior to occlusion, markedly reduced VF on reperfusion. In conscious dogs, both **12** and PGI_2 abolished VF caused by coronary occlusion (39). The effect of **12** was abolished by indomethacin, suggesting that activity depends on diversion of PGH_2 to PGI_2 (39). Neither **1** nor **2** had any effect on ventricular tachycardia or VF caused by programmed electrical stimulation in conscious dogs 3-7 days after surviving coronary occlusion and reperfusion, indicating that TxA_2 only has a role in arrhythmogenesis in the ischemic and immediate post-ischemic period (40).

The effect of TxA_2 synthetase inhibition on cerebral infarction has been examined. In anaesthetized cats with cerebral artery occlusion followed by reperfusion, **1** given before or after occlusion did not affect EEG changes, regional cerebral blood flow, or infarct size (41).

The effect of TxA_2 synthetase inhibitors on vasospasm caused by intracisternal injection of fresh autologous arterial blood in anaesthetized dogs has been studied to assess possible effects on the delayed cerebral vasospasm that occurs after subarachnoid hemorrhage. Intracisternal injection of **5** inhibited contraction of the basilar artery up to 6 hr after injection of blood (42). Neither **2** nor **5** was effective against delayed vasospasm when given intravenously (43), although **2** relieved cerebral arterial spasm in rabbits after intracisternal injection of fresh autologous arterial blood (44).

The effect of TxA_2 synthesis inhibition has been studied in animal models of renal disease. Both **2** (45) and **6** (46) prevented elevation of TxB_2 excretion and increased glomerular filtration rate (GFR) in rats with ablation of renal mass. The same compounds prevented reductions in renal blood flow and GFR in rats up to 3 hr after induction of nephrotoxic serum nephritis, but had no effect on GFR after 1 or 14 days (47).

Conflicting reports have appeared regarding the effectiveness of TxA_2 synthetase inhibitors in hypertension. Thus, treatment of young spontaneously hypertensive rats (SHR) with **13** for 3 weeks improved renal function and delayed the initiation of hypertension (48), but treatment with **6** for 5.5 weeks was ineffective (49). By contrast, **13** had little effect on established hypertension in SHR (48), but **6** was reported to cause a reduction in systolic blood pressure that was maximal after 10 days (51).

AA metabolites including TxA_2 may play a role in the pathophysiology of endotoxin shock (51). Injection of bacterial endotoxin into animals results in an early transient rise in TxA_2 production which is a likely cause of the ensuing thrombocytopenia and pulmonary hypertension (51). Compound **5** prevented the early pulmonary hypertension caused by infusion of *E. coli* endotoxin in conscious sheep, but did not affect the later changes in pulmonary vascular permeability (52). Compound **2** improved organ perfusion in early endotoxin shock in rats (53), but had no effect on mortality after endotoxin administration to baboons (54). Both **2** and **5** reduced the extent of microthrombus formation in kidney glomeruli of rats after injection of *E. coli* endotoxin (55), but neither **1** nor **6** prevented the metabolic, cardiovascular or thrombocytopenic effects of a 4 hr infusion of endotoxin in rats, and did not affect mortality (56).

Gastric mucosal ischemia resulting from vasoconstriction is a possible factor in the formation of mucosal lesions in response to various stimuli. TxA_2 synthetase inhibition with **2** or **6** protected rats against acidified taurocholate-induced damage (57, 58), and **5** reduced the incidence of gastric erosions in water-immersion stressed rats (59).

Clinical Evaluation of TxA_2 Synthetase Inhibitors - Inhibition of TxA_2 synthetase in man by dazoxiben (60), OKY-1581 (61), dazmegrel (62), furegrelate (63), CV-4151 (64) and pirmagrel (65, 66) has been demonstrated by measurement of serum TxB_2 levels after dosing. Measurement of increased serum 6-keto $PGF_{1\alpha}$ levels has been used to show

diversion of PGH_2 to PGI_2 (61, 64, 65). However, measurement of urinary metabolites is considered to give a truer indication of TxA_2 and PGI_2 synthesis *in vivo* as prostanoid synthesis resulting from activation of blood cells during sampling is avoided (60). Thus, dazmegrel reduced the excretion of 2,3-dinor-TxB_2, but increases in 2,3-dinor-6-keto-$PGF_{1\alpha}$ were only transient (62). An approximate doubling of urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ levels has been reported after dazoxiben (60), pirmagrel (66) and OKY-046 (67). Higher absolute levels of urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ were measured after TxA_2 inhibition with pirmagrel in patients with severe peripheral vascular disease who had a higher basal level of platelet activation and TxA_2 synthesis (68). However, these metabolite levels still reflect sub-threshold therapeutic levels of circulating PGI_2, although levels at a local site of platelet activation may be higher.

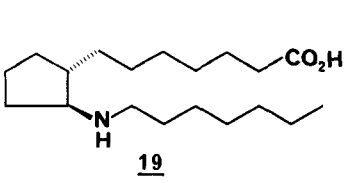
Evaluation of dazoxiben in stable angina has failed to show a significant effect (69, 70). OKY-046 was reported to have a small effect on exercise tolerance (71) but another study showed no benefit (72). In a 12 month study, both OKY-046 and OKY-1581 were claimed to reduce the frequency of anginal attacks (73). Both OKY-046 (74) and CV-4151 (75) were found to be effective in the treatment of unstable angina. Urinary levels of 2,3-dinor-TxB_2 were found to be normal in stable angina patients, but large increases, probably reflecting episodic platelet activation were observed in unstable angina patients, suggesting that TxA_2 may play a more significant role in unstable angina (76).

Infusion of OKY-1581 within 6 hr of the onset of acute myocardial infarction abolished the increase in plasma TxB_2 compared with controls, and creatine kinase release was markedly reduced (77). Administration of OKY-1581 after surgery for subarachnoid hemorrhage gave only a small reduction in vasospasm and ischemic symptoms, which were not statistically significant (78). Several trials have failed to show any effect with either dazoxiben (79-81) or dazmegrel (82) in Raynaud's phenomenon. Dazoxiben also failed to alter significantly the hemodynamic and pulmonary consequences of sepsis and adult respiratory distress syndrome (83), and did not improve renal function in patients with hepatorenal syndrome (84).

Possible reasons for the disappointing performance of TxA_2 synthetase inhibitors in clinical studies to date have been discussed (20, 85). There is evidence to suggest that complete inhibition of TxA_2 production may be required to prevent platelet activation, and doses of inhibitors used so far may not have been sufficient to prevent recovery of TxA_2 formation between dosing (85). More potent or longer-acting compounds may therefore be required. Also, accumulated PGH_2 may substitute for TxA_2 if diversion to PGI_2 does not occur (20, 85). Potentially, this problem may be overcome by combination with a TxA_2/PGH_2 antagonist, and it has been reported that dazoxiben and the antagonist BM 13,177, **34**, act synergistically in inhibiting collagen-induced platelet aggregation (20).

Thromboxane Receptor Antagonists

The majority of TxA_2 receptor (TxR) antagonists may be considered to be prostanoid analogues incorporating a typical α-side chain, but often with substantial modification to the ω-side chain. Structure-activity relationships for compounds related to 13-azaprostanoic acid



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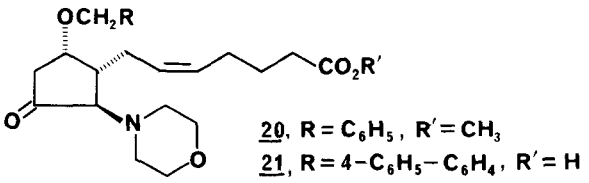
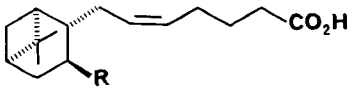
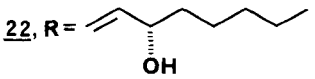


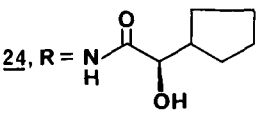
20, R = C_6H_5, R' = CH_3
21, R = 4-C_6H_5-C_6H_4, R' = H

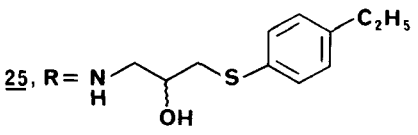
(13-APA, 19) have been reported (86). The location of the nitrogen atom and the trans orientation of the side chains were very important for good activity. Compound 19 was shown to be a specific inhibitor of intraplatelet Ca^{2+} release induced by U-46619, a TxA_2/PGH_2 mimetic (87), and also potentiated PGI_2 reversal of U-46,619-induced platelet aggregation (88). Other azaprostanoid TxR antagonists include AH 19,437, 20, and AH 23,848, 21, with the latter being approximately 100x more potent than the former (89). In human PRP, 21 selectively inhibited aggregation caused by submaximal concentrations of AA, PGH_2, TxA_2, U-46619 or collagen (90). In anaesthetized dogs 21 had no effect on blood pressure or mesenteric arterial blood flow, but it antagonised the vasoconstriction caused by a close arterial injection of U-46619 (90). 21 prevented the pulmonary hypertensive effect of bacterial endotoxin in cats (91) and protected anaesthetized dogs against ischemia-induced arrhythmias, as well as suppressing ventricular fibrillation following reperfusion (36). In man, orally administered single doses of 21 inhibited ex vivo platelet aggregation induced by U-46619 for at least 8 hr (92), and when given on a repeat dose basis for 10 days showed sustained inhibition of aggregation (93). The compound also inhibited platelet deposition onto arterial prostheses in patients with vascular disease (94) but had no obvious effect in stable angina (95). The synthesis of 13-azaprostanoids and their actions at TxA_2/PGH_2 receptors has been reviewed (96).

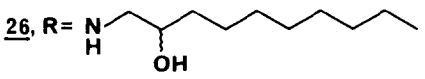
Pinane thromboxane A_2 (PTA_2, 22) appears to have significant TxR agonist properties in addition to its antagonist activity in several biological test systems (97). Modification of the ω-side chain gave SK II-144, 23, which was reported to be an antagonist of both $PGF_{2\alpha}$ and TxA_2/PGH_2 with no agonist activity (98). Other modifications of the ω-side chain produced ONO-3708, 24, (99), ONO-11105, 25, ONO-11119, 26 and ONO-11120, 27 (100) which were said to be pure TxR antagonists. Binding studies have been carried out with a ^{125}I-labelled derivative of 27 using washed platelets of man, dog and rabbit; the results obtained suggest that the TxA_2/PGH_2 receptor varies between species (101).

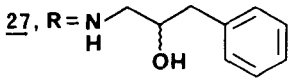








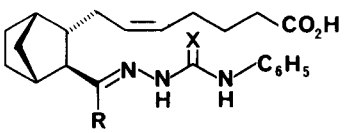




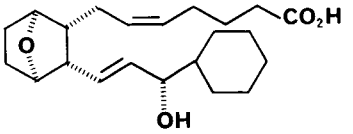
Photoaffinity labelling of the receptor has also been carried out with a close analogue of **27** (102), and it appears that the TxA_2 receptor in platelets is different from that in the vasculature (103). Based on this work it was proposed that the platelet receptor be designated $(TxA_2/PGH_2)_\alpha$ (α for aggregation), and the vascular receptor be $(TxA_2/PGH_2)_\tau$ (τ for tone). Previously, the differentiation of the receptors was suggested by the different affinity of the particular antagonist under study. However, work with **21** has suggested that these apparent observed differences could be explained equally either by partial agonism or by differences in receptor density and stimulus-response coupling (104). Studies suggesting considerable similarity between the two receptors have been carried out using EP-045, **28**, (105). This compound produced TxR blockade *in vivo* but its effects were short-lived. However, modification of the ω-side chain produced EP-092, **29**, which was more potent and had a longer duration of action (105, 106).

A series of TxR antagonists has been produced based on the 7-oxabicyclo[2.2.1]heptane ring system (107, 108). Two compounds typical of the series are SQ-27,427, **30**, and SQ-29,548, **31**, (109). Of the two, **31** has been the more extensively studied and has been shown to be a potent and specific TxR antagonist in human platelets, rat aorta and guinea-pig trachea (110). Compound **31** significantly prevented the extension of ischemic damage in the myocardium and improved survival following acute coronary artery ligation in rats, supporting a role for TXA_2 in the pathophysiology of acute myocardial ischemia (111). Blockade of TxA_2 by **31** during ischemia and reperfusion in anaesthetized dogs also resulted in a significant salvage of jeopardized myocardial tissue (112).

ICI-180,080, **32**, is an orally active TxR antagonist with a pA_2 of 7.5, as measured against U-46619 on rabbit thoracic aorta (113, 114). An analogue, ICI-185,282, **33**, has been reported to have a superior duration of action (115).



28, R=H, X=O
29, R=CH_3, X=S



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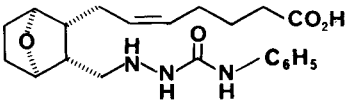

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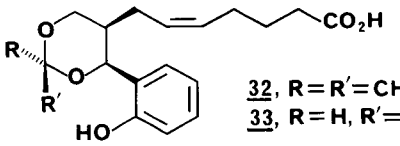
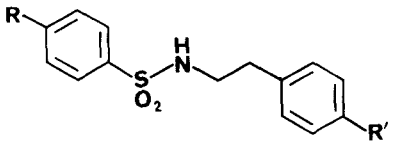


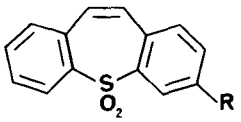
32, R=R'=CH_3
33, R=H, R'=CF_3

Sulotroban (BM-13,177, **34**) represents a marked structural departure from the prostanoid type of TxR antagonist. It inhibited aggregation of human platelets by AA, collagen or U-46619, and competitively inhibited the vasoconstriction induced by U-46619 or $PGF_{2\alpha}$ in rat and rabbit aorta (116). It was also effective in decreasing the damage associated with acute myocardial ischemia in cats (117, 118). In patients with atherosclerotic disease, a dose of 2 x 800 mg of **34** over 4 days significantly reduced *ex vivo* collagen-induced platelet aggregation

(119). Using **34** it was shown that TxR blockade in normal subjects does not influence either arterial blood pressure or digital blood flow, from which it was concluded that TxA_2, PGI_2, as well as other prostanoids, do not play a relevant role in modulating the systemic vascular response to cold (120). Pharmacokinetic investigations have shown that **34** has a terminal plasma half-life of 0.85 hr, which is similar to the half-life for platelet inhibition (121). This led to the conclusion that complete inhibition of TxA_2-dependent platelet function would require multiple doses of **34**. A close analogue, BM-13,505, **35**, has been found to be 3-5 times more potent (122) and to have a terminal half-life in man of 6.7 hr (123). Complete inhibition of *ex vivo* platelet aggregation to PGH_2 or U-46619 was achieved for 6 hr with a single dose of 200 mg (124). Compound **35** protected rabbits against glycerol-induced renal failure (125).

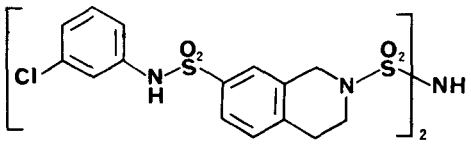


34, R = H, R' = OCH_2CO_2H
35, R = Cl, R' = CH_2CO_2H



36, R = CH_2OH
37, R = CO_2H

A dibenzo[b,f]thiepin-5,5-dioxide, L-640,035, **36**, and its carboxylic acid metabolite L-636,499, **37**, have been found to be antagonists of the action of various contractile prostanoids on smooth muscle (126) and platelets (127). SK & F 88,046, **38**, inhibited aggregation of human platelets to AA, collagen or U-44069, and antagonised TxA_2-mediated changes in airway mechanics in anaesthetized dogs (128).



38

Conclusion

Clinical studies with both TxA_2 synthetase inhibitors and antagonists have, overall, failed to show convincing efficacy in stable angina, and unstable angina may be a more appropriate target. An important role for TxA_2 in the pathophysiology of acute myocardial ischemia is indicated from studies in both animals and man, and further clinical work in this area is merited. However, despite extensive study, there is still uncertainty regarding the role of TxA_2 in other disease states. Further clinical studies, coupled with improved techniques for monitoring TxA_2 synthesis *in vivo* (129), will hopefully help to define more clearly the role, and relative merits, of TxA_2 synthetase inhibitor and antagonist therapy.

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Chapter 11. Peripheral Actions Of Dopamine Receptor Agonists

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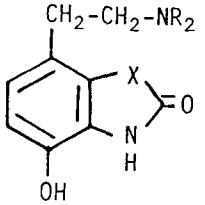
INTRODUCTION

Since the suggestion that discrete dopamine receptors may be present in the periphery (1,2), much progress has been made in the synthesis and pharmacological characterization of dopamine receptor agonists. At least two distinct subtypes of peripheral dopamine receptor have been identified and highly potent and highly selective agonists are available to stimulate both the vascular receptor, designated DA_1, which mediates vasodilation, and the neuroinhibitory receptor, designated DA_2, which modulates neurotransmitter release from sympathetic nerve terminals. While drug development efforts have concentrated on agonists, the identification of selective antagonists for the DA_1 and DA_2 receptors has greatly facilitated studies on the role of dopamine receptors in cardiovascular regulation.

RECENT DEVELOPMENTS IN THE IDENTIFICATION AND CLASSIFICATION OF DOPAMINE RECEPTOR AGONISTS

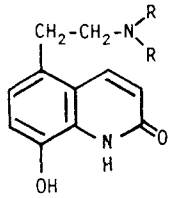
Selective Agonists at Dopamine Receptor Subtypes- The structure activity relationships for agonists and antagonists at DA_1 and DA_2 receptors have been discussed in several comprehensive reviews (3-6). Most of the recently identified agonists not covered in these reviews stimulate the DA_2 receptor; hence this section will concentrate on these agents. Several highly potent and highly selective DA_2 agonists have been identified. These include SK&F 103376, 1, (7,8), an aminoalkyl thiazolone structurally similar to SK&F 89124, 2, (9-11). Replacement of the methylene group by sulfur results in a marked enhancement in the neuroinhibitory potency, as measured in the isolated perfused rabbit ear artery, a useful model for in vitro determination of DA_2 agonist potency (12). SK&F 103376 has an EC_{50} of 0.028 nM in this assay (7) as compared to a corresponding value of 1.8 nM for SK&F 89124 (9) and 37 nM for dopamine (12).



1: R= n-C_3H_7, X=S
2: R= n-C_3H_7, X=CH
3: R= H, X=S

Both SK&F 89124 and SK&F 103376 contain an N,N-dipropyl phenethylamine. The presence of a tertiary nitrogen atom with at least one n-propyl group has been confirmed to be optimal for DA_2 agonist activity by many studies in several different structural series, including the phen-

ethylamines (13), and those represented by 2 (14), 4 (15), 5 (16) and 6 and 7 (5,17). It is interesting to note that 3, the N-unsubstituted analog of SK&F 103376, has weak, but pharmacologically significant activity at the DA_1 receptor (7,8). This represents the first example of a non-catechol having DA_1 agonist activity.



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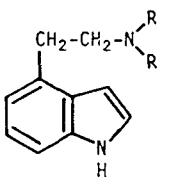

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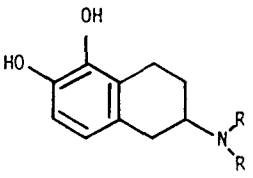

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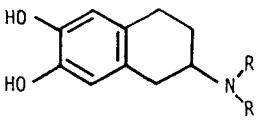
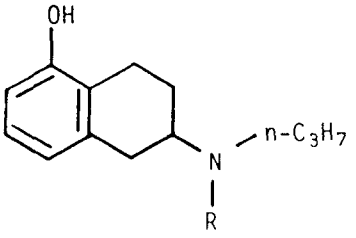
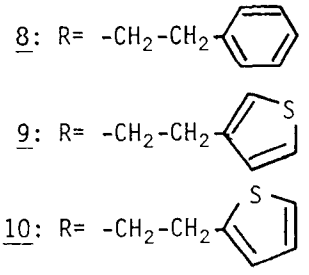


7

Surprisingly, a large aralkyl substituent often confers optimal potency (14,15). Compound 8, N-0434, is a highly potent and selective agonist at central presynaptic dopamine receptors (18). Although neither this agonist nor equally potent analogs in which the phenyl is replaced by thienyl 9, 10, N-0437, have been examined in peripheral dopamine receptor models, their ability to displace ^{3}H-spiroperidol binding at very low concentrations would suggest that they are likely to be potent neuroinhibitory agents (19,20).



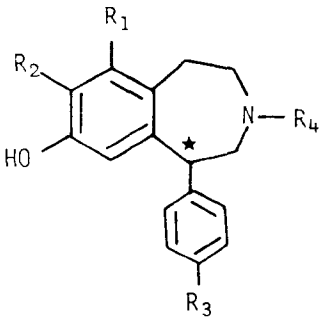


8: R= $-CH_2-CH_2-$

9: R= $-CH_2-CH_2-$

10: R= $-CH_2-CH_2-$

Fenoldopam, 11, and SK&F 38393, 12, continue to be the only examples of potent, selective agonists at the vascular DA_1 receptor. Although many members of this series have high DA_1 compared to DA_2 potency, SK&F 85174, 13, has potent agonist activity at both DA_1 and DA_2 receptors (21, 22). Other 6-halo, N-allyl analogs, 14,15, also have agonist activity at both receptor subtypes (23), and the N-methyl derivative of fenoldopam, 16, is suprisingly potent in the rabbit ear artery assay (24).



	R_1	R_2	R_3	R_4
11	Cl	OH	OH	H
12	H	OH	H	H
13	Cl	OH	OH	$-CH_2-\overset{H}{C}=CH_2$
14	Br	OH	OH	$-CH_2-\overset{H}{C}=CH_2$
15	F	OH	OH	$-CH_2-\overset{H}{C}=CH_2$
16	Cl	OH	OH	CH_3
17	H	Cl	H	CH_3 (R enantiomer)
18	H	Br	H	CH_3

Potential Further Subdivision of DA_1 and DA_2 Receptors- Although the DA_1 and DA_2 receptor concept explains most of the pharmacology of dopamine receptor agonists in the periphery, there are a few inconsistencies. Fenoldopam and SK&F 38393 are potent dilators of the renal and mesenteric vascular beds in anesthetized animals (25-28) as well as in conscious animals (27,29) and man (see below). This effect is clearly a result of DA_1 receptor stimulation, demonstrated even more convincingly with the availability of the selective DA_1 antagonists SCH-23390, 17, and SK&F 83566, 18, which block the vasodilator effects of the benzazepines at low concentrations (30,31). Nevertheless, fenoldopam and SK&F 38393 are very weak or inactive in a standard *in vitro* model for the DA_1 receptor, the rabbit splenic artery contracted with PGF_{2a} (26), although DA_1 mediated relaxation is produced if this tissue is contracted with norepinephrine (32). Fenoldopam will also induce vasodilation in the isolated perfused rat kidney (33), and will relax rabbit renal arterioles contracted with either norepinephrine or angiotensin (34).

The relative potency difference with fenoldopam and SK&F 38393 between the dog mesenteric vascular bed and rabbit splenic artery has been used to suggest that the DA_1 receptor can be further subdivided (26). This subdivision is supported by the marked potency difference for sulpiride as an antagonist in the mesenteric bed (relatively potent blockade) and isolated splenic artery (inactive).

Similar differences are seen when the antagonist potency of cis-alpha flupenthixol and fluphenazine are compared in two DA_2 receptor models, cat cardioaccelerator nerve stimulation (potent blockade by both antagonists) and the isolated rabbit rectococcygeus muscle (both essentially inactive). Although these differences suggest two subgroups within both the DA_1 and DA_2 receptor classes (26), the definitive proof of this concept will require comparison of agonist potencies and antagonist receptor dissociation constants in additional receptor models, to determine whether a consistent pattern emerges.

ADDITIONAL SITES OF ACTION FOR DOPAMINE RECEPTOR AGONISTS

In addition to the established sites of action for dopaminergic agonists, recent evidence suggests that dopamine receptors may play a role at other sites of cardiovascular regulation. It has been reported that fenoldopam, a selective DA_1 agonist, will increase renin levels following *in vivo* administration (35,36). Although this could be a reflex response to the fall in blood pressure, recent experiments using primary cell cultures show that fenoldopam has a direct action on the juxtaglomerular cell to increase renin secretion (36). Although the concentration of fenoldopam required to produce this effect (650 nM) was relatively high, a low concentration (0.9 nM) of SCH 23390 produced significant blockade, suggesting a DA_1 receptor mediated effect. DA_1 receptor stimulation also appears to have a direct action on the renal tubule to increase electrolyte excretion. This effect will be discussed in relation to the clinical effects of fenoldopam.

Although DA_1 receptor agonists do not inhibit adrenergic neurotransmission *via* an action on release from the varicosity, several reports suggest that a DA_1-like receptor may be involved in inhibition of ganglionic transmission. Both DA_1 and DA_2 receptor agonists will inhibit transmission through the lumbar, stellate and superior cervical ganglia of the dog (37-39) and will inhibit the generation of a compound action potential in isolated rat superior cervical ganglia (40). The action of

quinpirole, 19, a selective DA_2 agonist (see below) was antagonized by (S)-sulpiride, a selective DA_2 antagonist. A slight, but statistically significant, antagonism of the inhibitory effect of fenoldopam was produced by (R)-sulpiride, which has DA_1 antagonist activity (41); however, the more potent and more selective DA_1 antagonist SCH-23390 had no effect on fenoldopam-induced inhibition. These in vitro results are similar to those observed in the intact animal, where (R)-sulpiride, but not SCH-23390, will antagonize the inhibitory effect of fenoldopam on ganglionic transmission (37). However, SK&F 83566, which has essentially identical pharmacological activity as SCH-23390 in all systems examined to date (42,43), has been found to antagonize the inhibitory effect of fenoldopam on tachycardia induced by preganglionic stimulation in the dog (31).

Further evidence for a distinction between ganglionic and vascular dopamine receptors is provided by biochemical studies which show that neither dopamine nor fenoldopam will increase cyclic AMP levels in isolated rat superior cervical ganglia (40) in contrast to a significant effect in the rat renal artery (40), rabbit splenic artery (44) and rabbit renal artery (45,46). Hence, at this time it is not clear whether the ganglionic dopamine receptor stimulated by fenoldopam represents an atypical DA_1 receptor with respect to either agonist or antagonist recognition site or transduction between receptor occupation and effector response.

DA_1 RECEPTOR STIMULATION IN MAN: CLINICAL RESULTS WITH FENOLDOPAM

As noted above, fenoldopam (SK&F 82526) is the prototype for the selective DA_1 receptor agonists, and its pharmacology has been characterized extensively in a variety of animal models (4,27,28,30,47). The clinical evaluation of fenoldopam for a variety of cardiovascular indications is currently in progress.

Fenoldopam has been studied extensively as an antihypertensive drug. A single oral dose will reduce both supine and standing blood pressure in hypertensive patients (48-51) with no tolerance to the antihypertensive effect if dosing is continued for 4 weeks (48). Although fenoldopam has little or no effect on blood pressure in normotensive volunteers, renal blood flow is clearly increased; however, glomerular filtration rate is not affected (52-55). This effect is consistent with the known ability of fenoldopam to decrease renal vascular resistance via a DA_1 receptor mechanism. In addition to increasing renal plasma flow, fenoldopam can induce a diuretic effect in man, possibly due to a direct tubular effect to enhance sodium excretion (54).

It is likely that, as seen in animal models, the effects of fenoldopam to decrease vascular resistance are due to DA_1 mediated vasodilation. Plethysmographic studies in the human forearm have shown that fenoldopam can produce dilation of this vascular bed via a dopaminergic mechanism since the increase in forearm blood flow could be blocked by (R)-sulpiride (56,57). The ability of fenoldopam to dilate forearm vasculature suggests that the distribution of vascular DA_1 receptors, at least in man, may be more extensive than previously suspected.

In congestive heart failure patients, either oral (58,59) or intravenous (60) fenoldopam produced beneficial hemodynamic effects, decreasing blood pressure, systemic vascular resistance and increasing cardiac index. Heart rate was not increased significantly in these patients.

THERAPEUTIC APPLICATIONS OF DA_2 RECEPTOR ACTIVATION

Studies in hypertensive patients with relatively nonselective DA_2 agonists, such as N-n-propyl, N-n-butyldopamine (PBDA), bromocriptine and co-dergocrine (hydrergine) show a reduction in blood pressure which could be attributed to a DA_2 receptor mediated sympatholytic effect (61-63).

The efficacy of DA_2 agonists in congestive heart failure is supported by limited studies with PBDA, bromocriptine and L-DOPA, the metabolic precursor of dopamine (64-67). The beneficial hemodynamic effects of these agents were attributed to an inhibition of excess sympathetic activity. In the antihypertensive evaluation of co-dergocrine evidence of improved left ventricular function was also observed, based on an increased left ventricular ejection fraction (64).

DA_2 agonists may also be of clinical utility in angina pectoris, since stress induced increases in cardiac rate, with a concomitant increase in myocardial oxygen requirements, could be blunted. The ability of co-dergocrine to blunt stress-induced tachycardia in hypertensive patients 64) supports such a hypothesis. Via inhibition of cardiac norepinephrine release, a DA_2 agonist could have a similiar therapeutic profile as a beta-adrenoceptor antagonist (68), with the advantage that its sympatholytic effect is produced only under conditions of excess sympathetic activity.

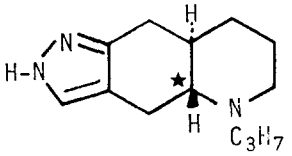
NEW DA_2 AGONISTS HAVING POTENTIAL CLINICAL UTILITY

The DA_2 agonists which have been studied in man to date are not optimal for testing the clinical utility of DA_2 receptor stimulation. PBDA, bromocriptine and co-dergocrine have activity at several other neurotransmitter receptors, and codergocrine is a complex mixture of ergot alkaloids. However, several of the selective DA_2 agonists which have been characterized extensively in animal models are being developed for human use.

Quinpirole- Quinpirole, LY 171555, 19, which has also been studied as the racemate, LY 141865, is a selective DA_2 agonist, designed to mimic the active pharmacophore of the ergot alkaloids. Quinpirole or LY141865 has been shown to reduce blood pressure and heart rate in anesthetized rats, dogs and primates (69-72). However, recent studies in conscious rats have shown quinpirole to produce a dose-related increase in blood pressure (73, 74). This effect was proposed to result from central DA_2 receptor activation, since the blood pressure increase could be blocked by metoclopramide or flupentixol, but not by domperidone, a selective DA_2 antagonist which does not penetrate into the central nervous system. The blood pressure elevation may be mediated *via* increased sympathetic outflow and by the release of vasopressin; after treatment with phenoxybenzamine, to block alpha-adrenoceptors, plus a vasopressin antagonist, quinpirole produced a reduction in blood pressure which was sensitive to blockade by domperidone. Evidence for vasopressin release by quinpirole, partially attenuating the DA_2-mediated hypotensive effect, was also observed in anesthetized rats, particularly under urethane anesthesia (72). In the very limited clinical experience to date with quinpirole, no decrease in blood pressure was observed in either normotensive or hypertensive subjects, with a slight increase in blood pressure, and elevated urinary catecholamine secretion in two individuals out of the six examined (75). This suggests that the paradoxical increase in

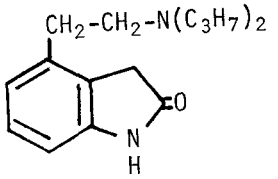
sympathetic nervous system activity seen with quinpirole in the conscious rat may also occur in man (75).

A central effect to increase sympathetic activity has not been observed in the clinical studies with the less selective DA_2 agonists, such as bromocriptine, PBDA or co-dergocrine. Whether the effects seen with quinpirole in conscious rats and perhaps in man reflect a general consequence of selective activation of central DA_2 receptors, or a property peculiar to quinpirole, awaits the further study of other selective DA_2 agonists.



19 (R enantiomer)

4-[2-(Di-n-propylamino)ethyl]-2-(3H)-indolones- As mentioned above, this structural class, whose members can be considered as analogs of N,N-di-n-propyldopamine (DPDA) in which the meta-hydroxyl is replaced by a cyclized amide function, are highly potent and highly selective DA_2 agonists (9,14,76). In contrast to DPDA and other catecholamines, the indolones are well absorbed following oral administration to the rat, especially SK&F 101468 20, (76). SK&F 101468 is hydroxylated in vivo to form SK&F 89124, which is 30-fold more potent as a DA_2 agonist (77); hence, although SK&F 101468 has intrinsic agonist activity at the DA_2 receptor, being only twofold less potent than dopamine as an inhibitor of adrenergic neurotransmission in the isolated rabbit ear artery (76), the majority of its in vivo activity may result from an active metabolite. Like quinpirole and DPDA, the indolones are effective antihypertensive agents in anesthetized animal models, producing a reduction in blood pressure and heart rate consistent with their sympatholytic profile (11).

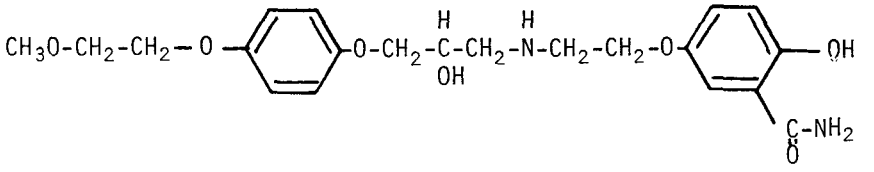


20

AGONISTS COMBINING DA_2 AGONIST ACTIVITY WITH ANOTHER PHARMACOLOGIC EFFECT

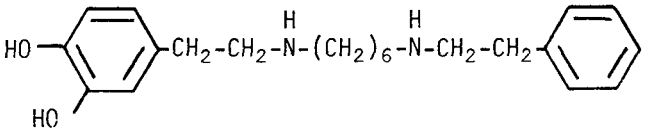
CGP 17582-This agonist, 21, which is not directly analagous to the other phenethylamine DA_2 receptor agonists, has both DA_2 agonist and $beta_1$-adrenoceptor antagonist activity. The DA_2 agonist activity is demonstrable both as inhibition of prolactin secretion or as a hypotensive effect in the anesthetized cat (78). Both of these effects were sensitive to blockade by sulpiride. In the anesthetized dog, intravenous infusion of

CGP 17582 produces a significant decrease in systemic vascular resistance. This effect was also sensitive to sulpiride blockade. CGP-17582, at an intravenous dose tenfold lower than that required to produce DA_2 agonist effects, produced a significant attenuation of exercise-induced tachycardia in the conscious dog, suggesting that the most potent pharmacologic activity of the molecule is beta-adrenoceptor blockade. This type of pharmacologic profile represents an interesting approach to the combination of beta-adrenoceptor blockade and vasodilator activity in the treatment of hypertension. Since DA_2 agonists will reduce indices of myocardial oxygen consumption, suggesting utility in angina pectoris (68), and $beta_1$-adrenoceptor antagonists are known to be effective, via their postjunctional sympatholytic effects (79) the combination of these two activities should make CGP-17582 an ideal candidate for evaluation as an antianginal drug.



21

Dopexamine-Dopexamine, 22, combines DA_1 and DA_2 receptor agonist activity with $beta_2$-adrenoceptor agonist activity. Dopexamine, like Sandoz 27-403, 23, contains two phenethylamine moieties, linked by an aliphatic chain. This type of structure can have quite high affinity for the DA_2 receptor, with 23 being fourfold more potent than dopamine as an inhibitor of neurotransmission in the rabbit rectoccygeus (80).



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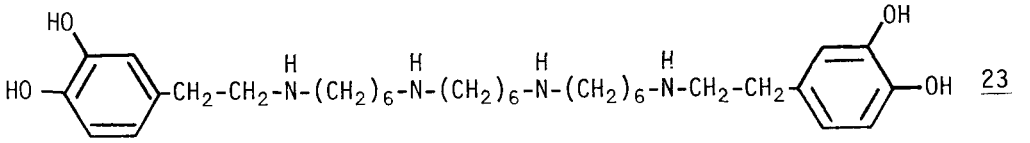

23

Dopexamine has agonist activity at DA_2 receptors, inhibiting adrenergic neurotransmission in the rabbit ear artery (sixfold less potent than dopamine) and anesthetized cat (fourfold less potent than dopamine At the DA_1 receptor, dopexamine is about threefold weaker than dopamine in decreasing renal vascular resistance (81). Dopexamine has 60-fold greater potency than dopamine in producing $beta_2$-adrenoceptor mediated relaxation of the guinea pig trachea, but has only very weak partial agonist activity at the $beta_1$-adrenoceptor, and no measurable agonist activity at either alpha-adrenoceptor subtype (81). In the anesthetized dog, dopexamine produced a hemodynamic profile consistent with activation of DA_1, DA_2 and beta-adrenoceptors, with a dose-related fall in blood pressure, renal and mesenteric vascular resistance, and an increase in heart rate and cardiac contractility (82).

In congestive heart failure patients, intravenous administration of dopexamine resulted in a significant decrease in systemic vascular resistance and a significant increase in cardiac index, stroke volume index and heart rate (83). This profile may be useful in congestive heart failure; however, further studies will be required to determine whether the tachycardia produced by dopexamine in dogs (82) and man (83) will restrict its applicability. It is interesting to note that at clinically effective doses, dopexamine produced no evidence of nausea or emesis in man, despite producing emesis in dogs at doses only slightly higher than the effective dose for inducing cardiovascular effects (81).

SUMMARY AND CONCLUSIONS

The peripheral dopamine receptors remain an attractive target for cardiovascular drugs. Although *in vitro* models show a clear distinction between the pharmacology of agonists selective for the DA_1 and DA_2 receptor subtype, the overall hemodynamic profile of selective DA_1 and DA_2 agonists in an intact animal is often quite similar (11). The most significant effect of either type of agonist is a fall in systemic blood pressure and total peripheral resistance. These are desirable therapeutic end points in cardiovascular disorders such as hypertension and congestive heart failure. The additional sympatholytic effect of DA_2 stimulation may offer an additional advantage in angina pectoris, *via* a reduction in cardiac oxygen demand.

The therapeutic utility of DA_1 agonists seems clear, based on the rapidly accumulating clinical data with fenoldopam. The efficacy of selective DA_2 agonists in man remains to be established, but the animal data, and the limited clinical data available with agonists having DA_2 activity, are encouraging. The ability to combine DA_1 and DA_2 agonist activity with other receptor agonist or antagonist activity in a single molecule also increases the likelihood of achieving the desired pharmacologic profile.

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SECTION III - Chemotherapeutic Agents

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Chapter 12. Quinolones

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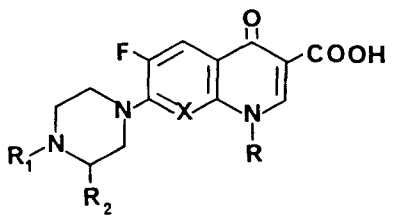
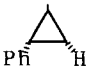
Introduction - The realization of the full market potential of norfloxacin (NOR), ofloxacin (OFL) and enoxacin (ENO) in Europe and Japan has spurred interest in the fluoroquinolones. The potential for oral use of the quinolones for infections which have traditionally required parenteral antibiotics is being emphasized. Although a large number of compounds have been synthesized in 1985 and 1986, only a few, such as AM-833 (1), NY-198 (2), AM-1091 (3), PD-117,558 (4) and A-62254 (5) have been selected for extended pre-clinical and clinical tests. Many fluoroquinolones have clinical efficacy rates as high as 90% and exhibit very few adverse effects. The search for new quinolones is focussed on the following areas: (a) Pharmacokinetic properties. Improvement of pharmacokinetic properties such as enhancing oral absorption, to achieve higher serum concentrations and to obtain a long serum half-life. (b) Water solubility. Many of the quinolones have low water solubility which presents a potential problem of crystalluria, as well as problems in the formulation for i.v. use. (c) Adverse reactions. Although the reported adverse effects to the quinolones are few, the nature of these effects is unpredictable and affects mostly the central nervous system (CNS). Researchers are presently investigating the pharmacological mechanism of this adverse effect. Simple animal models needed to test CNS side-effects are not currently available. An in vitro assay used to measure inhibition of binding of gamma-aminobutyric acid to mouse synaptic membranes has been established (6). In this assay, NOR, ciprofloxacin (CIP) and ENO have stronger inhibitory activity than their N-methylated derivatives. Based on these data, it is suggested that quinolones having carboxylic and unsubstituted piperazinyl groups might induce convulsions at lower concentrations than their substituted analogs. (d) Bone toxicity. The effect of quinolones on cartilage at the growing ends of bone limits their use to patients over 12 years of age. In vitro models are being studied to better understand the mechanism by which quinolones adversely affect cartilage growth (7). (e) Mode of action of quinolones. There have been reports of the ability of the quinolones to bind to DNA (8). Molecular biologists are continuing to study the mode of action of the quinolones, i.e., their binding to single-stranded DNA, the mechanism of inhibition of DNA gyrase activity and the interaction of quinolones with eukaryotic DNA (9-11). (f) Resistance. There have been reports of resistance development during treatment (12-14). To prevent resistance development the quinolones have been used in combination with beta-lactams and aminoglycosides for treatment of serious infections (14). New quinolones are being tested against resistant organisms in an effort to find significant activity against quinolone resistant bacteria. (g) Antibacterial spec-

trum. NOR, CIP, OFL, pefloxacin (PEF) and ENO have less than optimal activity against streptococci and enterococci, limiting their use in respiratory tract infections (15). Also, these compounds do not have clinically useful activity against anaerobes. New quinolones, such as CI-934, are reported to have improved activity against streptococci (15).

The fluoroquinolones have been reviewed extensively (15-19). This chapter discusses the new compounds reported in 1986 and the significant new findings for those quinolones reported prior to 1986.

Mechanism of Action Considerations - While the quinolones inhibit DNA gyrase activity, they have been shown to bind to single-stranded DNA and not to the the gyrase enzyme (8). They do not, however, bind to double-stranded DNA (9). The quinolones have been shown to bind to single-stranded DNA in a cooperative manner at low drug concentrations (9). The bacterial gyrase enzyme (topoisomerase II) is approximately 100 times more sensitive to inhibition than its eukaryotic equivalent (10). A proposed mechanism for the selective activity of the quinolones against bacterial DNA gyrase without inhibiting mammalian topoisomerase II has been described (20). The fact that greater than 100 mcg/ml of a quinolone is required to inhibit calf thymus 9S DNA polymerase alpha primase complex and the mutation rate was not increased in amber-revertant assays indicates that quinolones do not affect eukaryotic DNA. It has been shown that one excision-repair gene, RAD2, is inducible in yeast by nalidixic acid (NAL) (21). Inhibition of mitogen-induced mononuclear cell proliferation has been related to inhibition of DNA synthesis in these cells (22,23). A report on the effect of NOR on DNA metabolism in *P. aeruginosa* shows that at sub-inhibitory concentrations, DNA inhibition is followed by recovery synthesis. Both the recovery synthesis and the bactericidal effect of NOR are dependent on protein synthesis (24). NOR and NAL do not induce mutagenesis or inducible DNA repair in *P. aeruginosa* (24). The DNA gyrase from quinolone-resistant *Escherichia coli* has been isolated, and its supercoiling activity has been found to be 250-fold more resistant to quinolones than is the activity of the enzyme from the sensitive strain (25). This resistance has been shown to be the result of an altered A subunit using resistant and sensitive A and B subunits combined *in vitro* to reconstitute a holoenzyme.



1 $R=C_2H_5$, $R_1=H$, $R_2=H$, X=CH
2 $R=c\text{-}C_3H_5$, $R_1=H$, $R_2=H$, X=CH
3 $X=R\text{-}COCH_2CH(CH_3)$, $R_1=CH_3$, $R_2=H$
4 $R=C_2H_5$, $R_1=CH_3$, $R_2=H$, X=CH
5 $R=C_2H_5$, $R_1=H$, $R_2=H$, X=N
6 $R=p\text{-}FC_6H_4$, $R_1=CH_3$, $R_2=H$, X=CH
7 $R=p\text{-}FC_6H_4$, $R_1=H$, $R_2=H$, X=CH
9 $R=o,p\text{-}F_2C_6H_3$, $R_1=H$, $R_2=CH_3$, X=CH
10 $R=CH_2CH_2F$, $R_1=CH_3$, $R_2=H$, X=CF
11 $R=C_2H_5$, $R_1=H$, $R_2=CH_3$, X=CF
17 $R=NHCH_3$, $R_1=CH_3$, $R_2=H$, X=CH
22 $R_1=CH_3$, $R_2=H$, X=CH, $R_1=$ 

Norfloxacin (1) - The world-wide clinical experience with NOR has been reviewed (26). The review reports that 1,407 patients were treated for urinary tract infections, 365 patients were treated for gonorrhea and 39 for gastrointestinal infections. This report includes the prophylactic use of NOR against infection in 140 granulocytopenic patients and in 395

travellers to prevent diarrhea. The number of adverse effects is less than 3%, the adverse effects being nausea, headache, dizziness, rash, elevation of liver enzymes and eosinophilia. The successful use of NOR in treatment of traveller's diarrhea has been reported (27). NOR appears to be a promising drug for treating shigellosis, typhoid, salmonellosis, Campylobacter and Yersinia infections (26,28). NOR is active against Campylobacter pyloridis (29). Although NOR is 95% effective against gonorrhea when administered at a 400 mg dose twice a day, it is not effective against Clamydia trachomatis (30). NOR was approved for clinical use in the United States in November, 1986.

Ciprofloxacin (2) - CIP remains the most potent quinolone against Enterobacteriaceae and Pseudomonas. The clinical experience with CIP has been reviewed (31,32). Like NOR, CIP has proven effective in simple and complicated urinary tract infections, gonorrhea and gastrointestinal infections. In addition, it is effective in treating osteomyelitis, skin and soft tissue infections, surgical infections and infections in immunosuppressed patients. It is also effective against P. aeruginosa in cystic fibrosis patients. Mild and transient adverse reactions have been noted in less than 3% of the patients treated. CIP is less effective against Chlamydia than against gonorrhea where it is 100% effective (33,34). CIP has been reported to be less effective against streptococcal and pneumococcal pneumonia than against Haemophilus and Enterobacteriaceae. In a few cases, resistant P. aeruginosa and Serratia marcescens have been reported (31). Resistant organisms have been isolated more frequently when CIP is used in chronic infections. The spectrum of CIP also includes Rickettsia (35,36). CIP is active against C. pyloridis (29). CIP is more potent than OFL, NOR, and amifloxacin (AML) against Mycobacterium tuberculosis and more potent than OFL against Mycobacterium intracellulare (37). CIP is unique among quinolones in being bactericidal for stationary phase bacteria (38). CIP and azlocillin were synergistic against 30% of P. aeruginosa tested in vitro and in a neutropenic mouse model (39).

An i.v. formulation of CIP has been shown to be clinically effective (40). A hypotensive effect has been reported with rapid infusion of all fluoroquinolones tested (41). CIP is less active in urine (42). Its oral absorption is reduced by 20% if taken with food (31). It does not penetrate into the CSF in significant amounts (<10%) and is excreted by the kidneys by glomerular filtration (43). Although CIP is not excreted by the biliary route, as much as 15% of CIP is found in the feces even after i.v. administration, because it is secreted into the gastric mucosa (44). There is no significant accumulation of CIP in patients with normally functioning kidneys. The pharmacokinetics of CIP in patients undergoing chronic ambulatory peritoneal dialysis has been described in which the mean elimination serum half-life was 16.8 hours compared to 3.9 to 6.6 hours described in normal subjects (45). In 12 volunteers aged 71-86, the peak serum concentrations were significantly higher than in volunteers aged 19-25 years. Renal and non-renal clearance was reduced in the elderly patients (46). Therefore, it is suggested that the dosage intervals be increased to every 12 hours in elderly patients. Antacids have been reported to reduce the oral absorption of CIP and the serum level has been reported to be as low as 27% of the level taken without antacids (47,48). Caffeine elimination is increased only slightly (44). Theophylline interaction has been reported in patients with chronic bronchitis (44). In a mouse limb bud test developed to determine the effect of quinolones on cartilage differentiation, CIP was shown to have a severe adverse effect (7). CIP has no adverse effect on the anaerobic

intestinal microflora (49) and has been successfully used to eliminate gram negative aerobic bacteria in immunosuppressed patients (50).

Ofloxacin (3) - OFL is approved for use in Europe and Japan. The clinical results, pharmacokinetics and metabolism have been recently reviewed (51). It is being used successfully to treat urinary tract infections, gonorrhea, skin and soft tissue infections, respiratory tract infections, osteomyelitis and infections in immunocompromised patients. In some reports, OFL has not been effective in treating streptococcal and chlamydial infections (52). In rats, dogs and monkeys, OFL is metabolized by O-acyl glucuronidation, N-demethylation and N-oxidation. The absorption of OFL after oral administration is not significantly affected by food intake; the peak serum concentration is 3.7 mcg/ml when measured without immediate food intake and 3.1 mcg/ml when taken with food. Antacids decrease the oral absorption of OFL by 73%. Caffeine and theophylline elimination is not significantly affected by OFL (53). Dosage adjustment of OFL is required in patients with altered renal function (54). The use of OFL in ophthalmic infections is being evaluated (55).

A short and convenient synthesis of OFL by the use of 2,3,4,5-tetrafluorobenzoic acid has been reported (56,57). Starting from (S)- and (R)- alaninol, both enantiomers have been synthesized, and the more active levorotatory isomer has been found to be the (S)-isomer (56).

Pefloxacin (4) - PEF is approved for use in France and has been recently reviewed (58). It is available for hospital use in both the oral and parenteral forms. The efficacy of PEF in complicated urinary tract infections is reported to be 98%. Adverse effects occurred in 9% of treated patients and were mostly gastrointestinal, neurological and rashes (58). Patients on PEF have also been reported to show photosensitivity. PEF has been used to treat pneumonia. PEF has been found to be effective in an experimental meningitis model (59). The pharmacokinetics of PEF after oral and i.v. administration have been described (60). The mean elimination serum half-life after multiple doses is 13.9 hours after i.v. administration. PEF undergoes enterohepatic circulation and is primarily excreted by the liver. Renal clearance of PEF is low (approximately 8 ml/min). Thirty one percent of the dose is excreted in the urine as N-desmethyl (NOR) and N-oxide metabolites. Cimetidine has been reported to interfere with the formation of the N-oxide metabolite. The CSF/plasma ratio is 60%. PEF does not interfere significantly with theophylline metabolism (61).

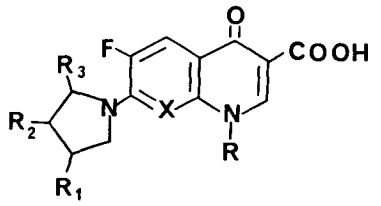
Enoxacin (5) - Recent results of clinical trials with ENO, for a variety of indications, have been published (62,63). It is effective for the treatment of simple as well as complicated urinary tract infections, respiratory tract infections, skin and soft-structure infections and gonorrhea. Seizures and convulsions have been reported in patients administered ENO. As with the other quinolones, ENO is less effective against Chlamydia than gonococcal infection.

ENO interferes with theophylline metabolism and caffeine elimination (61). The pharmacokinetics of i.v. administered ENO have been reported (64). The serum half-life of ENO is 5.1 hours after i.v. administration, compared to 6.2 hours after oral administration. Topical, ophthalmic and i.v. preparations of ENO are being evaluated (65). ENO is the only quinolone studied to date which has bioequivalent oral and i.v. preparations. ENO is expected to be marketed in early 1987 in Europe.

Difloxacin - (A-56619) (6) - Several studies have reported on the in vitro activity and efficacy in experimental animal infections of difloxacin (DFL) (66-70). In general, it is equal to OFL against staphylococci and streptococci and 2- to 4-fold less active against aerobic gram negative bacteria in vitro. DFL is the most active quinolone in vitro against Chlamydia (71) and is more active than ENO in experimental endocarditis (72). DFL is also the most active quinolone against Legionella and is more potent than other quinolones against intracellular bacteria (73,74). DFL has in vivo activity equal to or better than that of OFL and CIP because of its higher serum levels and longer serum half-life. DFL undergoes enterohepatic circulation and is metabolized to the N-demethyl (A-56620) and N-oxide forms. It is excreted in the bile and undergoes enterohepatic circulation. The mean serum half-life of DFL is 26 hours (75). DFL is more active at pH 6.5 then at pH 7.2 in vitro (66).

A-56620 (7) - This compound was found to be poorly absorbed after oral administration in phase I clinical trials.

A-60969 (A-61827 HCl salt) (8) - The in vitro and in vivo activity of A-60969 was reported previously (76). This compound is more active than CIP against anaerobes and streptococci. This compound is being developed in Japan (T-3262)and is in phase II clinical trials (77).



8 R=o,p-$F_2C_6H_3$, R_1=NH_2, R_2=R_3=H, X=N

12 R-C_2H_5, R_1=$CH_2NHC_2H_5$, R_2=R_3=H, X=CF

15 R=c-C_3H_5, R_1=$CH_2NHC_2H_5$, R_2=R_3=H, X=CF

16 R=c-C_3H_5, R_1=NH_2, R_2=R_3=H, X=CCl

18 R=c-C_3H_5, R_1=NH_2, R_2=CH_3 (trans), R_3H, X=N

19 R=c-C_3H_5, R_1=Cl, R_2=CH_2NH_2 (cis), R_3=H, X=N

21 R_1=N=CH-R_4, R_2=R_3=H, X=CH

23 R=o,p-$F_2C_6H_3$, R_1=NH_2(S), R_2=H, R_3=CH_2OH(R), X=CH

A-62254 (A-63004 HCl salt) (9) - A series of 1-difluorophenyl-6-fluoroquinolones has been reported (5). In this series, A-62254, having a 3-methylpiperazinyl group at the 7-position and a 2,4-difluorophenyl group at the 1-position, is one of the most potent analogs (5,78). The in vitro activity of A-62254 against staphylococci is equal to CIP and DFL. Against streptococci, A-62254 is as active as CIP and 2- to 4-fold more active than DFL. Against enterics and Pseudomonas, it is 3- to 4-fold less active than CIP and 2- to 4-fold more potent than DFL. The potency of A-62254 is the same at pH 6.5 and 7.2 in vitro. In mouse protection tests, A-62254 is more active than CIP and as active as DFL against staphylococci, streptococci and Salmonella. It is as active as CIP and DFL against enteric bacteria. It is 2-fold less active than CIP against Pseudomonas. Pharmacokinetic studies in mice show that the area under the serum curve for A-62254 is seven times greater than that of CIP and five times less than that of DFL.

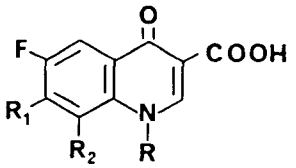
AM-833, Ro-23-6240 (10) - This compound is as active as NOR in vitro and 4- to 8-fold less active than CIP (1). It is as active as CIP against

mycobacteria (79,80). In mouse protection tests, in a granuloma pouch model and in a urinary tract infection model, AM-833 is more active than CIP and OFL, because it has superior pharmacokinetic properties (81). The peak serum level in man is 4.5 mcg/ml, and the mean elimination half-life is 8-12 hours (82). The protein binding of AM-833 is 27% (82). Pharmacokinetics of AM-833 in mice, rats, rabbits, dogs and monkeys have been reported (83). The serum half-life varies from 1.57 hours in rabbits to 9.42 hours in dogs. When administered i.v. to rats approximately half of AM-833 is excreted in the urine (42.9%) and bile (6.7%).

NY-198 (11) - The in vitro activity of this quinolone is similar to NOR (2). In experimental animal infections, NY-198 was 2- to 4-fold more potent than OFL and NOR. The metabolism of NY-198 in man has been described (84). NY-198 undergoes enterohepatic circulation (85). The C_{max} and AUC increase in a dose dependent manner. The steady-state levels are 0.5-2.0 mcg/ml after administration of 200 mg of NY-198 every 12 hours for 7 days. When 300 mg is administered every 8 hours for 7 days, the steady state levels are 2.1-5.5 mcg/ml. No adverse effects were noted with a single oral dose of 100 mg except for one case of stomach discomfort. NY-198 does not penetrate into the CSF and, therefore, may have lower rates of CNS side-effects than other quinolones.

CI-934 (12) - The synthesis of CI-934 has been published (86). This compound is 2- to 8-fold more active than CIP against gram-positive aerobic bacteria (87). Staphylococci and streptococci, except enterococci, are inhibited by <1 mcg/ml of CI-934 (88). No clinical results have been reported.

S-25930 (13) and S-25932 (14) - Quinolone S-25932 is more active against staphylococci and streptococci than S-25930 (89). S-25930 is more active than S-25932 against enteric bacteria and Pseudomonas. Both of the compounds are at least 4-fold more active than CIP against aerobic cocci and are significantly less active (2- to 16-fold) than CIP against gram-negative bacteria. Bacteria resistant to NOR and other fluoroquinolones are cross-resistant to S-25930 and S-25932 (89).



13 R_2R=-$CH_2CH_2CH(CH_3)$-, R_1=CH_3

14 R_2R=-$CH_2CH_2CH(CH_3)$-, R_1= 

24 R=c-C_3H_5, R_1=  , R_2=H

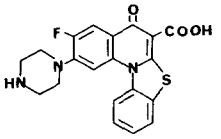
25 R=C_2H_5, R_1=  , R_2=H

PD-117,558 (15) - This compound is 8- to 10-fold more potent than CIP against staphylococci and streptococci including enterococci (90). The MIC_{90}s of PD-117,558 are 0.05 mcg/ml for staphylococci and pneumococci, and 0.1 mcg/ml for Streptococcus pyogenes and alpha hemolytic streptococci. PD-117,558 is 4- to 8-fold less active than CIP against enteric bacteria and Pseudomonas. Superior activity is also seen in vivo in mouse protection tests against staphylococci and streptococci (91). The ED_{50}s are 10 to 20 times less than those of OFL and CIP. In mouse protection tests, PD-117,558 is as active as OFL against gram-negative bacteria and less active than CIP against Pseudomonas (91).

The pharmacokinetics of PD-117,558 in rats and dogs, upon both oral and i.v. administration, has been described (92). In rats, the C_{max} is 1.0 mcg/ml, and the serum half-life is 3.4 hours after oral administration of 6.25 mg/kg. In dogs, the C_{max} is 1.7 mcg/ml, and the serum half-life is 8.4 hours after oral administration of 6.25 mg/kg. Concentration versus time profiles are similar after oral and i.v. administration.

AM-1091 (16) - AM-1091 has excellent gram-positive and gram-negative activity (3). In particular, this compound has improved activity against staphylococci and streptococci, including enterococci, and anerobic bacteria. The MIC_{90}s for all staphylococci and streptococci are 0.1 mcg/ml, and 0.39 mcg/ml for enterococci, whereas the MIC_{90}s of CIP for staphylococci and streptococci are 3.13 mcg/ml and 1.56 mcg/ml for enterococci. Enterobacteriaceae and P. aeruginosa are equally susceptible to AM-1091 and CIP. Bacteroides is 6-fold more susceptible to AM-1091 (MIC_{90} 0.1 mcg/ml) than to CIP (MIC_{90} 6.25 mcg/ml). AM-1091 is at least 10 times as active as CIP against staphylococci and streptococci and twice as active against enteric bacteria and P. aeruginosa in mouse protection tests after oral administration.

Other Quinolones - No significant developments have been reported in 1986 for AML (17), AT-3295 (18) or AT-3765 (19). The properties of AML have been reported (93). A series of novel tricyclic benzothiazolo[3,2-a]-quinolone antibacterial agents has been published (94). Compound 20 is the most potent member of that series and has in vitro activity comparable to NOR. The synthesis and structure-activity relationship of a series of arylfluoronaphthyridines (analogs of A-60969) has been published (95). In general, the naphthyridine analog is found to have better oral absorption than its quinolone counterpart. A series of quinolone imine antibacterial agents (21) has been described (96). Many of these derivatives (R=cyclopropyl, p-fluoro-phenyl and R4=p-fluorophenyl and p-cyanophenyl) have in vitro activity 2- to 8-fold greater than CIP against gram-positive bacteria and similar activity against gram-negative bacteria. These data indicate that a surprising tolerance to bulk is allowable for the 7-substituent.



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A study on chiral DNA gyrase inhibitors has been reported (97). The (-)-(1'S, 2'R) analog (22) is the more potent of the enantiomers. However, the degree of chiral recognition by most bacteria is small. A substantial degree of bulk tolerance is available at the N-1 position. Another chirality study on 7-(2-substituted-4-aminopyrrolidinyl) derivatives has been reported (98). The (2R,4S)-analog (23) is 15- to 20-fold more potent than the (2S,4S)-isomer. The synthesis of a highly potent quinolone E-3846 (24), an analog of irloxacin (IRL) (25), has been described (99). IRL is undergoing phase I clinical evaluation (100). The MICs of IRL for staphylococci range from 0.03 to 1.0 mcg/ml. It is as active as NOR against Enterobacteriaceae. The MIC of IRL is >2 mcg/ml against Pseudomonas. This compound is more active at slightly acidic pH than at neutral pH in vitro.

Summary - We have described the chemistry, microbiology, adverse effects and clinical efficacy of many quinolones which are at various stages of development. Future research will focus on areas that will correct the major deficiencies of quinolones, such as CNS adverse effects, effect on cartilage growth, resistance development and spectrum of activity.

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Chapter 13. Immunotherapy of Infectious Diseases

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Introduction - In the last several years research on the design, synthesis, preclinical and clinical evaluation of novel immunomodulating agents continued unabated. Simultaneously, new information regarding older and well established drugs has also been discussed.

The purpose of the present chapter is to provide a reasonably comprehensive review of the current research on immunomodulating agents and their role in the immunotherapy of infectious diseases, in particular. The ever expanding clinical application of immunosuppressive drugs has led to a significant increase in the number of opportunistic (nosocomial) infectious diseases. The latter, coupled with the spread of acquired immune deficiency syndrome (AIDS) and AIDS-related complex, and a large population of immunocompromised cancer patients, is turning the effective treatment of opportunistic infections caused by both bacteria and fungi, into a major challenge for scientists at all levels of drug research.

In the past year or so, a number of reviews have appeared regarding the immunomodulating activity of various drugs [1-6] including their mode of action [7] and application in veterinary medicine [8]. A novel method for the assay of immunomodulating drugs and its application to culture broths of microorganisms was developed [9] along with a new procedure for screening of immunomodulators by injection of laboratory animals and subsequent isolation of the peritoneal macrophages and evaluation of their 5'-nucleotidase activity [10].

One of the vital functions of the immune system is to provide defense against infectious diseases. Several populations of phagocytic and lymphoid cells, and a number of soluble factors, as part of the immune system, play an important role in providing such defense by either directly participating in the inflammatory response, or by regulating the intensity of the immune response and augmenting the potency of the phagocytic cells. One striking example of such participation is the role of T-lymphocyte cells in preventing opportunistic fungal infections of the skin and mucous membranes such as chronic mucocutaneous candidiasis [11]. The T-lymphocytes produce lymphokines which act locally to elicit inflammation and provide protection against a host of fungi, mycobacteria and viruses. For example, patients with an impaired immune system show a subnormal production of lymphokines by the T-lymphocytes in response to Candida antigens. This results in a major immunoabnormality that is usually treated by a combination of antifungal drugs (clotrimazole, ketoconazole or amphotericin B) and correction of the underlying immune defect by transfer factor [11].

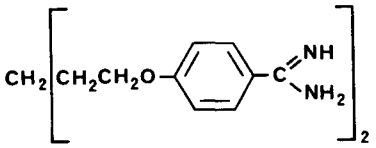
Since the last review of the immunotherapy of infectious diseases [12], a significant number of novel immunomodulating agents were synthesized, and new ways were developed to manipulate the immune response in order to prevent and treat infections. In the present review an attempt will be made to update some of the recent advances regarding

immunomodulating agents. The latter are arranged either according to the nature and increased complexity of their chemical structures, or by a specific therapeutic activity.

Aliphatics - An evaluation of the mechanism involved in the sodium diethyldithiocarbamate-induced immunomodulation was carried out by using its hydrophilic analog, sodium N-methyl-D-glucamine dithiocarbamate [13]. Other studies related to both the immunomodulating activity [14] of sodium diethyldithiocarbamate and a purification procedure that may render the drug suitable for treatment of AIDS, have also appeared [15].

Aromatics - The immunomodulating properties of pentamidine (1), used in the treatment of African trypanosomiasis, were studied [16]. Pentamidine inhibited the ability of mouse splenic lymphocytes (B-cells, in particular) to respond to mitogens.

A long-term low-dose and a short-term high-dose administration of bestatin (2) to normal and immunocompromised rats were used to evaluate its effectiveness in controlling infectious diseases [17]. A 2-stage protocol designed to monitor the immunomodulating properties of the drug during the treatment of a series of clinically relevant and sublethal experimental infections, has been proposed [18].



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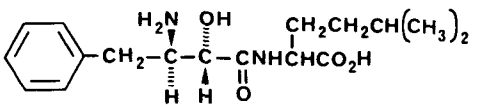

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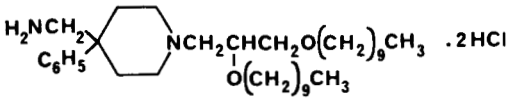
Saponins - When administered orally, a Quillaja saponin significantly potentiated the humoral response of mice given inactivated rabies vaccine and enhanced their resistance to subsequent intracerebral challenge with live rabies virus [19].

Heterocycles - The therapeutic efficacy of compound CP-46,665-1 (3), an immunostimulating synthetic lipoidal amine, was evaluated in Leishmania donovani-infected mice [20]. The drug, which was used in combination with antimony N-methylglutamine, led to a 10-fold decrease in infection compared to untreated mice.

The pharmacological profile of a novel immunomodulator, LS 2616 (4), was studied [21-23]. The drug enhanced the delayed-type hypersensitivity response of rats to Bordetella pertussis [21], as well as natural killer cell activity in mice [23]. Some 3,6-bis(substituted)-acridine derivatives (5) were reported to be effective in reducing the splenomegaly in Rauscher leukemia virus-infected mice [24], and in activating murine tumor-inhibitory macrophages in vitro [25].

The immunoregulatory effects of levamisole (6) were studied [26,27]. The drug was found effective in correcting the secondary immunologic deficit due to poliomyelitis virus [28]. Furthermore, at an oral dose of 30 mg/kg, given for 6 days, levamisole restored the immune response of Mastomys natalensis infected with Brugia malayi [29].



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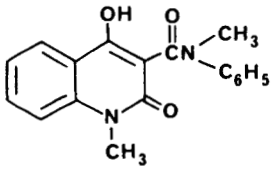

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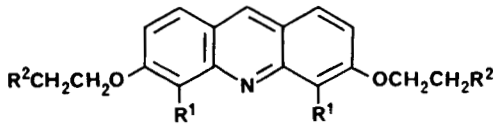

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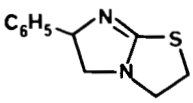

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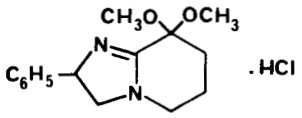
The imidazo[1,2-a]pyridine 7 was reported to be a selective T-cell immunorestorative agent [30,31]. The drug enhanced the T cell-dependent processes in immunosuppressed, but not in immunocompetent mice [31,32]. Furthermore, it consistently reversed the generalized immune depression of peripheral leukocytes obtained from patients with Down's syndrome; however, it did not affect the immune function of cells from healthy volunteers [33]. Compound 7 was also found active against murine hepatitis virus in vivo [34].



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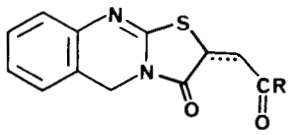

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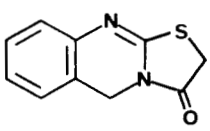

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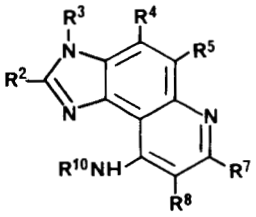

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A number of 2-substituted 2,3-dihydro-5H-thiazolo[2,3-b]quinazoline compounds (8,9) displayed potent immunosuppressive activity in the Kennedy plaque assay [35].

The imidazo[4,5-f]quinolines 10 enhanced the immune response of mice challenged with Pseudomonas aeruginosa [36].

Cimetidine (as well as cyclophosphamide and ibuprofen) increased the resistance of mice against a lethal polymicrobial septic infection after trauma (hind-limb crush injury and amputation) [111].

Antibiotics - Ampicillin was found to elicit in vitro an immunoenhancing effect on the expression of IgM receptors and the angiogenesis-inducing potential of human lymphocytes [37]. In mice, the drug stimulated the production of anti-sheep red blood cell-complement-antibody complexes [37]. The influence of cefoxitin and cefotaxime on interleukin-1 production by mouse peritoneal resident macrophages activated by opsonized zymosan was investigated [38]. The effects on the humoral and cellular immune responses of various cephalosporin antibiotics have been studied [39,40]. Several reviews discussing the immunomodulating properties of antibiotics have also appeared [41-43].

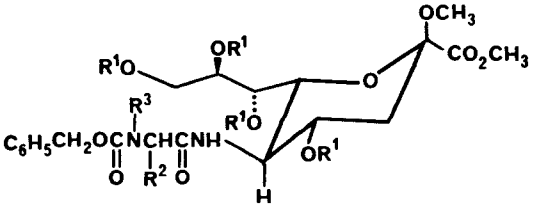
Antimalarials - The effects of quinine, chloroquine, pyrimethamine, mefloquine and quinacrine on the functions of human polymorphonuclear leukocytes (PMN) were examined in in vitro experiments [44]. In general, the antimalarial drugs depressed PMN functions such as iodination and locomotion reactions, hexose monophosphate shunt activity and the PMN adherence which are usually associated with antimicrobial activity of the cell [44].

Cationic Surface-Active Compounds - The immunomodulating activity of dimethyldioctadecylammonium bromide (DDA) [112] in relation to route and time of administration was investigated in mice [45]. DDA (as well as cyclophosphamide) potentiated the delayed-type hypersensitivity response of mice to inactivated enveloped viruses (measles, influenza PR8, and herpes simplex type 1) [46].

Germanium Compounds - At doses of 33-300 mg/kg, carboxyethylgermanium sesquioxide (GE-132) protected mice from the lethal effects of influenza A_2 virus; of the different routes of administration, the oral one provided the best protection [47]. The observed effect appeared to involve a potentiation of the host's immune function [48] rather than a direct virucidal action [47].

Mono- and Polysaccharides - The N-substituted neuraminic acid derivatives 11 were found to be immunosuppressive by activating the suppressor T-lymphocytes and inhibiting Ig production by mouse spleen cells [49].

A number of monosaccharide analogs (12) of lipid A were prepared and found to be immunostimulants [50].



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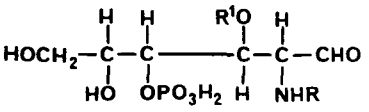

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Water-soluble chitosan oligomers and their N-acetyl derivatives, e.g. hexa-N-acetylchitohexaose, enhanced the immune responses toward bacterial and fungal infections [51]. The immunomodulating activity of a mixture of mannans extracted from C. albicans was investigated [52]. The mixture consisted of immunostimulatory and immunosuppressive components which were separated by molecular size or charge using chromatography.

Acylglycans isolated from Klebsiella showed a remarkable activity in stimulating the secretion of interleukin-1 and colony-stimulating factor [53]. At a concentration of 100 μg/ml, a Bupleurum kinmingense polysaccharide was found effective in augmenting the proliferation of mouse splenocytes incubated with various mitogens [54].

Two polysaccharides, RON [55] and RIN [56] isolated from rice bran, showed potent immunomodulating activity by improving significantly resistance to Listeria monocytogenes and Escherichia coli infections. An acidic fraction of baker's yeast mannan, acting as an immunoenhancer, markedly increased the survival of mice infected with Streptococcus aureus [57].

Milk macrophages obtained from healthy women, when incubated with a lipopolysaccharide, demonstrated an increased ability to phagocytize Candida albicans [58]. The reported priming of the macrophages by the lipopolysaccharide resulted in a greater release of O_2^- [58].

A Salmonella typhimurium lipopolysaccharide when complexed with ribosomes of Brucella abortus or Aspergillus fumigatus led to modulation of the antibody response of mice toward the lipopolysaccharide [59]. This finding may shed some light on the mechanism of action of some ribosomal vaccines - for example, if a lipopolysaccharide is complexed with ribosomes it may be converted to a T cell-dependent form of the antigen to which the mice can respond [59].

The immunomodulating activity of various polysaccharides has been discussed in several reviews [60-62].

Amino Acids and Small Peptides - Pretreatment of mice with forphenicinol [63] (10-1000 μg/day for 5 days, prior to infection) increased the resistance to Pseudomonas aeruginosa-induced septicemia [64]. The most potent effects (reduction of mortality and the number of bacteria in vivo) were observed at a dose of 50 μg and may be attributed to an increased activation of the neutrophils [64].

The immunopotentiating heptanoyl-γ-D-glutamyl-(L)meso-α, ε-diaminopimelyl-(L)-D-alanine (FR41565) (isolated from Streptomyces olivaceogriseus) decreased the incidence of diarrhea caused by Escherichia coli in suckling piglets [65].

Cyclosporin A - Mice treated prophylactically with cyclosporin A had an enhanced resistance to infection with Leishmania major [66]. The mice, which displayed a sustained delayed-type hypersensitivity and were resistant to further challenge with virulent L. major, appeared to have a modulated induction stage of their immune response toward the parasites [66]. A cyclosporin A-induced immunomodulation of chronic hypersensitivity pneumonitis in rabbits was investigated [67]. The drug elicited a transient suppressive effect on the T-cells, especially the helper/inducer and delayed hypersensitivity subsets [67].

Peptides and Proteins - TP-5, a thymopentin analog [68,69], increased the survival rate in an infectious guinea pig model with immunologic deficiencies [68]. The peptide improved the ability of neutrophils and macrophages to phagocytize and kill Pseudomonas aeruginosa. A number of immunomodulating thymopoietin peptides were prepared [70,71] and found resistant to degradation by peptidases [70].

A low molecular-weight macrophage suppressor factor (MSP) (present

in murine spleen cell culture supernatant) decreased in vitro the phagocytosis of Listeria monocytogenes by resident murine peritoneal macrophages [72].

Stress may suppress the immune system and increase the frequency and severity of viral and neoplastic diseases. Although the mechanisms of stress-induced modulation of the immune responses are not clear, it is thought that some neuropeptides may be involved in the process by affecting the role macrophages play in the host defense against infection and neoplasia [73]. Evidence of this was the finding that one neuropeptide, neurotensin, significantly enhanced the cytolytic capabilities of peritoneal macrophages activated by γ-interferon [73].

Purified thymus factor (PTF) was reported to stimulate the delayed-type hypersensitivity in guinea pigs and mice, and to increase the host resistance against infection by Klebsiella pneumoniae [74].

Human peripheral blood lymphocytes responsive to hepatitis B virus antigen (HBsAg) were cloned from the blood of HBsAg-seropositive adults at 30 days post-vaccination with purified HBsAg [75]. These cloned cells were found to be cytotoxic to human hepatocellular carcinoma cells.

The immunomodulating properties of various peptides and proteins have been reviewed [76-80].

Interferons (IFNs) - Human α- and β- (but not γ-) IFNs suppressed in vitro the replication of AIDS viruses (LAV, HTLV-III, and ARV-2) [81]. At the time of peak virus production, IFN-α preparations inhibited the replication (as measured by reverse transcriptase activity) in a concentration-dependent manner [81]. The antiviral and immunomodulating activities of a combination of recombinant murine α- and γ-interferons were examined in mice [82]. The combination protected weanling mice against infection with herpes simplex virus 1 or encephalomyocarditis virus in a synergistic manner.

Rat recombinant γ-IFN augmented the number of peripheral blood leukocytes and spleen cells and increased the phagocytic capacity of the macrophages in rats when injected intravenously [83]. Pretreatment of normal human monocytes with IFN-γ, interleukin-1 or tumor necrosis factor enhanced monocyte cytotoxicity [84]. In vitro incubation of Langerhans cells from AIDS patients with INF-γ resulted in an increase in the HLA-DR and OKT6 antigens [85].

The immunomodulating activity of IFNs has been discussed in several reviews [86-90].

Peptidoglycans - The immunopotentiating activity of various peptidoglycans was reviewed recently [91]. An immunopotentiating mixture consisting of 2 types of peptidoglycans was isolated from Lentinus edodes mycelium (grown on a xylose-rich solid medium) [92]. The peptide portion of the peptidoglycans contained acidic amino acids, while the polysaccharide portion contained arabinose, glucose, galactose, mannose and xylose. The reported peptidoglycans showed low toxicity and were effective in preventing a wide variety of infectious diseases [92].

Peptidoglycans from 2 strains of Listeria monocytogenes were mitogenic to mouse lymphocytes, activated macrophages in vivo (but not in vitro), and stimulated nonspecific immunity in vivo to Candida albicans [93]. They also elevated natural killer cell activity in vivo.

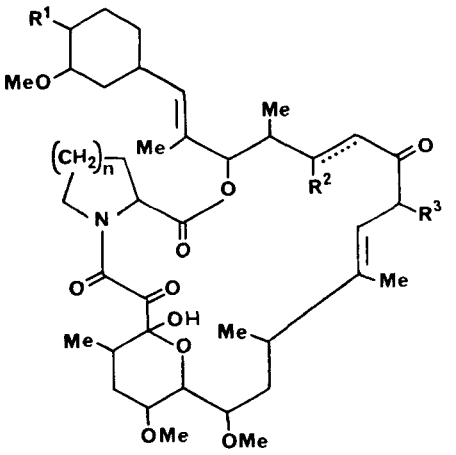
An immunopotentiating glycoprotein, B-EF (bone marrow-enhancing factor), was isolated from murine bone marrow and was determined to have a molecular weight of over 10,000 [94].

Microbial Products - Several studies on the activity of OK-432, a non-specific immunopotentiator isolated from Streptococcus, have appeared [95-100]. The induction by OK-432 of a delayed-type hypersensitivity to M24 streptococcal antigens and its modulation by synthetic adjuvants were investigated in a guinea pig model [101].

Staphylococcal enterotoxin A (SEA), a protein isolated from culture supernatants of Staphylococcus aureus, was found to be a potent T cell mitogen and an inducer of γ-IFN [102]. The protein significantly enhanced in vitro the natural killer cytotoxicity of human peripheral blood monocyte-depleted lymphocytes within 3 h of treatment at 37°C [102]. The immunomodulating properties of staphylococcal enterotoxins on the in vivo immune responses of C57BL/6 mice were examined [103]. Of the 5 serologic types A(SEA), B, C, D, and E(SEE), only SEA and SEE markedly inhibited the antibody response to sheep red blood cells. However, SEA did not affect the antibody response to a thymus-independent antigen, Salmonella flagella, but did affect the T cell-mediated immune response [103].

A number of immunomodulating bacterial products, isolated by lysis of Staphylococcus aureus, Escherichia coli and Klebsiella pneumoniae, were found effective against parasitic, bacterial and viral infections in mice [104]. The products elicited an IFN-inducing activity and stimulated phagocytosis by macrophages [104]. An immunomodulating extract isolated from Pseudomonas aeruginosa protected mice against an experimental Pseudomonas aeruginosa infection when injected intramuscularly [105].

A number of complex polycyclic derivatives (13) were isolated from Streptomyces hygroscopicus yakushimaensis culture broth and mycelial cake [106]. When tested for activity they showed immunosuppressive and anti-microbial properties.



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A number of reviews covering the immunomodulating activity of various products of microbial origin have been published [107-110].

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Chapter 14. Anti-neoplastic Agents

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Introduction - The previous review of antineoplastic agents in these reports covered 1984(1). For continuity, both 1985 and 1986 are summarized in this chapter.

Although the primary thrust of this review is to highlight agents which act directly on tumor cells, other, more subtle areas of cancer research are evolving. The biochemical basis for cell differentiation has been reviewed(2), as well as the potential for chemotherapy with this approach(3). Retinoids as antiproliferative agents and inducers of differentiation(4), and the early clinical experience with hexamethylene bisacetamide(5,6) have been summarized. The biochemical rationale behind metastatic invasiveness is being elucidated(7,8). As knowledge of the process builds, drugs which hold promise as antimetastatic agents are evolving(9).

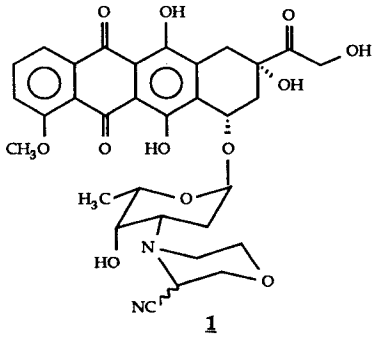
Two proteins which appear to be directly involved with the malignant process have been isolated and characterized. Angiogenin, a 123 amino acid protein which elicits blood vessel proliferation at 10^{-12} to 10^{-15} molar concentrations, has been isolated from human adenocarcinoma cell line HT-29(10). Its amino acid sequence(11) has been determined and its cDNA has been cloned(12). A second protein, p15E, originally isolated from Friend, Moloney and Rauscher leukemia viruses, is also associated with malignant cells(13). It appears to inhibit macrophage accumulation at tumor sites. A synthetic 16 amino acid segment based on a conserved region of p15E exhibits many of the immunosuppressive properties of the native protein.

Two recombinant alpha-interferons have been granted FDA approval for clinical use against hairy cell leukemia(14). Numerous clinical trials for use of other interferons against cancer are in progress. Human trials were reported which used IL-2-activated autologous lymphocytes(15) as well as high-dose IL-2 itself(16). Although objective responses were observed in both trials, the treatment is not without its critics because of its high cost and toxic side-effects(17). Tumor necrosis factor is summarized elsewhere in this volume. As more monoclonal antibodies are developed which recognize tumor-associated antigens, the potential for passive immunotherapy increases(18,19), as does the possibility of active immunotherapy either by vaccination with tumor antigens(20,21) or through anti-ideotype responses.

The ten-year NCI experience with the mouse xenograft model was reviewed, particularly emphasizing the model's usefulness for identifying potential cytotoxic agents which would not have been discovered with the more traditional assays(22). A four-volume set was published describing *in vitro* test systems useful for cancer research(23). DNA topoisomerase II has become established as a molecular target for cancer therapy; the mechanism by which double-strand scission is induced is not completely understood(24,25).

Anthracyclines - An *in vitro* system for correlating potential cardiotoxicity among anthracyclines was developed using neonatal rat cardiac myocyte cultures and measuring leakage of cytoplasmic enzymes in response to drugs(26). A series of anthracyclines was examined for their ability to increase oxygen consumption by rat liver microsomes(27). High oxygen consumption correlated with the appearance of common 7-deoxyaglycone metabolites and the ability, following chemical reduction, for individual compounds to induce formation of superoxide anion or hydroxyl radical. Good agreement was found between the inability of specific anthracyclines to cause increased consumption of oxygen by microsomes and the lack of cardiotoxicity in animals, but not with their level of antitumor activity. Anthracyclines useful in the clinic have been

summarized(28). The accumulated biological testing data on 5-iminodaunorubicin, N,N-dibenzyl-daunorubicin, and 3'-deamino-3'(3-cyano-4-morpholinyl)-doxorubicin (1, MRA-CN) have been reviewed(29). Unlike doxorubicin itself, MRA-CN caused DNA-DNA crosslinks(30), did not exhibit cross-resistance with the parent compound toward certain tumors, and has shown no cardiotoxicity potential. Moreover, MRA-CN is from 100 to 1400 times more potent than doxorubicin, depending upon the tumor test systems(31-33). Several attempts have been made to capitalize on the potency of MRA-CN through additional synthetic modifications(34-38).



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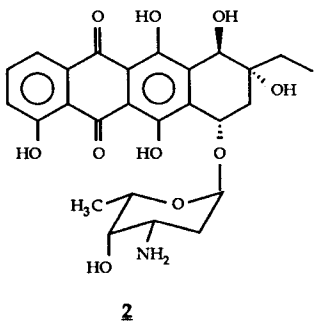
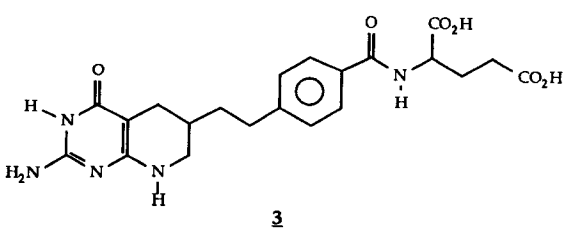


2

The N-benzyl-14-valerate of doxorubicin (AD 198) showed high antitumor activity against P388 tumor systems(39,40). The 2,6-dideoxy-2-fluoro-α-L-talopyranosyl derivatives of daunorubicin and doxorubicin have been synthesized(41). Both analogs exhibited better activity against L1210 than the parent drugs and resisted acid-catalyzed hydrolysis. A novel, highly potent anthracycline, oxaunomycin (2) has been isolated from a mutagenically altered strain of *Streptomyces* sp. D788(42). It has about 100 times the potency of doxorubicin or daunorubicin against L1210 cells.

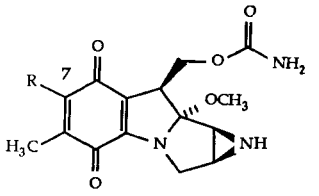
Antifolates - Clinical development of trimetrexate(43), BW-301U (piritrexim)(44,45), CB-3717 (N^{10}-propargyl-5,8-dideazafolic acid)(46,47) and 10-deazaaminopterin(48) as well as clinical use of antimetabolites in general(49) have been summarized. Methotrexate (MTX) and other antifolates were discussed in terms of their mechanism of action at the cellular level(50). A variety of new MTX and aminopterin derivatives were prepared and assessed for their ability to inhibit dihydrofolate reductase (DHFR) or thymidylate synthetase, as substrates for folate polyglutamate synthetase, or for activity against MTX-resistant cell lines. Modifications within the glutamic acid residue included synthesis of alkyl- and aryl-γ-esters(51,52), γ-fluorination(53), replacement by α,ω-diaminoalkanoic acids such as ornithine, 2,4-diaminobutyric, or 2,3-diaminopropionic acids(54-56), increasing the distance between folyl and glutamate residues(57) or replacement of the γ-carboxyl by phosphate(58) or sulfate(59) groups. In general, *in vitro* enzyme inhibition was observed with many of these derivatives, but the analogs did not show superior activity in mice compared to MTX. Numerous replacements or substitutions at one or more of the N^5, N^8 or N^{10} nitrogens have been reported(60-66). One series wherein both N^5 and N^{10} were replaced by carbons has yielded a compound with an apparent mode of action different from other members of the MTX series. The 5,10-dideaza-5,6,7,8-tetrahydrofolic acid 3 was a good substrate for folate polyglutamate synthetase and a good inhibitor of L1210 growth *in vitro*



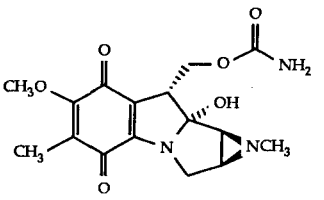
3

(roughly equivalent to MTX), but a poor inhibitor of DHFR or thymidylate synthetase (IC_{50} >10^5 and >10 times greater than MTX, resp.)(67). Only later was a new mode of action suspected, after *in vivo* testing revealed that **3**, unlike MTX, was curative in the 6C3HED lymphosarcoma, B-16 melanoma, C3H mammary adenocarcinoma, Lewis lung carcinoma and X5563 plasma cell myeloma tumor systems, and strongly inhibitory toward M-5 ovarian carcinoma and Madison lung tumors(68). The primary molecular site of action for **3** is believed to be inhibition of glycinamide ribonucleotide transformylase(69), an enzyme which catalyzes the first of two one-carbon transfers in purine biosynthesis(70).

Mitomycins - The clinical use of mitomycin C (MMC) and its derivatives was reviewed(71,72). Stereochemical relationships between MMA, B and C (**4**, **5** and **6**, resp.) were clarified based on a combination of the chemistry and circular dichroism spectrometry of a common degradation product(73), then related to a definitive X-ray structure of MMC reported earlier(74). All three compounds are proposed to arise from a common biosynthetic pathway. A practical two-step synthesis of MMA, a precursor for 7-substituted mitosanes, has been reported using MMC as starting material(75). The first synthesis of leucoaziridinomitosene (**10**), a proposed bioelectrophilic form of a mitomycin, has been published(76). Evidence was offered that a single-electron reduction is sufficient for bioactivation of MMC(77). Using the revised absolute stereochemistry of MMC(74) and computer modeling it was proposed that a non-intercalative initial interaction with the major groove of DNA is followed by covalent crosslinking between C_1 and C_{10} of MMC and O_6 or N_2 atoms of guanine residues(78).



4 R = CH_3O-
6 R = NH_2-
7 R = AcNH-
8 R = $HSCH_2CH_2NH$-
9 R = Me_2NCH=N-



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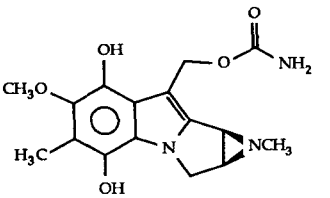


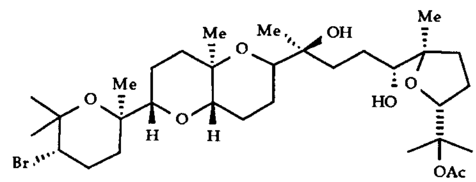
10

In an effort to identify mitomycin derivatives which are more potent, yet overcome tumor resistance to the parent drug, most programs have concentrated on either modifying the aziridinyl nitrogen or functionalizing through the C-7 amine, or both(79). Primary amines added to fermentation media of *S. caespitosus* produce both MMC and B analogs derivatized at C_7(80). N-Acetyl MMC (**7**, BMY-26605) is less myelosuppressive to bone marrow of mice(81). Modifications of the thiol function of **8** (RR-150) have led to improved pro-drug forms of this compound(82,83). Amidine derivatives represented by BMY-25282 (**9**) demonstrated marked superiority to MMC against P388 and B16 tumor models(84) and circumvented MMC resistance in a series of human carcinoma cell lines(85). Conjugation of MMC through its aziridine amino group to high molecular weight dextran *via* ε-aminocaproic acid residues yielded a slow-release form of MMC with increased cytotoxicity(86-88).

Natural Products - Numerous agents exhibiting promising antitumor activity have been isolated from marine or terrestrial sources, but only those with extremely high potency or those offering unique structural features are mentioned here. Assays which have made possible the detection of extremely minute amounts of these potent agents in fermentation broths have been reviewed(89). Additional members of the daphnane(90), bryostatin(91,92) and tricothecene(93) families have been reported. These agents exhibit *in vitro* ED_{50} values against P388 or L1210 leukemias in the ng/ml range; optimal *in vivo* doses are measured in μg/kg. Thyrsiferyl 23-acetate (**11**)(94), and some related compounds with activity against P388 leukemia cells were isolated from the red alga *Laurencia obtusa*. Several members of the halichondrin family, from the sponge *Halichondria okadai* Kadota, have been isolated and their structures determined(95,96). The most active compound in the series, halichondrin B (**12**), exhibited *in vitro* activity against B-16

melanoma cells and produced significant increases in life span *in vivo* against B-16, P388 and L1210 tumors.

The pharmacology of CI-920, a novel phosphate-containing antileukemic agent, has been reviewed(97). Another, somewhat more complex phosphate-containing antitumor metabolite has been isolated from the marine sponge *Discodermia calyx*. The structure of calyculin A (13) was determined from single-crystal X-ray diffraction(98). The structure(99) and relative stereochemistry(100) of sesbanimide A, a highly potent, low molecular weight antitumor agent isolated from *Sesbania drummondii* seeds, has been reported in prior years. The absolute stereochemistry of (+)-sesbanimide A (14) has now been confirmed based on two independent syntheses(101,102).



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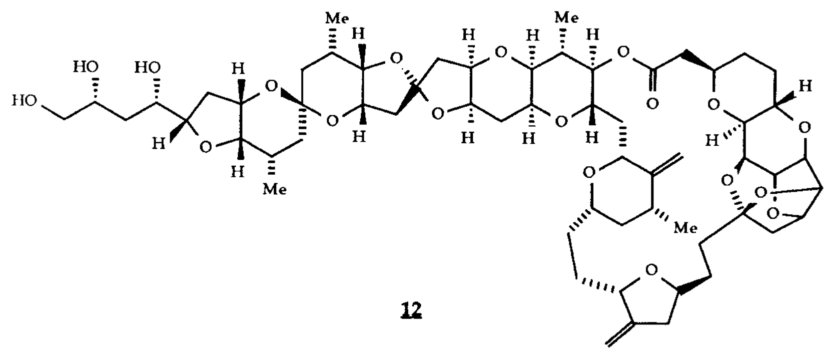

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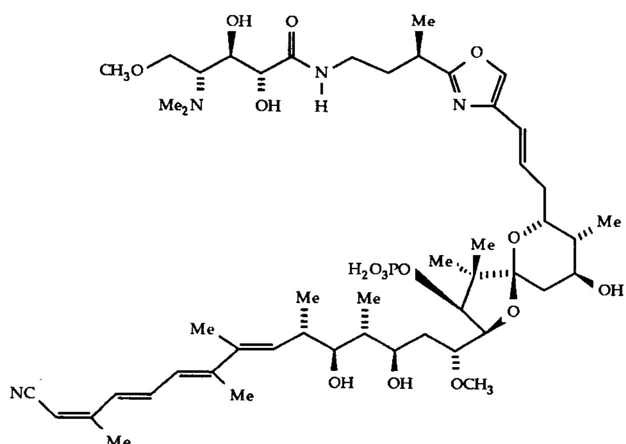

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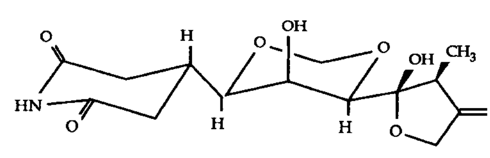
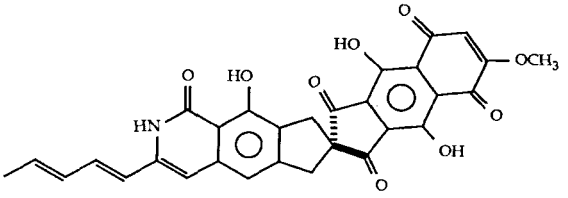


14

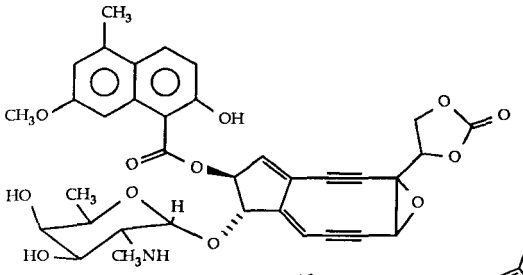
Biosynthetic assembly of fredericamycin A (15) in fermentations of *Streptomyces griseus* involves a polyketide pathway(103). All carbon atoms except for that of the methoxyl group are derived from acetate. A total synthesis of (+)-fredericamycin A has been achieved(104). Evidence has been presented that fredericamycin A exerts it anticancer effect by spontaneously forming an oxidized free radical which transfers an electron to molecular oxygen, generating a radical anion(105).



15

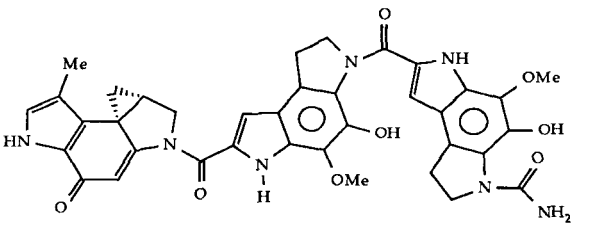
Interesting light-dependent cytotoxicities have been ascribed to ravidomycin, desacetyl-ravidomycin and gilvocarcin V(106). Even at massive concentrations of the agents no cytotoxicity is noted toward cells if cultures are grown in the dark. Maximum DNA damage was observed if cells were treated with the drugs plus light at 400 nm, whereas light of shorter, or especially longer wavelengths caused correspondingly less cytotoxicity.

Although the isolation and antitumor activity of neocarzinostatin were reported in 1965(107), the complete structure was elucidated only recently. The primary structure of the protective coat protein has been revised on the basis of GLC-coupled FAB mass spectrometry(108). It shows considerable homology with macromomycin and actinoxanthin. A gross structure for the neocarzinostatin chromophore has been proposed to contain a unique bicyclo[7,3,0]-dodecadiyne core flanked by α-D-N-methylfucosamine, naphthoic acid and ethylene carbonate moieties (16)(109,110). Neocarzinostatin-induced damage to DNA has been proposed to involve intercalation of the naphthoic acid residue and electrostatic binding of the amino sugar to a phosphate group; reaction of the dodecadiyne core with a thiol and then a molecule of oxygen leads to a DNA strand break(111).



16

The chemistry, mechanism of action, and biological properties of CC-1065 (rachelmycin, 17) were reviewed(112,113). Synthetic programs produced two related analogs, U71184 (18) and U73975 (19), which retain the high antitumor activity of CC-1065 against a diverse panel of tumors, but do not cause the delayed hepatotoxicity in mice observed with the parent(114,115).



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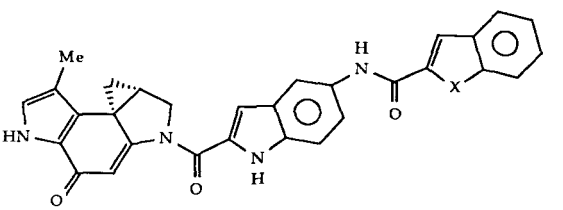
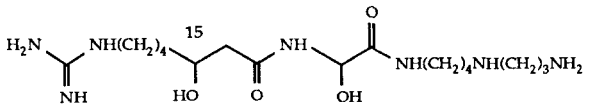


18 X = NH
19 X = O

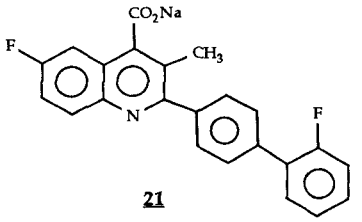
The antitumor activity and acute toxicity of spergualin (**20**) against murine tumor lines were summarized(116,117). Spergualin used in a medium poor in amine oxidase is inactive, suggesting that a metabolite is the active species(118). A more stable analog, 15-deoxyspergualin has been synthesized and is more effective against L1210 leukemia(119). It is also effective as an immunosuppressive agent in murine organ transplantations(120,121).



20

Four groups have isolated complexes of extremely potent, sulfur-containing antitumor antibiotics from fermentations, three from *Actinomadura* cultures, the fourth from *Micromonospora*. PD 114,759, PD 115,028, PD 119,193 and PD 119,707 (verectamycins A, B, C & D, resp.) were isolated from *Actinomadura verrucosospora* (ATCC 39363)(122). PD 114,759, the most potent member of the complex, is active *in vitro* against a panel of tumors (123). Other antibiotics with a similar spectrum of antitumor activities were isolated: *Actinomadura pulverasea* sp. nov. No. 604 produced FR-900405 and FR-900406(124), and a third complex of seven related compounds, named esperamicins, was obtained from *Actinomadura verrucosospora* strain H964-62 (ATCC 39334)(125). All three *Actinomadura* sets appear to have similar physicochemical properties and some may in fact be identical. The fourth set, containing at least 12 distinct, though related compounds, has been isolated from *Micromonospora echinospora* ssp *calichensis* NRRL-15839 and NRRL-15975(126,127). The compounds of this complex (designated LL-E33288) exhibit comparably high antibacterial and antitumor activities as noted above, but differ in structure by the presence of a bromine or iodine atom(128). Moreover, a delayed toxicity was reported for the series, manifesting itself two to four months after treatment(129). Only the structures of some degredation fragments from the four sets have been published thus far(122,124,125,128,130).

Synthetic Agents - Analog synthesis based on NCS 339768 yielded DuP-785 (**21**), an orally active 4-quinolinecarboxylic acid derivative with a broad spectrum of antitumor activities(131,132). DuP-785 exhibited anti-metastatic activity against the murine B16F10 tumor even when treatment was delayed 5 days(133). Antitumor activity among synthetic platelet activating factor (PAF) analogs was noted when the 2-*O*-acetyl group of PAF was substituted by less easily hydrolyzable functionality such as alkoxy. A series of rac-(2-alkoxyalkyl)- and -(2-alkoxy-alkenyl)-phosphocholines was prepared(134), but none offered a sufficiently wide spectrum of activity to warrant clinical development as an antitumor agent. Replacement of the C_1 oxygen by sulfur has yielded derivatives with improved activity against HL-60 promyelocytic leukemias and BG-1 and BG-3 human ovarian carcinoma cell lines compared to their oxygen counterparts(135). PAF analog BM 41,440 (**22**) was inhibitory toward the growth of 19 of 22 human tumor cell lines, acting by both interfering with phospholipid metabolism of cellular membranes(136) and by activating macrophages and NK cells(137).



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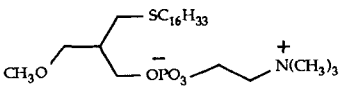
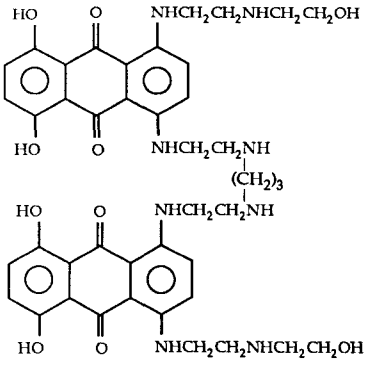


22

The discovery, preclinical development(138,139) and clinical antitumor activity(140) of mitoxantrone have been reviewed. As compared with doxorubicin, the lower cardiotoxicity of mitoxantrone correlates with greatly reduced induction of oxy radicals and lipid peroxidation(141-145). In an avascular rat cornea model the stimulated formation of a new blood vessel supply (angiogenisis) by and to malignant cells was inhibited by mitoxantrone and even more by bisantrene(146). Interactions with DNA by mitoxantrone and related compounds have

been studied by many methods(147-156). Computer modeling and other data indicated that in the strongest of several binding modes the tricyclic nucleus is intercalated in the minor groove of DNA, aligned perpendicularly to GC base pairs(157,158).



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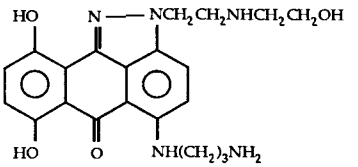

24

The terminal nitrogen atoms of mitoxantrone and analogs have been variously derivatized(159-161); other analogs such as **23** were prepared as potential DNA bis-intercalators(162). Some of these compounds were highly effective against P388 leukemia and B-16 melanoma, but none was as effective as mitoxantrone. A non-phenolic 1-azaanthracenedione was moderately active vs. L1210 leukemia in mice(163). Anthrapyrazoles such as **24** (CI-942) were less potent than mitoxantrone, but gave substantial percentages of "cures" against P388 and L1210 leukemias, B-16 melanoma, the M5076 sarcoma and the MX-1 mammary xenograft in nude mice. They had activity against other solid tumors and showed relatively low cardiotoxicity(164,165). Many analogs of **24** were also moderately to highly active, including compounds in which the carbonyl group was replaced by NH, S, O or Se moieties(166-169). Like doxorubicin, mitoxantrone and bisantrene(147,170), the anthrapyrazoles also bound strongly to DNA and initiated breaks in DNA(171,172). Strengths of DNA binding for various intercalators correlated poorly with antitumor activities in animals(158,170,173).

Difluoromethylornithine hydrochloride (DFMO) as a single agent and in combinations with other antitumor drugs has been reviewed through early phase II trials(174), as has use of ornithine decarboxylase as a target for chemotherapy(175). An irreversible inhibitor of this enzyme, (2R,5R)-6-heptyne-2,5-diamine (MDL 72175), mimics many of the activities of DFMO, but at a 200-fold lower concentration(176). Selenazole, the selenium analog of tiazafurin, is in general 5-17 times more cytotoxic than the parent compound(177). Other analogs were less effective(178,179).

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Chapter 15. Antiviral Agents

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The last few years have seen an enormous increase in antiviral research. The focus of this chapter will be on advances in chemotherapy against DNA and RNA viruses since 1983. Special emphasis will be on retrovirus inhibitors. Several general reviews on antivirals along with a number of a more medical bias have appeared (1-13).

DNA Viruses

The research in this area has concentrated mainly on the herpes family of viruses. Most published work has targetted either herpes simplex virus type 1 (HSV 1) or type 2 (HSV 2). Many nucleoside analogues which are active against HSV 1 or HSV 2 share a common mode of action in that they require activation to the monophosphate by the viral specified thymidine kinase (TK). The corresponding triphosphates, derived from these monophosphates, then inhibit the viral DNA polymerase.

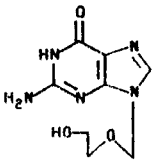
Acyclic Nucleoside Analogues

Acyclovir [ACV, 1] continues to be the only treatment for genital herpes infections. The oral formulation of ACV is effective against both primary and recurrent genital herpes, with minimal side effects, but the rate of recurrence returns to pretreatment frequencies once ACV is discontinued (14-18). Recent data suggests that oral ACV is safe and effective when taken continuously for up to a year (19). Topical ACV, while effective against primary herpes infections, is not efficacious when used for recurrent disease (20-22). This lack of efficacy may be due to the fact that in topical formulations ACV poorly penetrates human skin (23). Intravenous ACV, which is superior to vidarabine for herpes encephalitis (24), is currently being tested clinically for neonatal herpes (18). Resistance has not been an important clinical problem (18).

One of the limitations of oral ACV is low oral absorption (25). Desciclovir (BW A515U), a prodrug which is converted to ACV by xanthine oxidase, is reported to have superior oral absorption (26). This prodrug is probably effective only after conversion to ACV (27).

Ganciclovir [DHPG, 2] has shown good in vivo activity against HSV 1 and HSV 2 (28-33). It is the triphosphate of DHPG which inhibits viral DNA polymerase and subsequently viral DNA synthesis (34,35). While the in vitro activity of DHPG is comparable to ACV, the enhanced in vivo activity is attributed to the persistance of DHPG triphosphate in infected cells (36). DHPG also shows in vitro and in vivo activity against cytomegalovirus (CMV) and holds substantial promise for the therapy of CMV infections (37-43). DHPG triphosphate accumulates in CMV infected cells to a much higher degree than ACV

triphosphate. It is the triphosphate that inhibits the CMV DNA polymerase. In animals the adverse effects of DHPG are radiomimetic; in clinical trials the most serious adverse drug reaction has been neutropenia (42).



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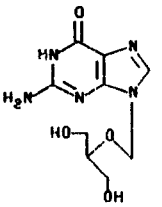

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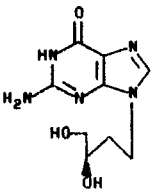


3

Buciclovir [BCV, 3] also exhibits in vitro as well as topical and systemic in vivo antiherpes simplex activity (44). BCV resembles the other acyclic guanosine analogs in its mechanism of action (45). Many butyl guanine analogues have been prepared and show interesting in vivo properties (46,47).

Two general reviews on the chemistry of acyclonucleosides have also recently been published (48,49).

Nucleoside Analogues

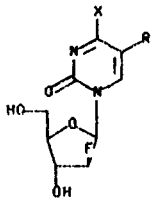
FIAC [4] has good activity against CMV as well as good clinical efficacy against herpes varicella zoster virus (VZV) in immunosuppressed individuals (50,51). FIAU [5] proved efficacious against VZV in monkeys (52). Although the pharmacokinetics of FIAU are good and the acute toxicity acceptable, protracted studies did indicate myelosuppresion and bone marrow depletion (53). Several papers on the SAR of fluoroarabinofuranosyl pyrimidine nucleosides have appeared (54-56).

Advances in the synthesis and antiviral properties of 5-vinylpyrimidine nucleosides have recently been reviewed (57). BVDU [7] was effective in a topical formulation against HSV 1 in animals and as an oral formulation against VZV in children (58,59). The unglycosylated base, 5-bromovinyluracil (BVU), has been reported to protect mice against a lethal disseminated HSV 1 infection (60). The BVU is probably converted to BVDU, or a phosphorylated product thereof, after the action of a nucleoside phosphorylase (61).

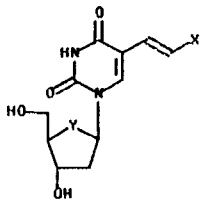
EDU [9] was effective against HSV 1 and HSV 2 only when given topically (62). CEDU [10], an analogue of EDU, is the most potent of the 5-haloalkyl pyrimidine nucleosides reported to date (63-65).

The 2',3'-diacetate appears to be the most promising of the prodrugs of ara-A reported (66). The efficacy of xylotubericidin treatment, both topically and systemically, against HSV 2 infection has also been described (67). The α- and ß-D-xylofuranosyl nucleosides of the

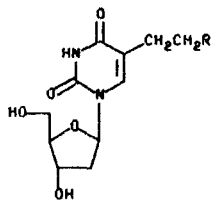
naturally occurring bases have also been prepared; a number of the ß-xylofuranosyl analogues show biological activity (68).



4 X = NH_2, R = I
5 X = OH, R = I
6 X = OH, R = Me



7 Y = O, X = Br
8 Y = CH_2, X = Br



9 R = H
10 R = Cl

Carbocylic Nucleoside Analogues

The neplanocins, which are structurally novel cyclopentene nucleosides isolated in 1981 (69,70), have recently been shown to possess antiviral activity (71). This activity results from inhibition of S-adenosylhomocysteine hydrolase and hence decreased RNA methylation and protein synthesis (72-74). Aristeromycin, the saturated analogue of neplanocin A, exhibits similar biological effects to neplanocin A (75). The 3-deaza analogues of both neplanocin A and aristeromycin have been synthesized and show more potent and selective biological activity (76-78). In vitro, 3-deazaneplanocin A is a highly potent inhibitor of purified S-adenosylhomocysteine hydrolase ($Ki = 5 \times 10^{-11}$) (79). The pyrimidine and acyclic analogues of neplanocin A have also been prepared (80-84). In addition, a number of acyclic aristeromycin derivatives have been synthesized as potential inhibitors of S-adenosylmethionine dependent methyltransferases but show little activity (85,86).

The carbocyclic analogues of 5-bromovinyldeoxyuridine (c-BVDU) and 5-iodovinyldeoxyuridine (c-IVDU) have been prepared (87-89). These compounds, although as selective as the natural nucleosides, are less potent (90). Cyclaridine, the carbocyclic equivalent of ara-A, has the advantage that it is not deaminated in serum, thereby increasing its bioavailability and thus its in vivo potency relative to its in vitro activity (91).

A general review on the synthesis and biological activities of carbocyclic nucleosides has recently been published (92).

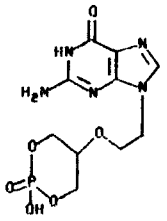
Nucleotide Analogues

Nucleotide analogues are nucleoside analogues with a phosphate group, or equivalent, and thus offer the advantage that they do not require activation by the viral TK. While the nucleotides may show activity against both TK^+ and TK^- strains of herpes virus as well as those viruses which lack a viral specified TK, they lose whatever selectivity the viral specified TK may confer on the original nucleoside.

The mono- and bisphosphate derivatives of DHPG have comparable activity to DHPG against HSV 1, HSV 2 and CMV, probably acting as

prodrugs. The DHPG phosphonate [12] shows activity only against CMV (93). The cyclic phosphate [11] has comparable activity in vitro and in vivo to DHPG against HSV 1, HSV 2 and CMV (94-96). It was also active against TK⁻ strains of HSV 1 not sensitive to DHPG, indicating that it is not a prodrug. An alternative mechanism may be involved in the action of the cyclic phosphate since the levels of triphosphate observed do not account for the antiviral potency of this compound (97). The phosphonate derivatives of ACV have also been prepared (98,99).

(S)-HPMPA [13] is a selective broad spectrum antiviral agent active against a number of DNA viruses by an unknown mechanism (100). (S)-HPMPA shows in vitro activity against HSV 1 and HSV 2 (TK^+ and TK^- strains), VZV and CMV as well as activity against retroviruses. (S)-HPMPA shows good in vivo topical efficacy against HSV 1. By contrast (R)-HPMPA and both the (R)- and (S)-3'-O-phosphonylmethyl derivatives lack antiviral activity.



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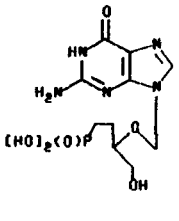

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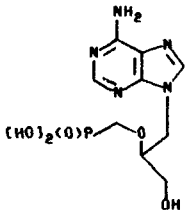


13

Other Antiviral Agents Against DNA Virus

The synergism of DHPG with various interferons against HSV 2 in mice has been reported (101-104), with the effective dose of DHPG being lowered approximately 10 fold. Similar synergistic effects against CMV are observed but over only a narrow range (105). 2-Acetylpyridine thiosemicarbazone (BW A723U) inactivates HSV 1 ribonucleotide reductase, and when used in combination it potentiates the activity of ACV (106). Several other thiosemicarbazones show in vitro activity, again by inhibition of ribonucleotide reductase (107,108).

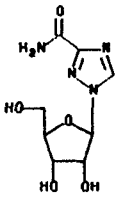
RNA Viruses

RNA viruses are responsible for the major respiratory diseases in humans. These include orthomyxovirus, respiratory syncytial virus (RSV), parainfluenza virus and rhinovirus.

Amantidine remains the only compound prescribed for the prophylaxis and treatment of influenza A in the U.S. (109). Available only as an oral formulation, it is not well absorbed, and has a serum half life of 14 hours. By contrast rimantidine (α-methyl-1-adamantanemethylamine hydrochloride), an experimental compound, is well absorbed with a serum half life approaching 30 hours. Also, rimantidine is more concentrated in respiratory secretions and has fewer adverse CNS side effects than amantidine. Clinical results with rimantidine show that if drug treatment is initiated early it is well tolerated and associated with significant clinical benefits against H_3N_2

type influenza A (110). In cell culture amantidine exerts its antiviral effect in two distinct concentration-dependent actions (111,112).

Ribavirin [14] has broad spectrum activity against a range of RNA and DNA viruses. It is especially active against influenza A, influenza B and RSV (113). Ribavirin is approved for treatment of lower respiratory tract RSV infections in hospitalized children (113,114). When given as a small particle aerosol, it was also effective against uncomplicated influenza A or B infections (115). The mode of action has been studied for influenza viruses and three possible mechanisms suggested (115,116).



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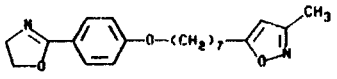


15

The novel cyclononanamine compound, ICI 130,685, has been reported to be more active than amantidine against H_3N_2 in animals (117). When ICI 130,685 was given prophylactically to volunteers for seven days it showed protection relative to placebo with few adverse reactions (118). A kanamycin derivative with a large acyl chain at the N-1 position is active against influenza A in mice with effects comparable to ribavirin and amantidine (119,120).

Picornaviruses, which include rhinoviruses and enteroviruses, are increasingly studied targets for antiviral chemotherapy. Using arildone (WIN 38020) as a lead, several compounds were synthesized and tested against picornaviruses (121). Disoxaril [WIN 51711, 15] has emerged as a potent inhibitor of human entero and rhinoviruses at nontoxic doses (122). Oral administration of WIN 51711 was able to prevent the development of paralysis and death in mice infected intracerebrally with lethal doses of human poliovirus (123). The compound acts by inhibiting early events in the replication cycle (124). The elucidation of the three dimensional structure of human rhinovirus 14 (HRV 14) (125) by X-ray has made it possible to study how WIN 51711 binds to HRV 14 in the crystalline form (126). This dramatic advance offers the potential to begin designing inhibitors at the molecular level.

A number of 3-substituted pyridazine derivatives also show good in vitro activity against rhinovirus infection (127). The receptor protein utilized by the major group of human rhinoviruses for attachment to susceptible cells has been isolated using monoclonal antibodies (128).

Retroviruses

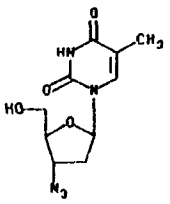
The discovery of human immunodeficiency virus (HIV) as the cause of acquired immunodeficiency syndrome (AIDS) has given research in this area great urgency (129-131). Approaches to AIDS treatment have concentrated on inhibition or interruption of the replicative cycle

of HIV. As HIV is a retrovirus, this replicative cycle involves a reverse transcriptase enzyme which transcribes the viral RNA genome to a proviral DNA in the cytoplasm. Reverse transcriptase (RT) is therefore the target of most chemotherapeutic approaches.

Suramin, a polyanionic acid, first shown to be a potent inhibitor of several animal reverse transcriptases (132) was later shown to impair the in vitro infectivity of HIV (133,134). In clinical trials, no clinical or immunological improvement was observed with suramin. In those cases where HIV replication was reduced to undetectable levels, viral replication was again detected some time after the suramin administration had stopped (135,136).

Retrovir [AZT, 16], a potent inhibitor of the in vitro replication and cytopathic effect of HIV has recently been approved for use by some AIDS or severe ARC patients (137,138). AZT is converted to its triphosphate by cellular enzymes and the triphosphate inhibits RT. The reverse transcriptase is 100 times more sensitive to AZT than the cellular DNA polymerase (136,139). Since it lacks a 3'-OH, AZT has the added advantage of being a chain terminator. AZT is absorbed through the gut and crosses the blood brain barrier (140). In a Phase I trial, AZT was given intravenously for 2 weeks and then orally for 4 weeks. Of the 19 patients studied, 15 had significant increases in their T-helper cells. While AZT appears well tolerated in the short term, its long term side effects are unknown. Studies in mice with murine leukemia virus indicate that chronic doses of AZT significantly prolong life but anaemia is a significant side effect (141). Unpublished clinical data do indeed suggest that this anaemia, along with a number of other adverse side effects are seen in patients. The guanine analogue of AZT, 3'-azido-3'-deoxyguanosine (AZG), also has in vitro activity against HIV (142).

Several dideoxynucleosides have recently been shown to have good in vitro activity against HIV (143). DDC [17] is the most active of the dideoxynucleosides tested, at concentrations of >0.5 μM, DDC completely protected ATH8 cells against HIV. DDC may have good metabolic stability since there is no evidence for either deamination or phosphorolysis of DDC (144). DDDC [18] is also a potent and selective inhibitor of HIV in vitro. The other unsaturated analogues have been prepared and tested (145-147).



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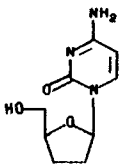

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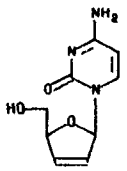

18

A number of other compounds have been reported to have activity against HIV. The tungstoantimoniate (HPA23) has been claimed to inhibit the growth of HIV in patients (148). Interferon (149), dithiocarb (150), AL-721 (151), rifabutine (152), forscarnet (153), D-penicillamine (154), and peptide T (155) have also been claimed to

represent interesting leads against HIV. The use of modified complimentary oligonucleotides, which have already shown potential as antiviral agents against HSV 1, has been applied to HIV infected cells with encouraging results (156,157).

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Chapter 16. Antifungal Agents

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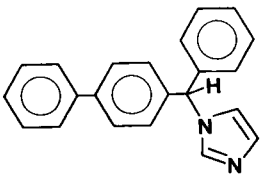
Introduction - Since the last review of antifungal agents in Annual Reports in Medicinal Chemistry(1), fungal infections have assumed increasing importance as a result of increases in organ transplants, cancer chemotherapy and acquired immune deficiency syndrome. Attention has continued to be focussed on azole antifungals and in particular on triazole derivatives. Itraconazole continues to show promise in the oral treatment of superficial and systemic infections(2,3). A recent development is fluconazole(4), a water-soluble triazole derivative, which offers, for the first time, the opportunity for both oral and parenteral therapy. In the field of topical azole antifungals, bifonazole has the advantage of once-daily application(5). Limited studies with liposomal amphotericin B(6,7) continue to suggest this method of formulation maintains efficacy whilst reducing toxicity. However, to date no commercial formulation is available. Terbinafine, an orally active allylamine derivative, has shown clinical efficacy in the treatment of dermatophytoses(8).

The mode of action of antifungal agents has been reviewed by Kerridge(9). Antimycotic sterol biosynthesis inhibitors have also been reviewed(10), as has the mode of action of azoles specifically(11). Azoles exert their antifungal activity principally through the inhibition of fungal cytochrome P-450-dependent lanosterol C14 demethylase and one reason for the shift in emphasis from imidazole to triazole antifungals, is the suggestion that they offer greater selectivity(12).

Following early reports that ketoconazole can inhibit various steps in mammalian steroidogenesis(13,14), several authors have examined this phenomenon in more detail. Further evidence on estradiol : testosterone ratios has been presented, as a possible explanation for the gynecomastia seen with this compound(15). It would appear that imidazole-containing antifungals e.g. miconazole, clotrimazole and ketoconazole, are potent inhibitors of several cytochrome P-450-dependent enzymes involved in mammalian steroid synthesis(16,17). The greater propensity for imidazoles to bind to mammalian cytochromes P-450 results in their influencing the metabolism of other xenobiotics cleared by these enzymes(18,19). This may also manifest itself as the antifungal modulating its own metabolism, e.g. the dose-dependent kinetics of ketoconazole(20). As outlined below, those triazole derivatives studied to date appear to be substantially free of these interactions.

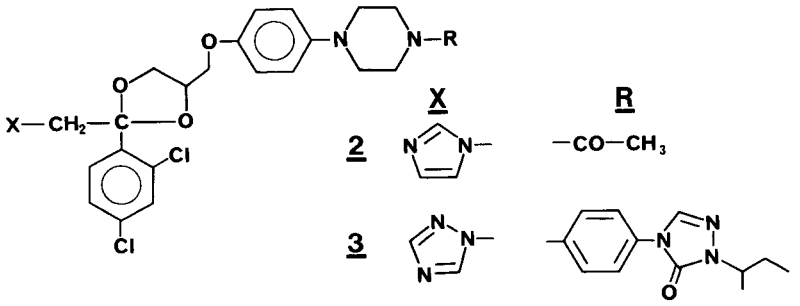
Bifonazole - Bifonazole (Bayer, Bay-h-4502, 1) has been developed as a once a day topical treatment of dermatophytosis and candidosis. The results of 97 clinical trials have been reviewed(5). Treatment for 4 weeks gave cure rates of 81-91% against tinea pedis due to T.rubrum

and T.mentagrophytes(21,22). Good local tolerance and cure rates of 70-100% against dermatophytosis, superficial candidosis and pityriasis versicolor were seen(5). This once-daily administration was reported to be as effective as miconazole given twice daily(23). Against T.versicolor, two week dosing gave 71-94% cure rates (24,25). Bifonazole has also been evaluated against superficial candidosis when a 4-week treatment produced a very good response in 80% of cases(26).



1

Itraconazole -A review of SAR in the imidazole and triazole series from which ketoconazole, 2, and itraconazole, 3, were derived has appeared recently(27). Extensive pre-clinical data have now been published on itraconazole. In vitro it is at least as potent as ketoconazole against a range of pathogenic fungi with notable additions to its spectrum being Aspergillus and Sporothrix(28). This improved activity against Aspergillus translates into better in vivo efficacy in animal models of systemic aspergillosis(29,30). In addition, itraconazole is fungicidal against Aspergillus and it has been suggested that this is responsible for its curative action in such models(31). Itraconazole is also effective in animal models of systemic candidosis(32), coccidioidomycosis(33), cryptococcosis(32,34) and paracoccidioidomycosis(35). Although penetration into the CSF is reported to be very poor in animals(36), enough drug is present to be efficacious in experimental, intracranial cryptococcosis(32,34).



X R

2 —CO—CH3

3

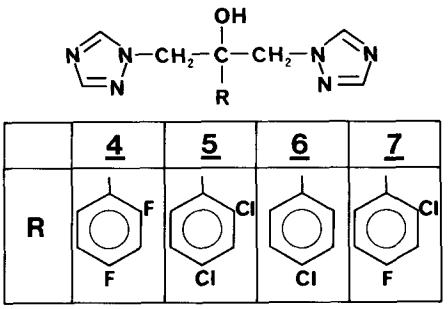
There have been several reports of early clinical results with itraconazole in the oral treatment of superficial mycoses. Efficacy has been demonstrated in dermatophyte infections of various body sites at doses up to 200mg/day for periods up to six weeks(37,38). Shorter courses of therapy are effective in the treatment of pityriasis(38,39). Very short treatment regimes (200mg, once daily for 2 days) are effective in vaginal candidosis(40). However, the teratogenicity of itraconazole in animals requires a strict contraindication in pregnancy(40).

Small, uncontrolled clinical trials of itraconazole, in particular in endemic fungal infections, have provided early indications of efficacy in a number of systemic mycoses, e.g. histoplasmosis, paracoccidioidomycosis(41,42) and sporotrichosis(42). A fuller review of clinical data has been presented, including the first evidence of efficacy in aspergillosis(43). A more recent publication, appears to confirm this early promise, although in few instances was the diagnosis of aspergillosis unequivocal(44).

In all the above clinical trials, the improved toleration of itraconazole over ketoconazole has been stressed. In particular, it would appear that itraconazole does not interfere with steroidogenesis in man(43).

Fluconazole - Fluconazole (UK-49,858, 4), a novel, water-soluble, orally-absorbed triazole derivative, was selected for development from a series of bis-triazole propan-2-ol derivatives(45). Optimal activity against systemic candidosis, dermatophytosis and vaginal candidosis in mice was seen when R was phenyl substituted by one or more halogen groups. The best compounds were 4 - 7 with 4 (fluconazole) being chosen for development on the basis of a long plasma half-life, high urinary recovery and the ready achievement of an i.v. formulation due to its water-solubility.

Fluconazole has been reported to be 10-100 times more active than ketoconazole against superficial and systemic Candida infections in mice, and against trichophytosis in guinea pigs(4). In systemic and intestinal candidosis and in immune-normal and immune-suppressed animals, fluconazole produced a superior curative effect to that of ketoconazole(46). It was also effective in animal models of systemic and intracerebral cryptococcosis(47,48) candida pyelonephritis(48) and pulmonary blastomycosis(49).



	4	5	6	7
R	F, F	Cl, Cl	Cl	Cl, F

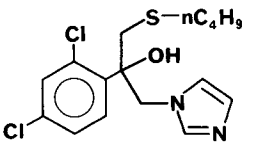
Fluconazole usually gave minimum inhibitory concentrations in excess of 100ug/ml against pathogenic Candida spp on standard mycological media(50) although it was much more active on defined media(51). The use of relative inhibition factors (RIF) indicated that fluconazole's in vitro activity was similar to other azole antifungals(50). Against Aspergillus spp and dermatophytes, the RIF values indicated low potency which contrasts with the efficacy seen in animal models, providing more evidence for the unreliability of in vitro tests on azole antifungals as predictors of in vivo efficacy.

Fluconazole has shown a number of pharmacokinetic properties in animals that distinguish it from other azole antifungals(52). Oral bioavailability was complete in mice, rats and dogs with even tissue distribution, including partition into the CSF(53). It had low protein binding and was stable to metabolism resulting in a long plasma

half-life, with 64-82% being excreted unchanged in the urine(52). Thus it was possible to correlate the *in vitro* activity of fluconazole, on a tissue-culture medium, with its activity *vs* vaginal candidosis in mice by taking into account its low protein binding and pharmacokinetic profile(51).

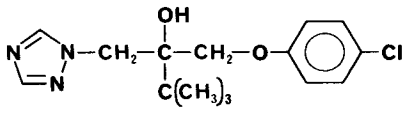
Evaluation in man has shown that fluconazole is 100% absorbed orally, has a long half-life and is excreted unchanged in the urine(54). In vaginal candidosis, 50mg/day for 3 days gave a high cure rate (85%) while fluconazole was also effective *vs* dermatophytosis at a dose of 50mg/day for 28 days. Good efficacy was also seen against candidosis of the esophagus(55), palate(56) and urinary tract(54). No side-effects have been reported with fluconazole which appears to be highly selective for the fungal C-14 demethylase enzyme(12) with no effects on testosterone biosynthesis(57).

Other Broad Spectrum, Orally Active Azoles - Little new information has been published on the orally active imidazole derivative SM4470, **8**. It is reported to be equivalent to ketoconazole in animal models of vaginal candidosis and dermatophytosis, but two fold more active in models of systemic candidosis(58,59). SM4470 was also active in a model of aspergillosis in which ketoconazole was inactive(59). No clinical data have been published to date.

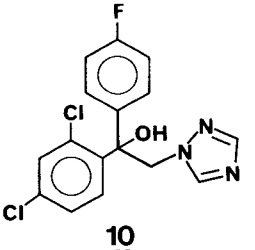


8

The *in vitro* activities of two experimental triazoles, vibunazole, **9**, and ICI-153,066, **10**, have been studied by several authors(58,60) and Fromtling has reviewed preclinical data on the former(61). Animal studies suggest both compounds are active against systemic fungal pathogens including *Aspergillus*(29), *Blastomyces*(62), *Candida*(63) and *Paracoccidioides*(64). In common with the imidazole derivative clotrimazole, vibunazole induces its own metabolism in mouse, rat, rabbit and dog(63,65), although unlike clotrimazole, not in man(65). Apart from a single report(66) of lack of efficacy in aspergillosis, no clinical data have appeared on vibunazole since 1983 and none at all on ICI-153,066.



9

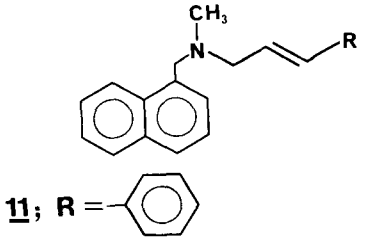


10

Other Antifungal Agents

Liposome encapsulated amphotericin B. There has been continued interest in the clinical development of liposomal formulations of amphotericin B with the aim of reducing toxicity. In addition to conventional amphotericin B/phospholipid liposomes, amphotericin B/sterol combinations have been shown to be effective(67). Liposome encapsulation should reduce the toxicity associated with amphotericin B and recent reports suggest this to be the case(6,7). However, trials have been limited to small numbers of patients who have not responded to other forms of antifungal therapy and results have not been remarkable(6).

Allylamines - SARs around naftifine, **11**, have been reported (68) and follow-up of this class of agent has produced terbinafine (SF 86-327, **12**) which was shown to be a potent inhibitor of squalene epoxidase in enzyme preparations from C.albicans and C.parapsilosis(69,70). The epoxidase from rat liver proved to be 2,000 times less sensitive than the fungal enzyme(69). In vitro studies showed that terbinafine had a broad spectrum of activity including B.dermatitidis, H.capsulatum and S.schenkii(71). The dermatophyte T.mentagrophytes is highly susceptible to terbinafine(70). In contrast, the yeast C.albicans is far less susceptible. The increased susceptibility of T.mentagrophytes was explained as being due to accumulation of squalene by the dermatophyte leading to cell death, while C.albicans is much more tolerant of squalene(70). Examination in guinea pigs(8) against trichophytosis showed terbinafine to be almost 100 times more potent orally than the earlier allylamine, naftifine. Progression into clinical trials showed that a 250mg oral dose twice daily for 4 weeks cured 27/33 patients with dermatophytosis. This cure rate was confirmed in a later study which also reported that terbinafine produced cures more rapidly than did griseofulvin and was also effective when administered topically as a 1% cream(72). Metabolic studies with naftifine, **11**, in man, rat, dog, rabbit and guinea pig have been reported(73).



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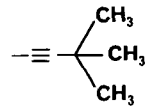
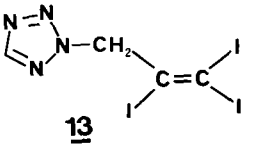
12; R = 



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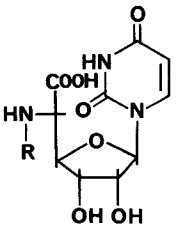
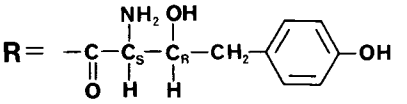
Others. The tetrazole derivative ME 1401 [**13**, previously known as CN-146 (1)] has been compared with other topical agents, including clotrimazole, tolnaftate and ciclopirox olamine, against a broad range of fungi(74). In general, ME 1401 showed comparable activity to clotrimazole and there was no cross resistance with any of the other agents. A novel analog, **14**, of nikkomycin was synthesised but,

despite high activity in the chitin synthase assay, it was inactive against C.albicans(75). The nucleoside chryscandin, **15**, has been reported to be active vs Candida spp but not against filamentous fungi(76,77). A study was published on a series of chryscandin analogs modified at the 3′-amino function with **16** being 16-fold more potent in vitro than chryscandin and superior in vivo to ketoconazole against a C.albicans infection(78). Phosphazomycin A was active against a broad spectrum of fungi but relatively weak vs Candida spp(79) while lucknomycin, a heptaene of uncertain structure, was evaluated in vitro against 403 isolates of C.albicans and shown to have similar potency to amphotericin B(80).





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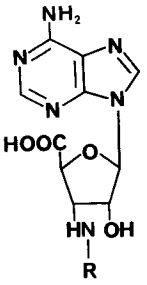
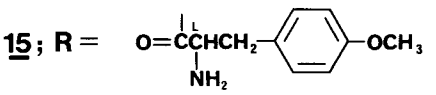
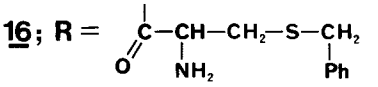
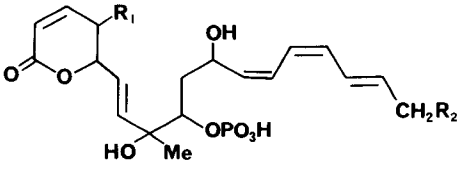


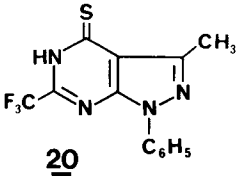




Fostriecin, **17**, and close analogs PD 113,270, **18**, and PD 113,271, **19**, which had previously been shown to possess antitumor activity, showed some activity against yeasts(81). Although poor vs Candida, they were active against the majority of Saccharomyces spp examined. A new pyrazalopyrimidine, **20**, proved to be active against both yeast and mycelial forms of C.albicans(82). Intravenously administered tuftsin, a naturally occurring immunomodulating peptide, was examined in prophylaxis of C.albicans infections in mice(83). Prolongation of survival was seen.



	R_1	R_2
17	H	OH
18	H	H
19	OH	OH



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Conclusion - Fungal infections in man remain an important cause of morbidity and mortality. Topical treatment of superficial infections is adequate in most cases. However, safe, orally active agents for use in more problematic diseases, e.g. extensive dermatophytosis or recurrent fungal vaginitis, are still needed. Despite the advances made in recent years, the number of drugs with proven efficacy against life-threatening systemic mycoses is small. Thus, the ideal agent, with potent, broad-spectrum activity and lacking side effects, has yet to be developed.

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Section IV: Endocrinology, Immunology and Metabolic Disorders

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Chapter 17 - Medicinal Chemistry Opportunities in Bone Metabolism and Osteoporosis

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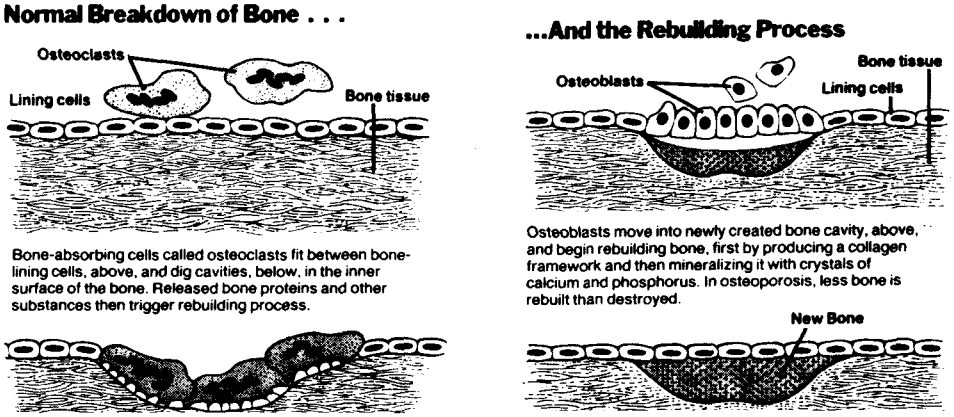
Introduction - Bone is a dynamic tissue which continuously undergoes remodelling (1,2). Approximately 10% of cortical bone (the dense, highly mineralized bone which comprises the shafts of long bones) and 20 to 40 % of the trabecular bone (cancellous bone found in the interior of vertebrae and in the medullary space of long bones) is remodelled each year in the healthy adult. In this process multinuclear osteoclasts resorb bone, releasing mineral and matrix peptide constituents. This resorption is linked to a rebuilding process in which osteoblasts replace eroded bone tissue (see below).



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Osteoporosis (OP) is a debilitating progressive loss of bone mineral which ultimately manifests itself in elevated fracture rates, generally among those over age 45 (3). OP is most prevalent among postmenopausal women, but affects both sexes. Current estimates suggest that 15 to 20 million persons in the U. S. are afflicted. More patients will be identified as the life span increases. Traditionally, modulation of the endocrine system has been the preferred approach to the management of OP (4-7). This report will provide an overview of topics in preclinical bone metabolism research as well as significant new developments in clinical management of OP.

Clinical Developments - The 1983 NIH consensus conference on OP has served to focus national attention on the clinical management of OP (8). This conference recommended the use of postmenopausal estrogen

replacement therapy, which has been shown to delay the onset and progression of OP (9-11). Coupled with adequate dietary calcium and exercise, this regimen represents the first line of defense against bone demineralization. However, replenishment of lost bone is not achieved by this strategy. Prophylactic benefit has also been derived from the use of progestagens such as norethisterone alone and in combination with estrogens (12-16).

Parathyroid hormone (PTH) acutely stimulates bone resorption in vitro, as well as activating bone remodelling in vivo (7). A therapeutic regimen consisting of PTH followed by 1,25-dihydroxyvitamin D3 (1,25D3; 1) treatment shows promise as a means to increase bone mineral (17). Progress in the area of PTH antagonists is likely to yield insight into this approach (18). Vitamin D metabolites such as 1 may play a direct, pivotal role in the development of OP (19,20). These metabolites regulate the function of differentiated human osteoblasts, monocytes and lymphocytes, serving to mediate bone metabolism at several levels (21,22). Calcitonin (CT), known to decrease the bone resorption process and have direct inhibitory effects on osteoclasts in vitro (23), has shown efficacy in one and two year trials (24,25). In addition to direct effects on bone resorption, CT may serve to increase bone formation by altering vitamin D3 metabolism (26). Significant drawbacks of CT therapy are the requirement for parenteral administration and potential antigenicity of the peptide; however, salmon CT has been successfully delivered intranasally. Human CT is available (27,28). CT levels are unaffected by estrogen therapy and it is thought that postmenopausal OP is not mediated by a primary CT deficiency (29,30).

In a two-year study, dietary calcium supplementation alone was insufficient to guard against the development of postmenopausal OP (31). Adequacy of calcium intake can, however, serve to return an individual to positive calcium balance (32). The impact of calcium deficiency on patient response to therapy is an important concern in the design of clinical studies (33). Calcium supplements have, however, been shown to increase the calcium balance of adolescent girls over a two week span, suggesting that adolescence is a critical period for skeletal calcium accretion (34). Nevertheless, the notion that increasing skeletal mass prior to menopause will protect against osteoporosis has been called into question (35).

Family history and skeletal attributes are imprecise, but are often as predictive as more sophisticated techniques in providing accurate OP risk assessment (36). While determinations of bone mineral content in longitudinal studies are useful, the information may come too late to benefit the individual patient. Assays (e.g., serum osteocalcin measurements) have appeared, only to be deemed more appropriate as indicators of the status of bone turnover (37). Several assays currently receiving attention are a luteinizing hormone releasing hormone (LHRH) induced menopause, alkaline phosphatase isozyme measurements, serum acid phosphatase activity, serum vitamin K levels, and T lymphocyte subset analyses (38-42).

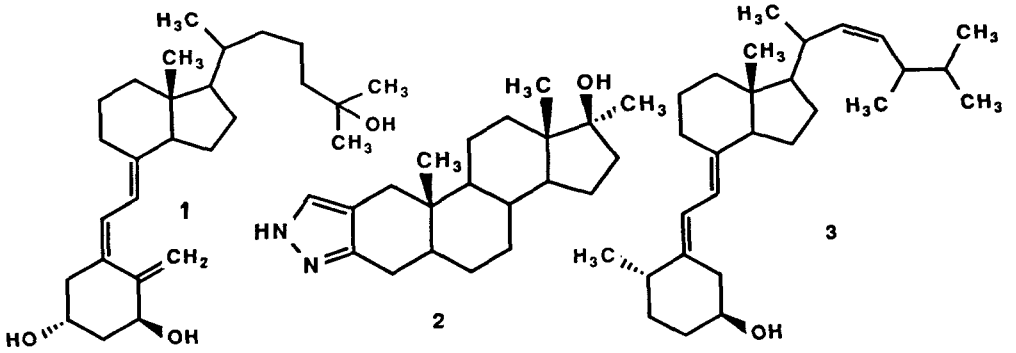
Preclinical Research - Understanding the mechanism by which mineral homeostasis and mechanical function are maintained is critical to the development of improved therapy for OP (43). The origin, development and differentiation of bone cells is an intensely investigated topic (44,45). It has been proposed that osteoblasts are key regulatory cells in bone. Observations concerning hormone effects support this notion

(46-48). Cytokines produced by macrophages or cells of the immune system may provide additional levels of control on bone remodeling (49-56). As more is learned about the development and differentiation of osteoclasts and the role of calcitropic hormones on bone cell activity, additional approaches to the modulation of mineral homeostasis will be realized. In this regard, bone matrix itself is a rich source of these activities and many peptide factors isolated from bone have been reported to modulate bone metabolism (57-59).

Animal Models - While there are many accepted *in vitro* assays for bone resorption and formation, preclinical *in vivo* models of bone metabolism are still being established (60,61). Studies in non-human primates indicate that ovariectomy produces a net decrease in both iliac and vertebral trabecular bone (baboons and cynomolgus monkeys, respectively) (62,63). Canines subjected to ovariectomy showed a lower bone mineral content in the iliac crest than controls, with the deficit ascribed to depressed bone formation, a result of decreased osteoblast activity (64). A limitation of these models is the time (months) required for the development of OP; thus, a significant challenge to investigators in the field is to develop shorter-term models of OP. Histologic changes in rodents induced by tenotomy or denervation occur within days, whereas ovariectomy induced bone loss occurs over a span of weeks (65-67). Such models have the potential to bridge the gap between *in vitro* and extended *in vivo* studies in larger species. Implants of bone matrix can be utilized to study endochondral ossification (68,69). Loss of bone mineral is accelerated in some strains of mice (without surgical or metabolic challenge), which may provide a model for senile OP (70). Correlation of these models with regard to human disease represents a major challenge.

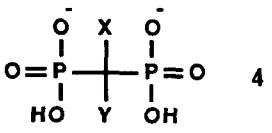
Pharmacologic effectors of bone metabolism - A large number of structurally diverse compounds have been reported to mediate bone metabolism. It is important to note the dose level used in all of the cases cited below because the level required to elicit a response is often well above or well below those required to elicit other types of pharmacologic activity.

Endocrine Agents - Endocrine mediators of bone metabolism are generally sex or calcitropic hormones (71). However, recent reports of bone sparing effects of corticosteroids and the modest to very low risk of OP in patients on low dose prednisolone for rheumatoid arthritis are encouraging (72-74). In the case of anabolic steroids the results are mixed. Dehydroepiandrosterone failed to ameliorate ovariectomy induced OP in rodents (75) yet stanazolol (2) caused a net increase in total body calcium in a 29 month study in postmenopausal OP women (76). Bone



formation rates were marginally increased in the stanazolol treated group relative to baseline measurements, but both test and control groups were treated with calcium, and showed a slight increase relative to baseline. Dihydrotachysterol (3), an analog of 1, improved the ability of marrow stromal cells from ovariectomized rats to proliferate in vitro, as well as increasing osteogenic parameters in implant grafts (77). In the same model system, estrogen increased the in vitro colony forming ability of marrow stromal cells, but did not increase the in vivo osteogenic properties of these cells (78).

Bisphosphonates - The bisphosphonates (4), carbon analogs of pyrophosphate, have been extensively explored (79,80). They generally inhibit both the crystallization and dissolution of calcium phosphate in vitro.



4

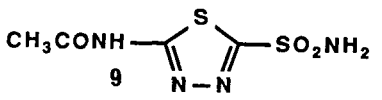
	X	Y
5	OH	CH_3
6	Cl	Cl
7	F	F
8	OH	$(CH_2)_2NH_2$

Etidronate (5) is the strongest inhibitor studied. When the substituents are halogens (6,7) one sees strong inhibition of bone resorption. 7 inhibits bone resorption and remodeling without inhibiting mineralization (81, 82). Bisphosphonates are reported to inhibit macrophage migration (83). Studies on the ability of 6 and 8 to inhibit the formation of resorption lacunae and PTH induced secretion of lysosomal hydrolase suggest the site of action to be the osteoclast as opposed to the osteoblast (84). 8 is also useful in treating juvenile OP, a disease in which the primary defect is unattenuated activity of metaphyseal osteoclasts (85).

Fluoride - A useful therapeutic agent would increase bone mass as well as decrease the fracture rate. Fluoride may represent such an approach to the clinical management of OP. In the spayed beagle dog, histologic evaluation of bone showed a number of fluoride effects (86). These include activation of bone turnover, and alterations of the differentiation and activity of both osteoblasts and osteoclasts. Increased osteoblastic activity in vitro as well as bone formation in vivo are results of fluoride exposure. However, fluoride dose and the availability of mineral constituents must be properly maintained to avoid the development of an osteosclerotic skeleton (87). In one study, only those patients developing skeletal fluorosis showed a decrease in vertebral fracture rate (88). Fluoride may alter trace mineral metabolism (Zn, Mn) within bone, effects that may have long term consequences (89,90). Some untoward side effects of sodium fluoride therapy may be alleviated by the administration of monofluorophosphate (MFP), which has an activity profile similar to that of sodium fluoride in vitro. Workers have demonstrated the ability of alkaline phosphatase in chick skeletal tissue to hydrolyze MFP, thus providing free fluoride at skeletal sites (91).

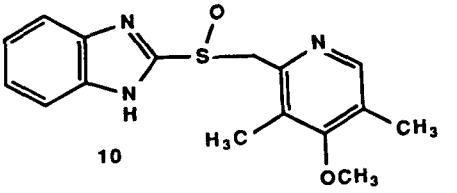
Enzyme Inhibition - The involvement of the enzyme carbonic anhydrase [EC4.2.1.1] in the bone resorption process has been extended by observations suggesting that limb disuse atrophy may be alleviated by administration of carbonic anhydrase inhibitors (92). An insufficiency in carbonic anhydrase was identified as the primary defect in osteopetrosis, a disease characterized by insufficient bone resorption (93). Carbonic anhydrase has been detected in the osteoclast in physiologically important quantities (94). The enzyme is associated with the active resorbing zone of the osteoclast after exposure to PTH, and

becomes dispersed throughout the cell after exposure to CT (95). An increase in the acidity of osteoclasts is stimulated by PTH, a process partially inhibited by carbonic anhydrase inhibitors (96), such as acetazolamide (9). 9 inhibits both prostaglandin E2 and 1,25D3 stimulated bone resorption (97,98). It is noteworthy that improvement of carbonic anhydrase inhibition is not necessarily well correlated with a similar improvement in bone resorption inhibition (99). Thiazide diuretics, which are recognized for calcium retention properties, are also carbonic anhydrase inhibitors; these compounds have received some attention as potential antiosteoporotics (100,103). Other complications of thiazide therapy may arise, however, such as reduced intestinal calcium absorption (104,105).



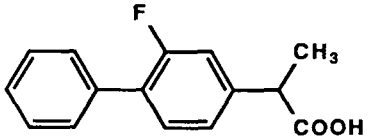
9

Examination of the mechanism by which osteoclasts generate an acidic environment has demonstrated the presence of an ATPase in the membrane of these cells (106,107). This enzymatic activity appears to be functionally and temporally associated with the resorption process (108). Omeprazole (10), a pharmacologic inhibitor of K+/H+ ATPase known to inhibit gastric acid secretion, inhibits bone resorption in vitro (109).



10

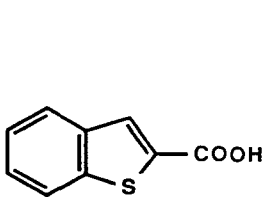
Collagenase is synthesized and secreted as procollagenase by osteoblasts in response to resorption stimulators. Collagenase inhibitors are synthesized and secreted as well (110,111). In an elegant series of experiments, the ability of isolated osteoclasts to degrade bone substrate was markedly enhanced by pre-incubation of bones with calvarial cells or with exogenous collagenase (112). Calvarial cells alone are able to degrade osteoid, but not mineralized tissue. Taken together, these observations support the notion that osteoblastic cells may physically predispose bone mineral to further digestion by osteoclasts (see also the accompanying chapter on osteoarthritis).



11

Prostaglandin E2 is a stimulator of bone resorption and thus may contribute to bone loss, particularly when associated with inflammation (e.g., in periodontal disease; 113,114). Flurbiprofen (11), has been shown to be a potent inhibitor of alveolar bone loss in beagles (115). Clinical studies are in progress.

Thiophene carboxylic acids - Thiophene-2-carboxylic acid (TCA) was originally described as a hypoglycemic antilipolytic agent with hypocalcemic and hypophosphatemic activity (116). Subsequent studies showed that TCA inhibited resorption of neonatal mouse calvaria in vitro (117). BL5583 (12) and BL5593 (13) have been shown to be active against heparin-accelerated OP (118). Recent work has extended this class to include aza analogs, indole-2-carboxylic acid and carbazole-1-carboxylic acid (14), as inhibitors of PTH stimulated bone resorption. The most potent compound studied (12) has been shown to transiently inhibit cellular Ca++ uptake (119), yet not alter intracellular cAMP (120). Interestingly, in a separate study, the Ca++ channel blocker diltiazem (15) was shown to be ineffective as an inhibitor of unstimulated bone resorption, yet was effective as an inhibitor of 1,25D3 stimulated bone resorption (as judged by ^{45}Ca and hydroxyproline release; 121).



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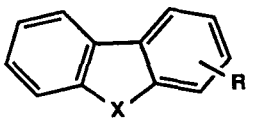
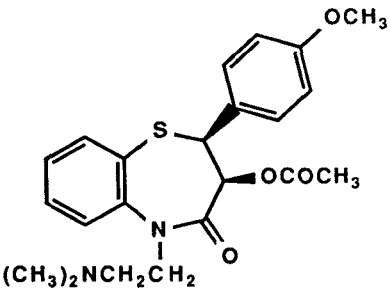
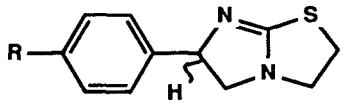


	X	R
13	S	2-COOH
14	NH	1-COOH



15

Lysosomal Enzyme Release Inhibitors (LERI) - LERI (chloroquine, hydroxystilbamidine, dapsone, and levamisole (L-16) have been demonstrated to inhibit bone resorption in vitro (122). Unlike its anthelmintic activity, levamisole's ability to inhibit resorption is not stereospecific (123). Dexamisole (D-16) and bromotetramisole (17) have similar activity. Levamisole and its congeners inhibit lactate production and alkaline phosphatase (124,125). Both enantiomers of (17) are approximately 10 fold more active than levamisole as inhibitors of ^{45}Ca release from cultured explants. These compounds may act directly on osteoclasts to inhibit resorption, rather than by a receptor mediated event. Phenothiazine derivatives have also been shown to inhibit alkaline phosphatase in osteoblastic cells (126). For example, promethazine (18) not only inhibits resorption (127) but leads to retention of bone mass in aging mice (128). Promethazine effects have been attributed to direct inhibition of macrophage activity (e.g., prostaglandin release and phagocytosis).

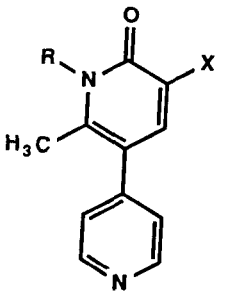


16 R = H
17 R = Br

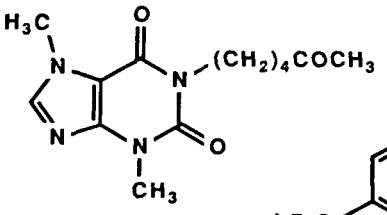
18

Incidental Reports - The compounds reported here have been the subject of single papers on activity in bone metabolism. For the most part, the mode of action is not well understood.

cAMP phosphodiesterase has been identified in bone tissue (129). Several reports have appeared on the effect of PDE inhibitors on bone metabolic processes. The cardiotonic agent milrinone (19) has been demonstrated to elicit an increase in bone resorption in vitro (130), in contrast to amrinone (20), an inhibitor of resorption. Pentoxifylline (21), a methyl xanthine derivative, increased Ca++ uptake and cAMP production in rodent osteoblast-like cells (132). The levels required for Ca++ uptake were well below those needed to increase cAMP production (133). This was construed to suggest a mechanism not dependent on PDE inhibition. BL3459 (22) and BL4160 (23), were reported to inhibit heparin induced OP in mice (134). Ipriflavone (24) is reported to augment the ability of estrogen to elicit CT secretion in the response to a serum calcium challenge (135). In rats with glucocorticoid induced OP, 24 suppressed resorption in the metaphyses and possibly inhibited bone loss in the diaphyses (136). Gallium nitrate has shown utility in the amelioration of hypercalcemia associated with malignancy (137,138).



	X	R
19	CN	CH_3
20	NH_2	H



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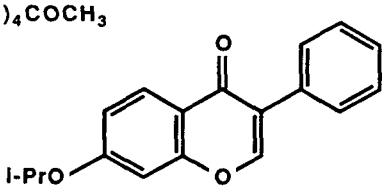

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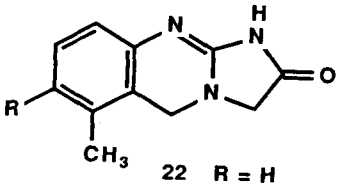

22 R = H
23 R = Cl

Summary - In the 5 years since the last review of bone metabolism appeared in this series, our knowledge of basic bone metabolism has greatly increased (4). Practical in vitro and in vivo models are now available to serve as a basis for the study of synthetic mediators of bone metabolism. Thus, the development of novel therapeutic approaches to the clinical management of disease represents a significant opportunity in medicinal chemistry.

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Chapter 18. Osteoarthritis as a Target for Drug Intervention

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Introduction

Definition, Epidemiology, and Symptoms of OA - Osteoarthritis (OA) is a group of disorders that share a common end stage of mechanical and biological joint failure. The disease is characterized structurally by deterioration of articular cartilage and formation of new bone at articular surfaces (1). OA is the most frequent of all mammalian joint diseases and produces significant morbidity. The disease is highly age-dependent, and certain types of OA show an hereditary disposition (2).

Osteoarthritis may be limited to one joint, although often several joints are involved and sometimes the disorder is generalized (1). Pain is the cardinal symptom of OA, initially being associated with motion but eventually persisting even at rest (2). Other symptoms include localized stiffness, tenderness, crepitus and mild joint enlargement (2). Cartilage changes include surface disruption and ulceration (3). Gross bone deformity is associated with advanced disease and synovial changes occur (4).

Treatment - Current chemotherapy of OA centers on relief of pain (5). The chronic use of steroidal and non-steroidal anti-inflammatory agents in this context continues to cause concern over the long-term effect of some of these agents on joint tissues (6-7). As the factors underpinning the deterioration of joint structures become clearer, the opportunities to modify rationally the disease process with drugs will increase. There is evidence that the pathological processes of OA are reversible (8). In the early stages of the disease, there are biochemical signs of a repair response by chondrocytes that might be further stimulated by drugs. Thus, the scene is set for the search for an anti-OA drug; there are disease models with clear signs that some aspects of the degenerative processes are subject to drug modification. In addition, rapidly improving clinical diagnostic and monitoring techniques and some biochemical hypotheses exist.

Biochemical Changes of Cartilage in OA - In normal adult cartilage, chondrocytes do not divide. They are embedded in an extracellular matrix of proteoglycan (PG) and collagen. The chondrocytes synthesize both of these macromolecules, as well as the enzymes that degrade them. In OA cartilage, chondrocytes proliferate in clones near ulcers and fissures and disappear in other areas (1,9). Changes also occur in the quantity and quality of both PG and collagen. PG content is decreased early in the disease, suggesting an increased rate of degradation as the rates of PG and collagen synthesis are elevated (10). The loss in PG starts in the superficial layers of cartilage (11). Collagen content decreases only late in the disease process. The rates of PG and collagen synthesis eventually decrease as the disease progresses

(10). Both the changes in PG content and PG synthesis correlate with the histological progression of the disease (11). The increase in PG synthesis results in a focal increase in PG content in the pericellular areas of chondrocyte clones (12).

The structure of PG may be altered in OA although this is not always seen (13). PG monomers are smaller and more easily extracted from OA than from normal cartilage, possibly reflecting destruction of the collagen network or fragmentation of PG (10). PG from OA cartilage shows a lower degree of aggregation and smaller aggregates (10,14). Changes in the carbohydrate portion of the PG structure have also been reported (10).

Techniques to Monitor Effects of Drugs

New Techniques with Potential for Monitoring Therapeutic Effects - The recent application of several techniques to the area of OA is now offering promise for the development of objective assays of cartilage degradation. Highly sensitive assays will be most likely to detect early changes at the stage that is probably most sensitive to drug intervention. Magnetic resonance imaging, ultrasound, ^{99m}TcHMD bone imaging, and bone scintigraphy have been used recently to monitor osteoarthritic changes in joint tissues (15-18). The application of immunological assays to the detection of PG-derived molecules in biological fluids may provide a technique that is feasible and sensitive enough for monitoring the course of cartilage destruction in OA (19-21).

Animal Models Used to Assess Drug Effects - Several experimental models of OA have been used to assess compound efficacy. Since no drug is known to alter effectively OA pathology clinically, it is not yet possible to evaluate which of the models is most likely to predict human response.

Meniscectomy produces laxity of the knee. Along with mimicking the changes of OA, this procedure induces changes in animals in weeks, a time period short enough for compound evaluation to be feasible. In the Hulth procedure the medial meniscus and both cruciate ligaments are excised. In the rabbit, cloning, flaking and fissures, and osteophyte formation occur in a progression that resembles that of OA in man (22). PG changes, biomechanical changes and synovial changes also occur (23-25). Partial medial or lateral meniscectomy in the rabbit also results in cartilage changes resembling those of OA in man, including cell and matrix changes (26,27). Some of the metabolic changes seem to be a nonspecific response to arthrotomy (26). Cartilage removed from the knee three days after partial lateral meniscectomy releases twice as much PG into culture medium during the next day in organ cultures as nonsurgical controls (28).

Joint immobilization produces some of the degradative changes observed in human OA (29). Instead of cell proliferation typical of OA, the chondrocytes of immobilized joints undergo degeneration and death. Immobilization impairs the movement of nutrients and metabolic waste products between cartilage and synovial fluid, which may cause the adverse cellular effects (30).

Degradation of Joint Cartilage

Mechanism of Degradation of Joint Macromolecules - Degradative enzymes have been hypothesized to mediate cartilage destruction. Chondrocytes are potentially the source of enzymes in OA, as these cells release proteases *in vitro*. Attention has been focused on the role of proteases since enzymes that degrade the carbohydrate structures of PGs have not been detected in OA cartilage (2,10,31-33). As the amino acid sequence of PG protein is identified, the cleavage sites of proteases can be sought and used to confirm the roles of specific proteases in OA (34). Inflammation is a minor and inconsistent aspect of OA and presumably not a major source of proteases in OA (4). Cathepsins are probably not important in the pathology of OA (32).

PG degradation occurs early in the disease, evidenced by cartilage softening and increased PG extractability prior to the onset of clinical symptoms (31). PG loss from the matrix is known to stimulate PG synthesis, enhance collagen degradation and increase diffusion of other potentially degradative factors into cartilage (35-37). PG degradation is an active process that ceases with cell death (38).

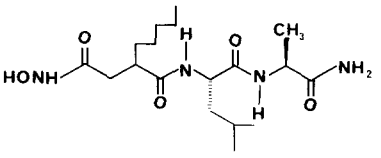
The vascular changes in OA probably do not mediate the cartilage changes but may only reflect an accompanying synovitis (39). Unchecked local protease activity may lead to the vascular invasion from subchondral bone in OA and may explain osteophyte formation (1,40). Some evidence exists for a role of neutral proteases from chondrocytes in this process and implies that protease inhibitors may block endochondral ossification (41).

Neutral Metalloproteoglycanase (NMP) - This enzyme is present in cartilage usually in latent form, has a molecular weight of about 30,000 daltons, is calcium-dependent and is inhibited by metalloprotease inhibitors but not by inhibitors of other classes of proteases (42,43). A three- to ten-fold increase in NMP activity is present in human OA cartilage (44,45) and NMP is secreted by OA chondrocytes (46). The degree of enzyme elevation correlates with the severity of the disease (44). A spectrum of PG degradation products is produced by this enzyme (45). Serine protease activity is much lower than NMP activity (44). Endogenous NMP inhibitors are tightly bound to PG in human cartilage (47). NMP that resembles the enzyme from OA cartilage has been isolated from normal human cartilage and mammalian explant and chondrocyte cultures (48-50).

An acidic metalloprotease is also elevated about 3-fold in osteoarthritis (51). This enzyme resembles NMP in most aspects except its pH optimum.

Several hydroxamic acid peptides inhibit rabbit and bovine NMP (43,52-54). Hydroxamic acid derivatives such as U24279 (1) completely blocked PG release from cartilage explants that were removed from rabbit knees after lateral meniscectomy and cultured for one day (28). These compounds also blocked PG release from cartilage cultures stimulated to release PG with retinoic acid, interleukin-1 (IL-1), or lipopolysaccharide (55). These compounds did not seem to be toxic to the cartilage in this study and did not block retinoic acid or IL-1 from interacting with the cells. Thiol peptides such as U19346 (2) and carboxyalkyl peptides also inhibit NMP (52,53,56-57). They are

less potent than the hydroxamic acids and do not block PG release from cartilage cultures stimulated with retinoic acid (55).



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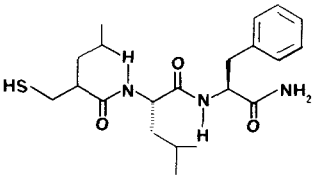

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NMP activity was elevated in cartilage of dogs following transection of the anterior cruciate ligament, although total PG content did not change. Prednisolone treatment blocked the increase in enzyme activity (58). The effects of the NMP (and collagenase) inhibitors EDTA (ethylenediaminetetraacetic acid) and EGTA (ethyleneglycol-bis-(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid) on cartilage degradation were assessed in a surgical model of OA in the rabbit (33). At doses of EDTA that do not suppress PG synthesis but traverse cartilage, NMP levels were significantly reduced in 75% of both operated and nonoperated rabbits. EDTA treatment resulted in a reduction in matrix staining loss but not in PG content measured biochemically (33). Treatment with suramin, 8-(3-benzamindo-4-meta-1-benzamindo)naphthalene-1,3,5-trisulfonic acid, resulted in the inhibition of spontaneous PG release from cartilage in vitro, possibly by inhibiting neutral protease (59).

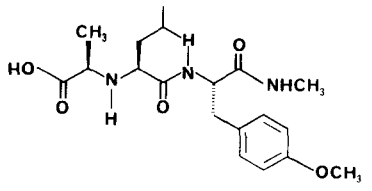
Collagenase - Collagenase may play a role in OA. The uptake of water by OA cartilage implies that the integrity of collagen fibrils has been destroyed, allowing PG to expand and absorb water (1). Although only a small decrease in collagen cross-links may cause this effect, changes in collagen structure are minimal in OA, as described above. In addition cartilage fibrillation or scarring and collagenase treatment, alone or in combination, do not cause progressive changes that resemble OA (60,61). Collagenase in human OA cartilage and animal cartilage is bound to an inhibitor (62). The amount of inhibitor-bound enzyme correlates with the severity of the disease and increases until the final stage of cell death, when it decreases (33). Collagenase is also associated with the early stages of OA in the dog (63).

Rabbit chondrocytes produce a latent collagenase in vitro which has a larger molecular weight than NMP, does not degrade PG, and cleaves collagen at the site typical of mammalian collagenases (42). Otherwise, it resembles NMP. The amino acid sequences of human NMP and collagenase show 55% homology (64).

It is possible that endogenous TIMP (tissue inhibitor of metalloproteinases) plays a major role in controlling NMP and collagenase activities (65). Recombinant techniques have allowed full expression of TIMP-cDNA by fibroblast tumor cells (66). Since the structure of cartilage TIMP resembles that from fibroblast cell cultures, it is possible that these studies will provide sufficient material to study in OA models (67).

Peptide hydroxamic acids and thiols inhibited chondrocyte collagenase activity (52,54,57). Several carboxyalkyl peptides that inhibited NMP were inactive as inhibitors of rabbit chondrocyte collagenase, although patents have appeared showing compounds of this class

(for example, 3) as potent inhibitors of rheumatoid synovial collagenase (28,43,52-56). Related hydroxamates such as 4 are also potent inhibitors of this enzyme (54). CL 205,241 (5), an inhibitor of collagen degradation, is being evaluated in a rabbit model of OA (68). This compound inhibits collagen degradation through its binding with the collagen substrate, rather than by directly inhibiting the enzyme (68).



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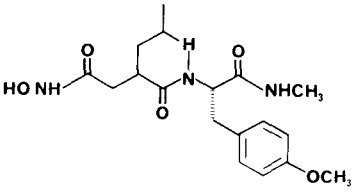

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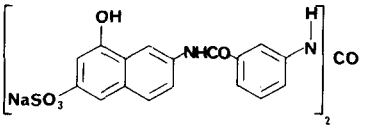

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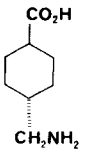

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Plasminogen Activator - The role of plasminogen activator in OA is unclear. Plasminogen activator may be released by senescent chondrocytes (69). It cleaves plasminogen to yield plasmin, which catalyzes PG degradation. Synovia from OA patients release an inhibitor of plasminogen activator into culture (70). Tranexamic acid (6), a potent inhibitor of plasminogen activator, shows a cartilage-protective effect in two surgical models of OA in the rabbit (71,72).

Free Radicals and Hydrogen Peroxide - Oxygen-derived free radicals may also play a role in OA. They degrade PG and hyaluronic acid *in vitro* and may be generated from oxidative reactions in lysosomes (73). Treatment of cartilage with hydrogen peroxide and copper leads to PG and collagen degradation (73). Hydrogen peroxide treatment leads to increased sensitivity of proteins to proteolysis and inhibition of PG synthesis (74). Orgotein, an anti-inflammatory metalloprotein from bovine liver, has superoxide dismutase activity, which destroys superoxide radicals. This activity may be its mechanism of action in horses with orthopedic problems although it failed to provide cartilage protection in two meniscectomy models of OA in the rabbit (71). Mannitol, an hydroxyl radical scavenger, was reported to be efficacious on pain and functional disability and safe in a clinical trial involving patients with severe OA (75).

Factors Altering PG Aggregation - PG molecules bind to hyaluronic acid, forming large aggregates that are less susceptible to proteases than non-aggregated PG. Factors that delay or disrupt PG aggregation would potentially enhance PG degradation and diffusion from the matrix (38). A decrease in PG aggregation is associated with OA, as described above. The affinity of PG for hyaluronic acid can be enhanced, for

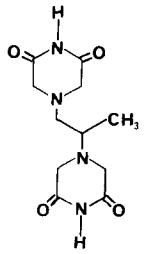
example, by alkali treatment, which possibly enhances disulfide bond formation or displaces a masking agent (76). Hyaluronic acid injections into OA joints have been efficacious in some trials, possibly by promoting PG aggregation, as hyaluronic acid probably does not play a major role in joint lubrication (1,77).

Factors That May Stimulate Degradative Mechanisms in OA - Presumably, in OA the chondrocytes themselves release enzymes or other mediators of cartilage degradation. The factors that stimulate chondrocytes to release these mediators provide another tier to target chemical intervention (78). Bone and mechanical changes can lead to cartilage changes that resemble OA (1,33). Matrix fragments and crystals present in OA joints may be factors that stimulate synovial release of IL-1 or induce protease release by chondrocytes (79,80).

Interleukin-1 - The synovial membrane in OA shows only a minimal infiltrate of inflammatory cells (4). Synovial cells alone (free of inflammatory cells) and chondrocytes release IL-1, which in turn stimulates cartilage matrix degradation (81-85). Articular chondrocytes, which contain IL-1 receptors (86), secrete NMP and collagenase in response to IL-1 treatment (87-90). This effect appears to be specific for chondrocytes, as numerous other factors that alter the effects of IL-1 on lymphocyte proliferation did not alter the chondrocyte response to IL-1 (90). IL-1 may elicit its effect by stimulating the synthesis of a protein that activates collagenase and NMP rather than stimulating the synthesis of the enzymes themselves (91). The PG released from IL-1-treated cartilage is degraded (92). IL-1 treatment results in a reversible inhibition of PG synthesis although the structure of the newly synthesized PG is normal (93).

Chondrocytes treated with IL-1 also release phospholipase A_2, while plasminogen activator release is reduced (88-90). Phospholipase A_2 may cause matrix degradation through the release of cytolytic lipids (89). Phospholipase A_2 also induces prostaglandin E_2 synthesis in IL-1-treated chondrocytes (89). Lipoxygenase activity may play a role in mediating the IL-1 effect on chondrocytes in vitro, but cyclooxygenase does not seem important (94). Recombinant IL-1, injected intra-articularly, induced cartilage PG loss that could not be explained solely by leukocyte accumulation (95). Prostaglandin E_2 production was not increased after IL-1 treatment in vivo (95).

Arteparon (glycosaminoglycan polysulfate) inhibits the effect of synovial culture medium on cartilage degradation without affecting cartilage degradation in the absence of synovial medium or affecting IL-1 production by synovia (96). Hydrocortisone, prednisolone, and piroxicam inhibit the effect of synovium on cartilage by blocking IL-1 production in vitro, while chloroquine reduces cartilage degradation without altering IL-1 production (83,97-99). Razoxane (7), retinol, and dexamethasone each inhibit collagenase production and stimulate TIMP production by chondrocytes cultured with synovium or its conditioned medium (100-102). Bisphosphonates such as 8 inhibit mononuclear cell factor-mediated release of collagenase and proteoglycanase by chondrocytes (103). The hydroxamic acid peptides that inhibit NMP and collagenase block cartilage PG release that is stimulated by IL-1 (55). Numerous other antiarthritic drugs fail to inhibit IL-1-induced cartilage degradation in vitro (104).



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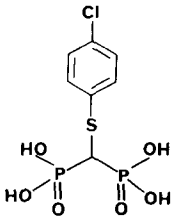

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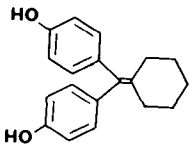
Endocrine Factors - Only indirect evidence suggests that endocrine factors may play a role in OA (3). Estrogen receptors have been demonstrated in articular chondrocytes (105). Tamoxifen, an antiestrogen, reduced the frequency of ulcers and severity of pitting in two meniscectomy models of OA in the rabbit, although osteophyte formation and metabolic changes were not altered (58,106-107). PG synthesis in vitro was suppressed by cyclofenil diphenol (9), a weak nonsteroidal estrogen (108). The antiestrogenic effects of tamoxifen (10) have recently been proposed to be mediated by a novel growth-promoting histamine receptor (109). Histamine H2 receptors have been demonstrated on chondrocytes. Treatment with the H2 blocker, cimetidine, produced symptoms of severe arthritis in ulcer patients which disappeared when treatment was ended (110,111). Low somatomedin-C levels in plasma have been demonstrated in patients with OA of the hip (112). Chondrocytes have receptors for somatomedins and produce these growth factors in vitro, which stimulate chondrocyte clonal growth and PG synthesis (113-115).



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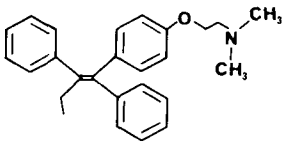
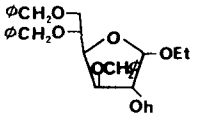


(10)

Factors That Stimulate Cartilage Repair - Along with compounds that block cartilage degradation, several agents have been evaluated in OA that may stimulate cartilage synthesis and repair. Orally administered glucosamine sulfate improved OA symptoms and was well tolerated in two clinical trials (116,117). Intra-articular injections of Arteparon resulted in a gradual improvement of clinical symptoms of OA (118). Arteparon administration to rabbits following medial meniscectomy resulted in marked reductions in histological scores of cartilage lesions and reduced neutral protease activity (119,120). In the immobilization model Arteparon treatment resulted in improved mobility after splint removal and improved radiological parameters and macroscopic appearance of cartilage, while PG extractability and aggregability remained near control levels (121-123). Pentosan polysulfate (SP54) also showed efficacy in the immobilization model while Rumalon (a biological glycosaminoglycan-peptide complex) was reported to be beneficial in clinical trials and to protect cartilage from the degradative damage induced by anti-inflammatory drugs (124-126).

Two major compilations of drug intervention studies in OA disease models have appeared (127,128). Tribenoside (11), benoxaprofen (12), and triamcinolone stand out as small molecular weight agents having beneficial effects, by undetermined mechanisms, in at least two different disease models (71,129-133).



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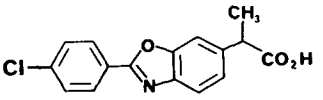

(12)

Conclusions

As new theories emerge to explain changes in molecular biology in OA, medicinal chemistry will provide the potential to probe these theories with the hope of providing therapeutic utility. Novel enzyme inhibitors may prove to be the most effective way of evaluating the roles of various enzymes in OA as the activities of these enzymes are low, but devastating, over a period of time.

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Chapter 19. Agents for the Treatment of Peptic Ulcer Disease

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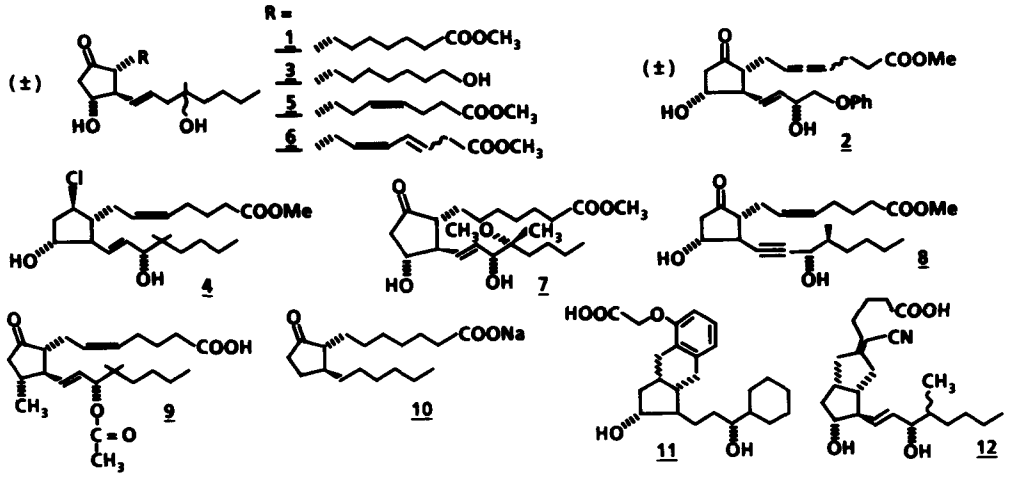
Etiology - Peptic ulcer disease (PUD) is defined as pathology, lesions and ulcers of any portion of the gastrointestinal tract exposed to acid-activated pepsin. Formerly, peptic autodigestion was thought to be the mechanism underlining the pathology. While it seems clear that peptic activity as well as acid are contributing factors, current reasoning incorporates additional factors into the etiology of the disease. Many articles and reviews have been devoted to the issue of gastroduodenal mucosal defensive factors (1-4). Bicarbonate secretion, quality and quantity of mucus production and release, blood flow, cell junctions and ion pumps are some functions thought to enhance mucosal protection (2). Although no single element may account for mucosal protection, all may be potential contributing factors. Insight into mucosal protective mechanisms were provided by studies performed by Robert (5). These studies led to the introduction of the term "cytoprotection" generally accepted to mean protection of the gastric mucosa by prostaglandins (PG's) at doses lower than those required to inhibit acid secretion. Histological studies revealed that in spite of PG administration, 100% ethanol destroyed the mucosal surface epithelial cells (6). Protection of cells deeper in the proliferative cell zone of the mucosa and subsequent rapid restitution of surface cells were observed. Others have observed protection of intestinal mucosa by PG's (7) and protection with agents chemically unrelated to PG's (2). While debate exists over the definition of cytoprotection, few doubt that gastric and intestinal mucosa possess protective mechanisms against damaging agents. Aggressive factors in PUD including acid, pepsin, bile and infectious agents are thought to be in balance with protective mechanisms. A deficiency in the defense system or an overwhelming of it may result in a shift of balance toward predisposition to PUD. The balance concept provides not only a rational explanation of the effectiveness of drugs used to counteract aggressive factors, but also offers the opportunity to explore therapy with new agents, such as PG's, that stimulate defensive mechanisms.

Prostaglandins - Preclinical and clinical efforts continue with a large number of synthetic PG analogs. The development, chemistry and status of most of these compounds have been reviewed (8). The mechanism of the gastric antisecretory effect of E-type PGs has been established with the identification and characterization of a PG receptor on the cell membrane of canine parietal cells (9,10). The receptors show high affinity and strict stereospecificity for E-type PGs but not for histamine, cimetidine or PGI and PGF compounds. Receptor binding was saturable and reversible and showed a high correlation to IC_{50} values for acid inhibition. In general, duodenal (DU) and gastric ulcer (GU) healing rates with PGs have been equivalent to those of histamine H-2 blockers (11). In addition, clinical emphasis is being directed to conditions not addressed by acid inhibition alone. By virtue of their mucosal protective properties, PGs have potential therapeutic indications in preventing gastroduodenal mucosal injury caused by NSAIDS, diet and life styles (e.g., alcohol, smoking, and stress). PG's may also have utility for treating refractory ulcer and preventing ulcer recurrence (12,13). The benefit of cytopro-

tective PG's in the treatment of PUD has been questioned (14).

Misoprostol (1) has been widely approved for marketing. Proceedings from two symposia (4,15) and a review (16) have detailed misoprostol's pharmacological and therapeutic effects, safety and its mucosal protective properties in animals and man. Misoprostol (200 mcg, qid) was as effective as cimetidine (300 mg, qid) in healing both GU and DU after 4 weeks of treatment in multinational trials. It was safe and well tolerated in these studies; diarrhea, the major adverse reaction, was usually mild and self-limiting. In a refractory DU study, misoprostol was significantly better than placebo in patients who had taken H-2 antagonists for 10 weeks or more (17). The protective effect of misoprostol against gastric damage caused by tolmetin and aspirin was established in humans (15). Against an ethanol insult in man, misoprostol was significantly more effective than cimetidine or placebo in protecting the gastric mucosa from injury (4,18). Potential effects on the pregnant uterus has become an issue with misoprostol and other antiulcer PG's. Although no effect was observed in animal studies with misoprostol, two recent studies have demonstrated uterotonic activity in pregnant women (19).



Enprostil (2), currently marketed in Mexico, is an effective and long acting antiulcer agent in animals (20) and man (21). Against DU, 4 week treatment with enprostil (35 mcg bid) produced a healing rate of 65%, compared to 39% for placebo. The only side effect observed was mild and self-limiting diarrhea (22). In comparative DU studies with histamine H-2 blockers, enprostil showed equivalent 4 week healing rates as cimetidine (23) but was significantly less effective than ranitidine (24). In a GU trial, healing rates of 70% and 82% were noted after 6 weeks of treatment with 70 mcg and 35 mcg bid doses, respectively, but only the 35 mcg dose was statistically different from placebo (25). In a comparative GU study, enprostil was equal to ranitidine in 6 and 8 week healing rates (26). Enprostil increased uterine contractility but a 35 mcg dose did not cause abortion in first trimester pregnant women (27).

Rioprostil (3) is an orally active antisecretory and mucosal protective agent with no evidence of contragestational, cardiovascular or pulmonary activity in animals (28). In clinical trials, rioprostil reduced both basal and pentagastrin stimulated gastric acid secretion (29). A single night time dose of rioprostil (600 mcg) was comparable to ranitidine (300 mg) in healing DU at 4 weeks (30). Diarrhea incidence was

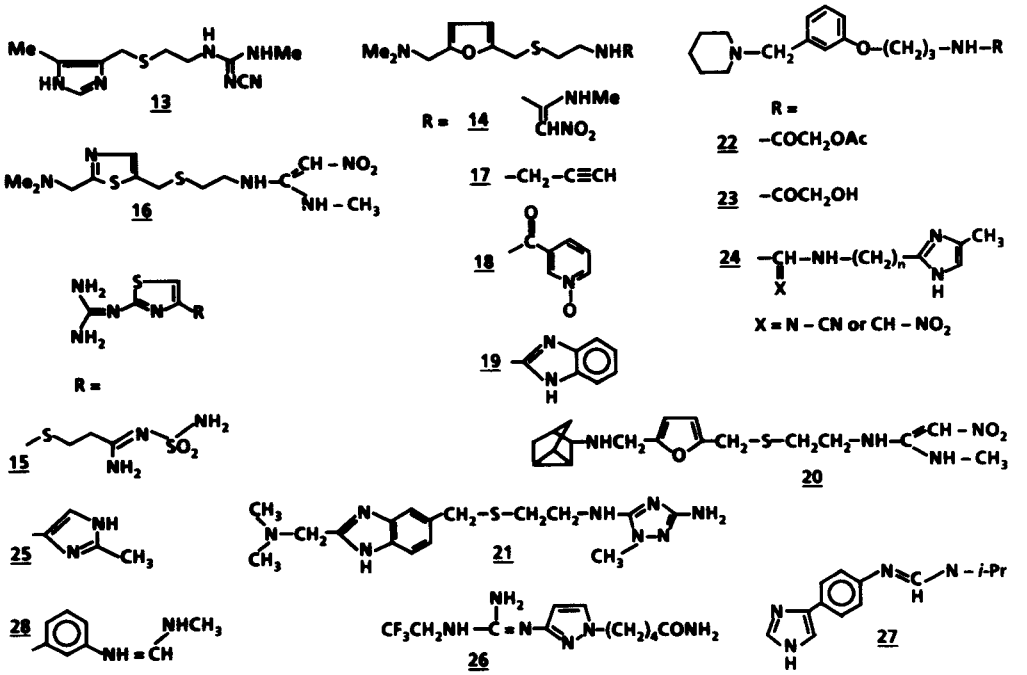
low. Nocloprost (4), currently in Phase II clinical trials, reduced both meal stimulated gastric acid secretion and aspirin-induced gastric bleeding. In SAR studies, the presence of a 5,6 cis double bond was essential for good mucosal protective activity while the corresponding 16-OH, 16-Me analog was considerably less active (31). Enisoprost (5) significantly suppressed histamine stimulated acid and pepsin secretion and was well tolerated in man at doses up to 400 mcg (32). Of 7 alpha-chain diene analogs of misoprostol, the most active antisecretory compound was a 1:1 mixture of 5Z,3Z and 5Z,3E isomers (6). The mixture showed antisecretory potency equivalent to enisoprost in dogs, but was considerably less diarrheogenic (33). A stereospecific synthesis of the active component (5Z,3E) was published (34). The synthesis, stereo-chemical assignments and animal pharmacology of mexiprostil (MDL-646, 7) and its stereoisomers were described (35). FCE 20,700 (8) inhibited basal acid output but not pentagastrin stimulated acid secretion at a single dose of 500 mcg in humans. This dose prevented the drop in gastric potential difference by aspirin, but 750 mcg tid did not reduce indomethacin induced gastrointestinal lesions (36). RO 22-6923 (9) inhibited histamine stimulated acid secretion for up to 8 hours in dogs after an oral dose of 250 mcg/kg (37) and was an effective mucosal protectant in animals (38). Rosaprostol (10), a prostanoic acid derivative marketed in Italy, was effective (2 gms/day) in reducing piroxicam and other NSAID induced GI side effects (39,40).

U-68,215 (11) was a long acting mucosal protectant and orally active gastric antisecretory agent in animals but was not diarrheogenic or uterotonic (41). Replacement of the cyclohexyl ring of U-68,215 with a tetrahydropyran ring reduced mucosal protective and antisecretory activity (42). Nileprost (12), the parent member of a series of gastric antisecretory and mucosal protective prostacyclins (43), reduced acid concentration against pentagastrin stimulation in volunteers at a single p.o. dose of 250 mcg, and reduced aspirin induced gastric bleeding (100 mcg) (44).

Histamine H-2 Antagonists - Cimetidine (13) and ranitidine (14) dominate the peptic ulcer market. Modification of dosage size and timing has demonstrated the effectiveness of once-a-day dosing; a single 300 mg dose of ranitidine given at bedtime showed significantly greater effectiveness than 150 mg bid (45-47). Studies with cimetidine, (46,48-50) and famotidine (15) demonstrated similar results. Single 300 mg doses of ranitidine and nizatidine (16) given with the evening meal were even more effective than bedtime administration (50,51). Relapse rates were higher with cimetidine than with sulcralfate, colloidal bismuth and antacids (52). Maintenance therapy with cimetidine and ranitidine equally protected against re-ulceration; antacid therapy was less effective (53). Histamine antagonists prevent the occurrence of stress-induced ulcers (54); however their use in combination with antacids may be preferred (55). The cytoprotective properties of H-2 blockers are controversial, but protection through release of endogenous PG's has been postulated (56-60).

Famotidine (15), launched first in Japan two years ago, was marketed in the U.S. in late 1986. At 40 mg/day given bid or daily 15 was effective in DU (61) and GU (62,63). A maintenance dose of 20 mg once a day prevented recurrence of DU (63). Like ranitidine it does not have antiandrogenic effects (64) or inhibit liver enzyme metabolism (65), effects noted with cimetidine. Unlike cimetidine (66) it provided relief

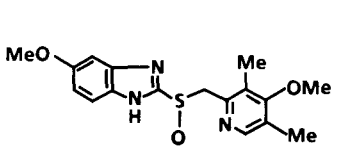
of non-ulcer dyspepsia (67). Nizatidine (16), a thiazole analog of ranitidine, was effective in DU (68) but relieved pain only after 8 weeks. It was as effective as ranitidine in inhibiting mean 24 hour acid output and pepsin secretion (69). ORF 17578 (17), the propargyl analog of ranitidine, was ten times more potent and longer acting in pylorus-ligated rats (70). CM-57755 (18) reduced acid secretion in man at equivalent doses of cimetidine (71). The benzimidazole analog (19) was more potent than ranitidine in guinea pig atria (72). Replacement of the dimethyl amine by a fused tricyclic amine yielded D-166376 (20) which increased the potency 2 fold in the Shay rat (73). Triazole 21 (IK 82029) inhibited water immersion stress ulcers in rats at doses equivalent to cimetidine (74). TZU-0460 (22) was effective in GU and DU and is expected to be launched in Japan this year. The deacetylated derivative HOE 062 (23) was equipotent to 22 in animal models (75). A number of conversions of the amide to heterocycles were successful but the activity did not differ from 22 or 14 (76-79). The TZU-0460-histamine hybrid molecules (24) were quite potent; lengthening its side chain to 3 methylene units increased potency (80). A single dose of the famotidine analog, zaltidine (25), suppressed basal and pentagastrin-stimulated acid secretion in both healthy and DU subjects (81). The fluorinated derivative ICI 162,846 (26) was also effective at low doses (82). Mifentidine (DA-4577, 27) was more potent than cimetidine or ranitidine at inhibiting dimaprit-induced gastric and duodenal damage in guinea pigs (83). Low doses in man reduced basal and pentagastrin-induced acid secretion. The conformation, ionization and tautomerism of 27 and the famotidine-mifetidine hybrid, DA-4643 (28) were discussed (84,85).



The nature of the H-2 receptor was investigated by analysis of receptor binding. Analysis of a pyridyltriazole series through QSAR indicate the importance of lipophilic factors (86). From conformational analysis of a number of H-2 antagonists, a working model of the H-2 receptor was formed (87). Several N-alkyl derivatives of 27, unlike 27, 13 or 14, bind irreversibly at a different locus on the receptor (88). Dipole moments of a number of cimetidine analogs indicate the importance

of dipole orientations (89). A series was prepared whereby the imidazole, guanidino and isothiourea moieties were systemically exchanged. These groups were not isosteric for the H-2 receptor (90).

H+/K+ ATPase Inhibitors - Two symposia proceedings (91,92) and a review (93) have summarized SAR and extensive preclinical and clinical data on omeprazole **(29)**. Clinical trials have demonstrated that omeprazole is a remarkably effective treatment for DU. In a comparative trial (94) with ranitidine (150 mg bid), omeprazole (20 mg single morning dose) healed significantly more patients at 2 and 4 weeks with fewer days of pain noted by omeprazole patients. Omeprazole (20 mg, single morning dose) was equal to ranitidine (100 mg bid) in healing GU at 2,4 and 8 weeks (95) and was effective in controlling gastric acid secretion in Zollinger-Ellison syndrome (96) and Barrett's ulcer (97) patients who were resistant to H-2 receptor antagonists. In clinical trials, omeprazole has been well tolerated, but recent findings that high doses of omeprazole caused enterochromaffin-like cell hyperplasia and carcinoid tumors in rats have intensified the controversy about its safety (98-100). Omeprazole is not the active inhibitor of the H^+/K^+ ATPase enzyme but is transformed within the acid compartment of the parietal cell to an inhibitor molecule which reacts covalently with an essential SH group on the enzyme (101). In vitro, omeprazole is reversibly transformed in acidic media to the sulfenamide **30** which can react with thiols to form disulfides **31**, thus representing a model for the covalently linked enzyme-drug complex (102,103).



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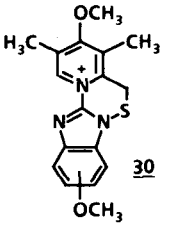

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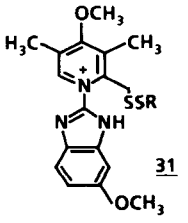

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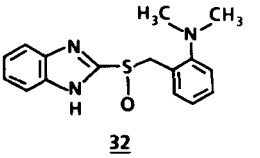

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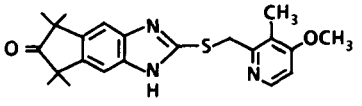

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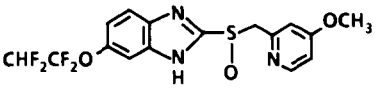

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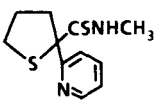

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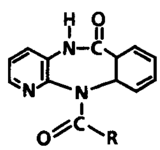
NC-1300 **(32)** (104,105), RO 18-5362 **(33)** (106) and B 831-56 **(34)** (107), one of a series of fluorinated benzimidazoles (108,109), are potent and long lasting inhibitors of gastric acid secretion in animals and act by mechanisms similar to omeprazole's. Mucosal protective effects against a variety of insults were also described for these compounds. RP 40749 **(35)**, which may act by inhibition of the H+/K+ ATPase enzyme, healed 100% of DU ulcers in an open trial after 4 weeks treatment (150 mg, once daily) with no side effects observed (110). Prolonged administration of high doses of RP 40729 caused neurological toxicity and muscular atrophy of the lower limbs in rats. The use of this compound in humans has been discontinued but other derivatives are under development (111).

Muscarinic M-1 Receptor Antagonists - Pirenzepine (**36**), a selective muscarinic M-1 receptor antagonist, reduced basal and stimulated acid secretion in animals and man. The lack of M-2 binding is evidenced by the decrease of anticholinergic effects. Pirenzepine showed efficacy in

DU (100-150 mg daily) (112,113) equivalent to 1 g/day of cimetidine . At these doses anticholinergic side effects were noted. Since acid secretion in the parietal cell is regulated by histamine, muscarine and gastrin receptors, it has been speculated that inhibition of two receptors would have a synergistic rather than an additive effect. A combination of cimetidine (200 mg) and pirenzepine (25 mg) bid was more effective at reducing intragastric acidity than either 400 mg of cimetidine or 50 mg of pirenzepine both given bid (114). Similar effects were demonstrated with ranitidine-pirenzepine combinations (115). Pirenzepine may have greater cytoprotective effects than the histamine antagonists since it, like the PG's, prevents gastric mucosal lesions induced by ethanol, HCl, NaOH and taurocholate. Cimetidine prevented only those induced by ethanol (116-119).

Telenzepine (37) is 4-10 times more potent than pirenzepine (36) as an inhibitor of acid secretion in rats and dogs. The lack of heart rate effects indicate that it also is selective for the M-1 receptor (120). Telenzepine inhibited basal gastric secretion in man as well as secretion stimulated by sham feeding (121), pentagastrin (122) and peptone (123) at doses ten times less than pirenzepine. The selectivity of the muscarinic receptor appears to reside on the piperazine moiety. AF-DX-116 (38) showed selective M-2 antagonism while replacement of the piperazine by a dialkylamino alkyl chain (39) removed all selectivity (124).



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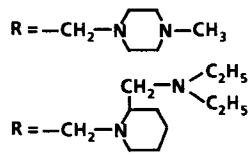
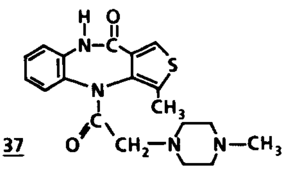


39 R = —$(CH_2)_nN(R_1, R_2)$



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Mucosal-Coating Agents - The efficacy of mucosal-coating agents, has been established (125-126). Sucralfate, a sulfated disaccharide-basic aluminum sulfate complex, forms an adherent coating with proteinaceous material at ulcerated mucosal sites. The coating may provide a barrier to hydrogen ion diffusion, reduce peptic activity and adsorb bile salts (127-128). Animal and clinical studies have reported that sucralfate protects the mucosa against damaging agents including alcohol (129) and aspirin (130). Sucralfate may also protect the gastric mucosa by stimulation of endogenous PG production (129). Another property of sucralfate is its ability to adsorb other agents (131,132), suggesting its proposed protective activity may result from preventing irritating agents from reaching the mucosa. The variety of proposed actions of sucralfate have complicated the understanding of its mechanism of action. Sucralfate, by virtue of minimal absorption, presents few complications. Only 2-3% of patients reported constipation, and decreased serum phosphorous with increased aluminum is only of concern in uremic patients (133).

Peptides - Two reviews on peptide regulation of acid secretion (134) and peptic ulcer disease (135) were published. The following table shows the different effects of icv vs iv dosing which emphasizes the role of receptor location for control of acid secretion (136).

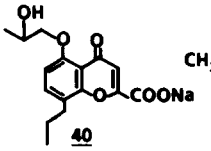
Effect of Peptides on Acid Secretion

Route	CRF	GRP	Endo[a]	Gast[b]	Neur[c]	TRH	Som[d]	CGRP	VIP	Sub P
icv	dec	dec	dec	inc	dec	inc	inc	dec	-	-
iv	dec	inc	-	-	-	dec	dec	dec	dec	dec

[a]Endorphans [b]Gastrin [c]Neurotensin [d]Somatostatin

Somatostatin, a tetradecapeptide, given i.v. inhibited acid secretion in animals and man and was effective for peptic ulcers and hemorrhagic gastritis (137,138). Lack of oral activity and side effects e.g. increased postprandial plasma glucose levels, gallstones and steatorrhea, limit its clinical use. Shorter chain cyclic analogs, although less potent, orally inhibited acid secretion with increased duration of action (139,140). Somatostatin has a cytoprotective effect on the gastric mucosa (141).

Miscellaneous - FPL 52694 (**40**), a mast cell stabilizer (142), produced a significant decrease of overnight basal acid secretion in dyspeptic patients after one week of treatment (143). FPL 52694 prevented ethanol induced gastric lesions in rats, probably through endogenous PG stimulation (144). Spizofurone (AG-629, **41**) (145-146), TEI-5103 (**42**) (147,148), BTM-1086 (**43**) (149), and mezolidon (KM-1146, **44**) (150) inhibited ulcer formation in a wide variety of animal models. Esaprazole (**45**) reduced aspirin induced gastric mucosal potential difference fall in patients with non-ulcer dyspepsia (151). RU-38086 (**46**) showed both gastric antisecretory and mucosal protective activities in the rat and cat (152). Sofalcone (**47**) enhances PG levels by both stimulating synthesis and inhibiting 15-hydroxy-PG dehydrogenase (153,154). In a controlled trial, sofalcone (100 mg tid) healed GU in 73% of patients (155).



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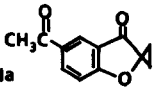

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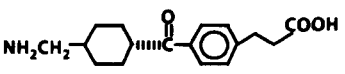

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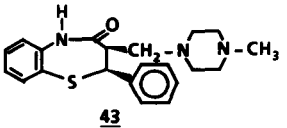

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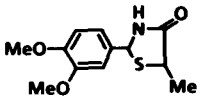

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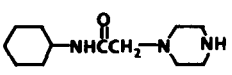

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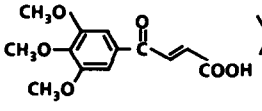

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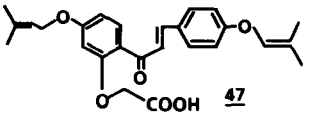

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Conclusion - Over the past decade, the introduction of new, effective drugs for peptic ulcer disease therapy has increased dramatically. However, the different drugs are not equally effective in all patients, and some patients remain refractory to treatment. In addition, the high relapse rates after successful treatment remain a significant problem and challenge for the future.

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Chapter 20. Dermatological Agents

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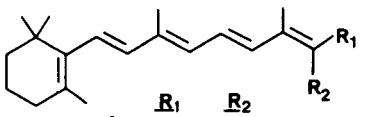
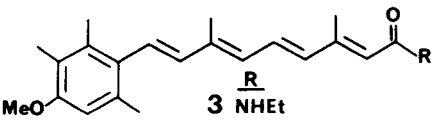
Introduction--Dermatology continues to undergo a major shift away from being a field self-admittedly preoccupied with description and taxonomy toward becoming a highly integrated medical discipline better termed the dermatosciences. This field encompasses biology, pharmacology and pathophysiology and is aimed at the development of more sophisticated and more effective therapies for diseases of the skin (1). This report will update the previous review in this series (2), focusing on newly developed treatments for acne, psoriasis, acute skin inflammation and alopecia.

Acne--The generally accepted model of acne development involves enhanced sebum excretion under androgen control, hypercornification of the sebaceous duct and its subsequent colonization with P. acnes, and eventual production of inflammation by chemotactic factors secreted by P. acnes (3). Most mild to moderate cases of acne can be held in check and eventually cleared by the use of topical benzoyl peroxide or either orally or topically administered antibiotics, although questions regarding their efficacy and relative safety have been raised (4).

The development of three generations of synthetic analogs of retinoic acid (1) as treatments for acne and other skin diseases marks the most dramatic advance in dermatopharmacology in the last decade (5). Isotretinoin (13-cis-retinoic acid, 2)



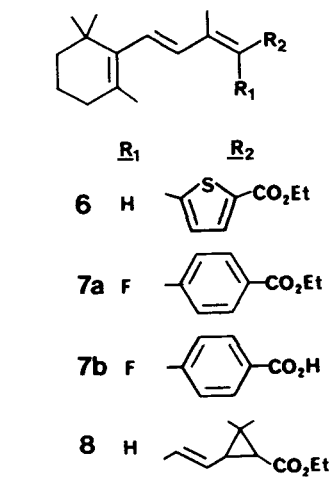
	R_1	R_2
1	COOH	H
2	H	COOH

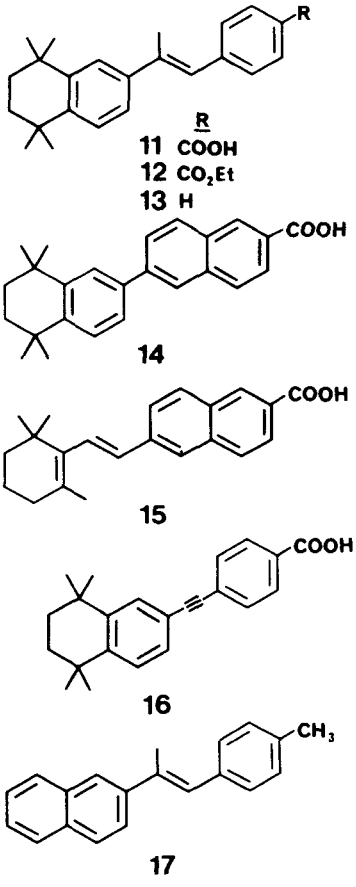


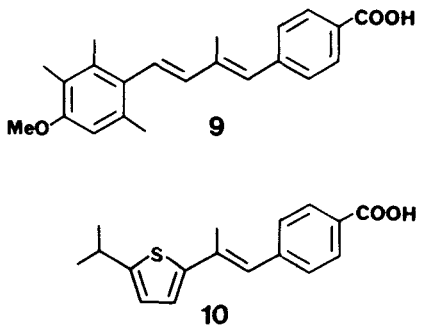
	R
3	NHEt
4	OEt
5	OH

is now firmly established as the treatment of choice for severe refractory conglobate and nodulocystic acne, due to the completeness and longevity of remission in almost all cases (6). The pharmacological profile of 2 suggests that skin surface lipid composition is altered and bacterial microflora levels are lowered as a result of reduced sebaceous gland size and sebum production, possibly due to interference with endogenous vitamin A metabolism (7). Isotretinoin therapy is always accompanied by side effects involving the mucocutaneous system which mimic hypervitaminosis A syndrome. These rarely lead to drug withdrawal, however. Decreased leukocyte and neutrophil levels, increased serum triglyceride and cholesterol levels, and decreased high-density lipoprotein levels have been observed, but were clearly dose-dependent and reversible (8,9). Because of reports of teratogenicity, administration of 2 is limited to severe, therapy-resistant modulocystic and conglobate acne, and is strictly contraindicated in women of childbearing potential in the absence of effective contraception (10). The successful clinical use of 2 and motretinide (3) in acne, coupled with similar results with etretinate (4) and etretin (5) in psoriasis (11), has prompted a search for other retinoid analogs with more favorable therapeutic ratios. Of a series of analogs of 1, 6, 7a and 7b were most active as inhibitors of ornithine decarboxylase (ODC), an

indication of potential keratolytic activity (12). Cyclopropane 8 was designed as a side chain analog of 1 (13). Second generation analogs related to 4, but targeted for acne, include 9 (14) and CBS-211-A (10) (15). The free acid (11) was found to

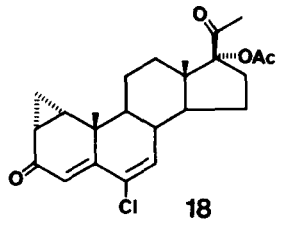






be the most active of a series of carboxylic and sulfonic acid analogs assayed as keratinization and differentiation inhibitors in cultured chick embryo skin (16). Later studies, however, showed that the corresponding ester 12 was less active than 2 in sebum suppression or treatment of acne (17). The unsubstituted analog Ro 15-0778 (temarotene, 13), inactive in the chick embryo assay, is highly active as an inhibitor of sebum secretion, and also decreases the size of sebaceous glands in castrated rats (18). The naphthyl arotinoid 14 has also been found to be more active than 15 as an ODC inhibitor (19). Other arotinoids under consideration include 16, reported to be active in suppression of hamster trachea keratinization (20), and 17, claimed as an antiseborrheal agent (21).

An alternative approach to the control of acne involves the regulation of elevated serum androgen levels, the hormonal imbalance known to be a primary cause of increased sebum production. The basic biochemistry of androgens and their chemical control has been described (22). The interest in antiandrogen therapy has been prompted by the successful uses of cyproterone acetate (18) (23,24) and spironolactone (19) (25) in women with acne. While 18 is essentially inactive topically, there are conflicting reports on the efficacy of topically administered 19, since outward improvement of the acne condition was noted (26), but was not accompanied by a lowering of the sebum excretion rate (27). Among a series of des-A-steroids exhibiting widely varying affinities for the androgen receptor, inocoterone (20) showed a strong local antiandrogenic activity in



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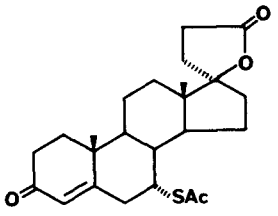

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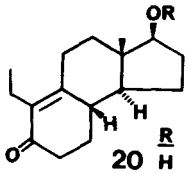
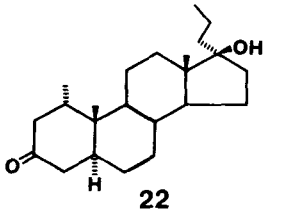


R
20 H
21 Ac

hamsters (28), while the corresponding acetate, RU-38882 (21), showed greater activity than 18 on the rat sebaceous gland (29). Topically administered 17α-propylmesterolone (SH 434, 22) reduced both sebaceous gland size and sebogenesis in hamsters, without influence on testosterone plasma concentrations or testes weights (30). The androstadiene derivative 23 also exhibited a similar profile on topical or subcutaneous administration (31). The smallest steroid-like fragment to display antiandrogen activity is cyoctol (24), shown to block dihydrotestosterone binding to androgen receptors (32). Criteria for antiandrogens operating by specific androgen receptor blockade without 5α-reductase inhibitory activity have been proposed, along with the disclosure that Org 7476 (25a) and Org 7294 (25b) display the desired profile upon topical administration (33). 5α-Reductase inhibitors recently proposed as antiandrogens include azaandrostenone 26 (34) and 27 (35). Ketoconazole, an inhibitor of testosterone biosynthesis (22), has also been reported to be effective in oral acne therapy, but potential hepatotoxicity exists. Further studies via topical administration are needed (36). Topical cimetidine has also been reported to ameliorate some manifestations of acne, although no reduction in sebum excretion was noted (37).



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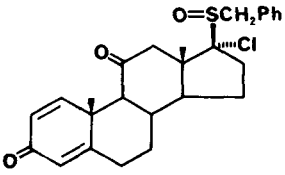

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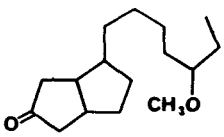

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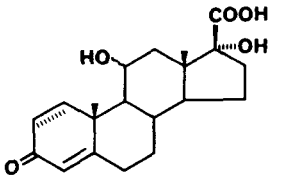
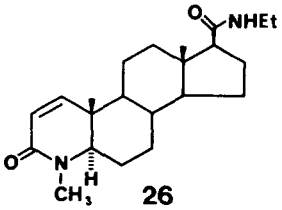


25a Δ-1,11β 25b 11-α



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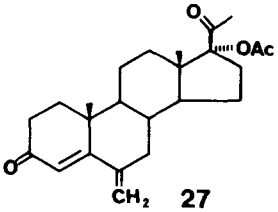

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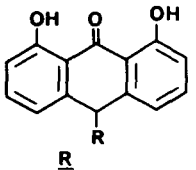
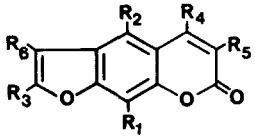
Psoriasis--In contrast to the advances in acne, treatment of psoriasis remains palliative, providing only temporary remission to this oftentimes physically and emotionally debilitating disease. Reviews on the current state of psoriasis research reiterate the problems encountered in clinical therapeutics, but consistently end with statements demonstrating a reliance on future advances to provide safe and consistently effective therapy (38-43). Topical steroids, oral antimetabolites, and topical application of coal tar, alone or in combination with UV irradiation, remain in use, despite their well-known drawbacks (42). More prevalent is the topical administration of anthralin (28) which provides effective clearing of psoriatic plaque and extended time to relapse (44). However, irritation, staining of skin, clothing and bedding, and dissatisfaction with ointment or paste formulations have contributed to low patient compliance (45). The exact mechanism of action of 28 in psoriasis remains obscure (44), but is likely mediated by its propensity to form free radicals (46,47). The ability of 28 to interfere with

DNA synthesis, but its non-mutagenicity in the Ames test (48), indicate a complicated influence on the biochemical pathways implicated in the pathogenesis of psoriasis. Chemical modifications of 28 designed to eliminate untoward effects include butantrone (29) (49). Results of a recent clinical trial, however, report that 29 produced significant staining and irritation, with minimal clinical response (50).

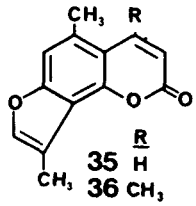


28 R = H
29 R = CO*n*-Pr



R_n=H, except:

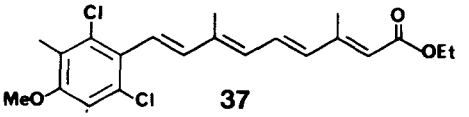
30 R_1=OCH_3
31 R_2=OCH_3
32 $R_{1,3,4}$=CH_3
33 R_5=CO_2Et
34 $R_{1,3,4}$=CH_3; R_6= 



35 R = H
36 R = CH_3

The use of oral 8-methoxypsoralen (8-MOP, 30) plus UV-A irradiation (PUVA) continues to be an effective treatment for psoriasis, but its benefits must be weighed against the long term side effects such as cutaneous carcinoma, melanoma, systemic malignancy, skin aging, immunologic alterations and eye damage, now becoming evident after a decade of use (51,52). The use of topical 30 (53) or other furocoumarin derivatives have been evaluated as replacements for oral 30 in PUVA regimens. Both 5-MOP (31) (54) and trioxsalen (32) (55) displayed favorable results, while 3-CPS (33) produced a severe dermatitis and a low resolution rate (56). AMT (34) was found most effective of a series of 8-MOP derivatives compared as inhibitors of mitogen-induced lymphocyte proliferation (57). Various methylangelicins (58,59), furocoumarin isomers shown to form only monophotoadducts with DNA, have been prepared and evaluated as replacements for 8-MOP. Of these, the 6,4'-dimethyl (35) and 6,4,4'-trimethyl (36) analogs have shown activity superior to 30 without induction of skin erythema (60).

Of retinoids examined for their effects in psoriasis, etretinate (4) was found most effective, although displaying a side effects profile similar to that of 2 (61). Both 5, the primary metabolite of 4, and Ro 12-7554 (37) were comparable to 4 in efficacy and safety (62,63). The clinical efficacy of E-5166 (38), an inhibitor of UV-B-stimulated epidermal ODC (64), has been proven in a pilot study (65). Arotinoid analog 11 showed evidence of activity against psoriasis (66), but was judged overall to be of less therapeutic value than 4 (67).



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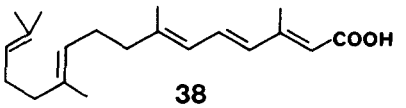

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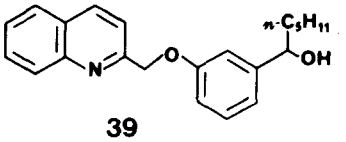
The regulation of abnormal arachidonic acid (AA) release and metabolism has become a prime target for pharmacological intervention in psoriasis, since the products of both the cyclooxygenase (CO) and lipoxygenase (LO) pathways have been implicated in the pathogenesis of the disease (68). Elevated levels of prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, leukotriene B_4 (LTB_4), LTC_4, LTD_4 and multiple hydroxyeicosatetraenoic acids (HETEs) detected in psoriatic skin chamber fluid or lesions (69-71) might indicate a general stimulation of AA metabolism. Further, the relative amounts of PG products versus free AA HETEs and LTs is low, indicating a diversion of AA metabolism to the LO pathway. This is supported by the exacerbation of psoriasis by the CO inhibitor indomethacin (72).

In addition, levels of 15-HETE, an inhibitor of both 5- and 12-LO, are selectively decreased, favoring a shift to the potent inflammatory and chemotactic LTs and 12-HETE, respectively (73). Since both 15-HETE (74) and PGE_2 (75) themselves have been found to improve psoriasis, manipulation of the misregulated LO pathway via 5- or 12-LO inhibition or specific stimulation of 15-HETE biosynthesis may prove beneficial. In a study of eight known antipsoriatic agents, a correlation was observed between LO inhibition and antipsoriatic activity, suggesting LO inhibition as an underlying mechanism of action (76). Anthralin, but not its metabolites, have been shown to be inhibitors of both 5-LO (77) and 12-HETE production (78). The use of the AA-induced mouse ear edema model (79-81) has allowed the identification of a number of topically active 5-LO inhibitors. Both Rev 5901 (39) (82,83) and lonapalene (RS-43179, 40) (84,85) were potent and selec-



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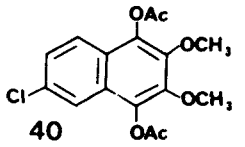

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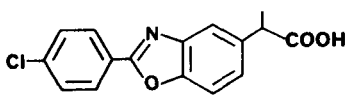
tive inhibitors of 5-LO; the former has been chosen for clinical trials in LO- and LT-mediated disease (86), while the latter has already displayed clinical efficacy comparable to steroid therapy in the treatment of psoriasis (87). 5-LO inhibition has also been demonstrated for ketoconazole (88), moderately active against scalp psoriasis (89).

Although CO inhibitors themselves exacerbate psoriasis (90), combination CO-LO inhibitors may be effective in psoriasis treatment. The original reports concerning oral benoxaprofen (41) confirmed its activity in psoriasis (91,92), but studies were halted upon withdrawal of the drug. Mixed CO-LO inhibitors since identified for consideration as potential treatments include BW-540C (42) (93,94),



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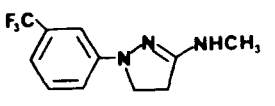

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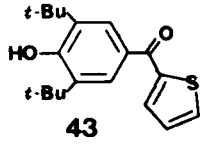

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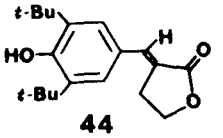

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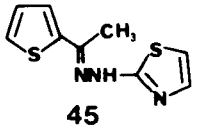

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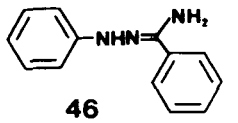

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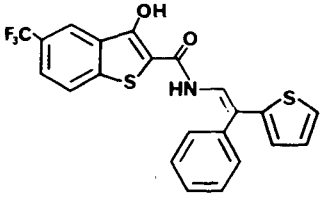

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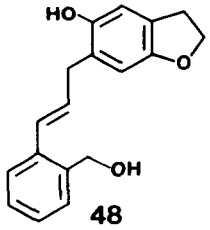

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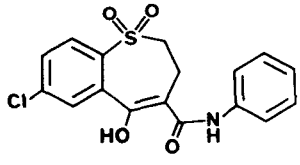

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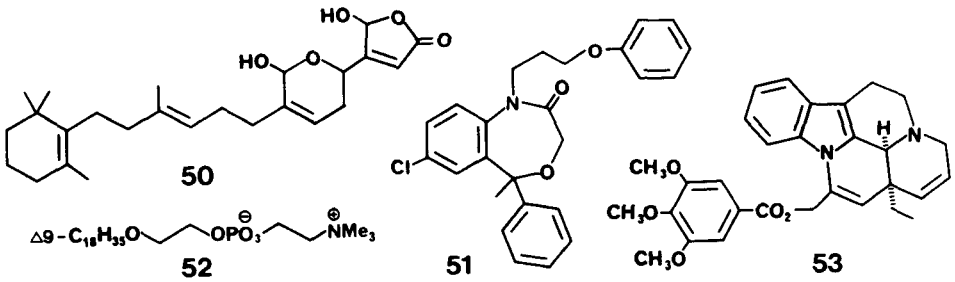
R-830 (43) (95), KME-4 (44) (96,97), CBS-1108 (45) (98), CBS-1114 (46) (99), L-652,343 (47) (100), L-651,896 (48) (101-144) and enolicam (49) (103). Differences in the CO-LO inhibition ratios within this series should help define the relative therapeutic contribution of these two mechanisms.

The inhibition of abnormally elevated phospholipase A_2 (PLA_2)-mediated release of AA (104,105) provides another entry to psoriasis treatment. Both

in vitro (106) and systemic (107) 4 inhibit PL activity. Of the recently reported PLA_2 inhibitors, however, only manoalide (50) (108) and 51 (109) exhibit indications of topical activity. Compound 52 was the most potent of a series of



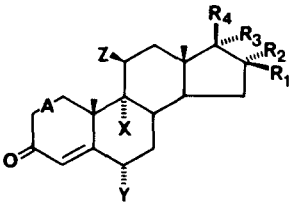
inhibitors based on active site modelling of bovine pancreatic PLA_2 (110). Elevated PLA_2 activity has also been coupled to an augmented ß-adrenergic adenylate cyclase response induced by glucocorticoids (111), a range of antipsoriatic and antimetabolic agents (112), and the retinoid analog 38 (113). Phosphodiesterase (PDE) inhibition provides another possible biochemical intervention leading to elevation of cyclic AMP (cAMP) levels. Among newer PDE inhibitors, the apovincaminol derivative RGH-4417 (53) has been targeted for the topical treatment of psoriasis (114).

Elevated levels of calmodulin (CM) have been detected in both involved and uninvolved psoriatic epidermis (115,116), raising the possibility of CM-inhibitor therapy for psoriasis with agents such as chlorpromazine (117). After treatment with 28, 4, PUVA and other standard regimens, significant overall reductions in CM levels were observed in lesional epidermis (118), while 28 itself was also shown to be a potent competitive antagonist of CM (119). The response of scalp psoriasis to ketoconazole cited above has also been ascribed to possible CM antagonism (120). Manipulation of intralesional calcium levels themselves has also been investigated as a potential treatment. Oral 1α-hydroxyvitamin D_3 was shown to be moderately effective in a small clinical trial (121), while 1,25-dihydroxyvitamin D_3 inhibits the growth of psoriatic fibroblasts in vitro (122).

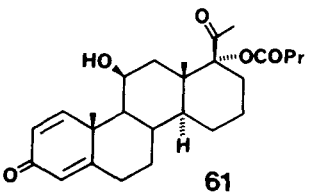
Continued reports on the successful treatment of psoriasis with the immunosuppressive agent cyclosporin A (CSA) (123,124) have prompted investigations into its mechamism of action. Although the effect of CSA on T-lymphocyte-induced abnormal keratinocyte proliferation is consistent with its immunosuppressive activity (125), the dose required for psoriasis treatment is significantly lower than that for other applications, such as graft rejection, suggesting a different mechanism of action (124). This observation was confirmed in a study showing that CSA does not inhibit epidermal cell growth at antipsoriatic therapeutic levels (126). One possible alternative explanation is that CSA exerts its effects by CM antagonism, a property which may be crucial to its action on T-lymphocytes, and amplified in psoriasis due to elevated CM levels (120). Topical application of CSA has also been suggested on the basis of activity in a model of contact hypersensitivity (127), and because of the possible reduction of side effects associated with systemic immunosuppression (128,129).

Interferons (IFNs) represent another class of modulators of T-lymphocyte induced keratinocyte activation and have been postulated as a potentially useful therapy (130). In a small study, r(recombinant) IFN-gamma, but not rIFN-alpha, was found to induce expression of a particular human leukocyte associated (HLA-DR) antigen in uninvolved and involved psoriatic epidermis after intralesional injection, without any effect on DNA synthesis (131). However, isolated clinical reports have shown conflicting results with rIFN-alpha (132,133), and a total lack of effect with rIFN-gamma (134).

Acute Skin Inflammation - Steroids remain the first line of topical treatment in acute skin inflammation almost regardless of the cause, and despite their well-known side effects of dermal atrophy and adrenal-pituitary suppression upon systemic absorption. Nevertheless, their proven value in treatment of various forms of dermatitis continues to prompt the search for more potent agents with reduced systemic potency. The structural homology among potent steroids is high, and has allowed the development of a structure-activity profile for antiinflammatory activity (135). The human vasoconstriction assay used to assess the antiinflammatory potential of candidate agents has been linked to inhibition of phospholipase A_2 (136) which in turn is mediated by lipocortin (137). Recent representative steroid structures exhibiting favorable to marked topical antiinflammatory activity and low systemic effects include 54 (138), 55 (139), 56 (140), tipredane (57) (141), timobesone (58) (142), RU-24643 (59) (143) and JO-1222 (60) (144). Similar profiles were reported for domoprednate (61) (145), naflocort (62) (146), and the pyrazolo analog (63) (147). Regardless of improvements in steroid therapeutic indices, the search for nonsteroidal agents topically active in the treatment of acute inflammatory dermatoses and operating by mechanisms independent of corticoid receptors remains a high priority. BRL-20459 (64) is active topically in inflammation models, but does not inhibit PG synthetase or cause thymic involution (148). The CO inhibitor OZP (65) displays activity as an antiinflammatory and analgetic agent, but is devoid of GI or other systemic effects due to rapid metabolism (149). Other compounds displaying indications of topical activity include 66 (150), 67 (151), and 68 (152).



	A	X	Y	Z	R_1	R_2	R_3	R_4
54	=	F	F	OH	H	O, O, Pr		CO_2Me
55	=	F	=O	OH	CH_3	H	OCO_2Me	$COCH_2Cl$
56	=	Cl	F	OH	CH_3	H	OCOEt	CO_2Me
57	=	F	H	OH	H	H	SEt	SMe
58	=	F	H	OH	CH_3	H	OCOMe	COSMe
59	=	Cl	H	Cl	H	CH_3	H	$COCH_2O_2CCH(C_6H_n)_2$
60	—	H	H	OH	H	H	OCOPr	$COCH_2OCSEt$



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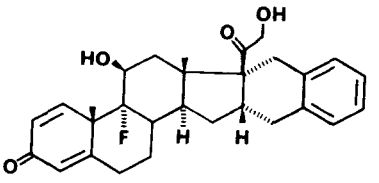

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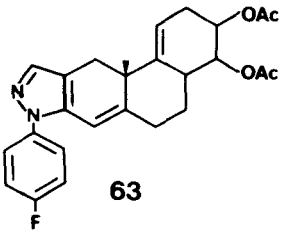

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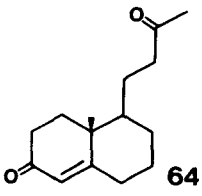

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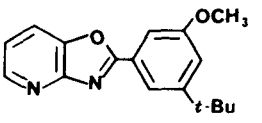

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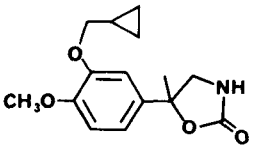

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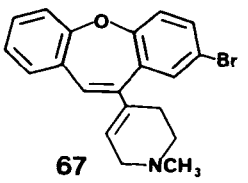

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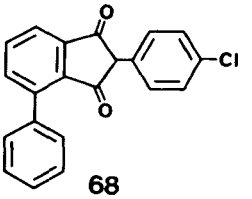

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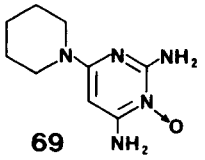
Alopecia (Baldness) - Alopecia areata, a non-scarring localized, and often sudden, hair loss, has been treated in recent years with contact allergens (153), but this therapy is viewed as too radical for routine use (154). However, observations during clinical trials of the potent antihypertensive agent minoxidil (69) showed that, among a number of systemic side effects, a reversible hypertrichosis (spontaneous hair regrowth) occurred in most patients after one month or more of treatment. A number of follow-up studies using topical formulations of 69 have produced widely varying results, probably due to inconsistencies in formulation types and strengths, length of treatment and endpoint evaluation (155). Common to these studies are the findings that a dose-response relationship does exist, the best results being obtained with a 5% solution (156). However, 69 lacks efficacy in more severe or extensive disease, including alopecia totalis (complete baldness) (157,158): in a controlled trial, 69 showed no advantage over contact allergens or placebo (159). Better results, however, have been obtained in the treatment of androgenetic alopecia (male pattern baldness) (160,161), especially in early stages (162). Dose-response studies for male pattern baldness indicate that a 2% solution is the preferred treatment modality (163). There have been no reported systemic side effects associated with treatment since 69 is only poorly absorbed (164) through the skin. Although the mechanism of action of 69 is not known, increased follicular microcirculation blood flow (165) and direct effects on both DNA synthesis and the growth pattern of keratinocytes (166,167) have been investigated. If the activity of 69 is due to its antihypertensive and vasodilatory properties, other topically active agents of this class should display similar results. It has been reported that the topically active vasodilatory PG viprostol (70) and the antihypertensive agent diazoxide (71) are both under consideration for this indication (168).



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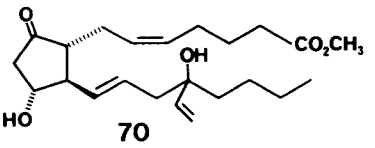

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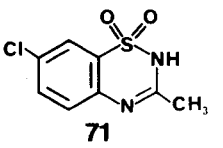

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Conclusion - Chance observation of dermatological side effects of various drugs, coupled with fundamental advances in the understanding of the complex biochemical pathways that cause or are caused by altered dermal metabolism, have pointed the way to cost effective and cosmetically acceptable regimens conducive to patient compliance. These agents, ideally possessing both high efficacy and low toxicity, will provide the urgently needed therapies for diseases of the skin for which only palliative treatment is currently available.

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Chapter 21. Pharmacologic Intervention in Diabetes Mellitus

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For the most part drugs currently available and under development for the treatment of diabetes mellitus are aimed at improving glycemic control. This goal is consistent with recent guidelines issued by the American Diabetes Association (1). Whether improved glycemic control alone will be sufficient to prevent the long-term secondary complications of diabetes is not certain; a multicenter clinical trial addressing this question is in progress (2).

Since last reviewed in this series (3), general review articles on hypoglycemic agents have appeared (4,5) and the physiological role of glucagon and somatostatin in diabetes was reviewed (6).

MODULATION OF GLUCO-REGULATORY HORMONES

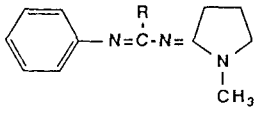
Glucagon/Somatostatin Analogs - Although most synthetic antihyperglycemic compounds stimulate insulin release or mimic some aspect of insulin action, agents which modulate glucagon and somatostatin secretion and action are also being studied (3,5). A glucagon receptor antagonist, [1-N$^{\alpha}$-trinitrophenyl-histidine,12-homoarginine]-glucagon, reduced the hyperglycemia of streptozocin-diabetic rats when infused intravenously (7). A structure-activity study of the N-terminal region of glucagon has been published (8). A number of somatostatin analogs, aimed at selective inhibition of glucagon secretion, have been synthesized (3). Sandostatin (SMS 201-995, D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr[ol]) (9,10) reduced postprandial hyperglycemia and glucagon levels of insulin-dependent diabetic (IDDM) subjects when injected with insulin (11,12). Reduced postprandial hyperglycemia and glucagonemia in IDDM patients have also been reported for WY 41,747 (Des-Ala^{1},Gly^{2}-[His-^{4,5},D-Trp^{8}]-somatostatin) as a parental insulin adjunct (13,14). L-363586 (Cyclo[N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe]), the most potent of a large series of cyclic hexapeptide somatostatin analogs, displayed oral efficacy in ob/ob mice and alloxan-diabetic dogs, even though bioavailability was very low (15).

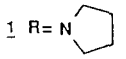
Proinsulin - A potential therapeutic role for proinsulin, the biosynthetic precursor to insulin, has been proposed. Although markedly less potent than insulin, human proinsulin had a prolonged action and preferentially suppressed hepatic glucose production compared to insulin in normal and diabetic humans (16).

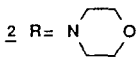
Sulfonylurea Insulin Secretagogues - In 1984, two "second generation" sulfonylureas, glyburide and glipizide were marketed in the U.S. (4,17,18). In spite of differences in plasma half-lives (4), volumes of distribution (4) and milligram potencies (17) of the newer sulfonylureas, glyburide, glipizide, gliclazide and glibornuride, no clear therapeutic advantages over older drugs such as chloropropamide or tolazamide have been demonstrated. In addition to causing increased pancrea-

tic secretion of insulin, sulfonylureas have been shown to increase tissue sensitivity to insulin (19,20). This latter effect can be demonstrated in a number of ways including measurement of increased insulin-mediated glucose metabolism (21,22).

Guanidine Insulin Secretagogues - The orally active hypoglycemics pirogliride (McN-3495, 1) and linogliride (McN-3935, 2) were selected for development from a large series of substituted guanidines (23). The pharmacology of 2 has been reviewed (3). In phase II clinical evaluations, 1 effectively reduced fasting and postprandial glucose levels and glucosuria in noninsulin-dependent diabetic (NIDDM) patients who had previously not responded to combined sulfonylurea and diet therapy (5). Side effects led to the replacement of 1 by 2 in clinical trials (5,24). In preclinical studies, 2 stimulated insulin secretion in vivo and had hypoglycemic activity in normal mice, rats and dogs and diabetic rodents (24). As observed with 1, analog 2 displayed a different insulin secretory mechanism from tolbutamide in isolated pancreatic islets (25). Preliminary clinical studies with 2 in NIDDM subjects have noted hypoglycemic efficacy concomitant with elevated insulin and c-peptide levels (26-28).

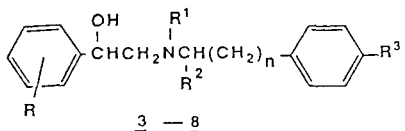


1 R= 

2 R= 

Adrenergic Insulin Secretagogues - Adrenergic, cholinergic and neuropeptide regulation of pancreatic endocrine secretion in both animals and man has been recently reviewed (29). Pancreatic tissues contain both alpha and beta adrenoreceptors, which inhibit and stimulate insulin secretion, respectively. Clinical studies with isoproterenol (30) and phentolamine (31) indicate that selective adrenergic compounds may provide potential novel agents for diabetic therapy (31,32). Interestingly, sulfonylureas have been reported to displace alpha- and beta-adrenergic radioligands from pancreatic islet preparations (33).

Beta agonist arylethanolamine analogs display both antidiabetic and antiobesity activities (34,36). As thermogenic antiobesity agents, 3-8 increase metabolic rate (37-41) through propranolol-inhibitable activation of both brown (37,40) and white (40-43) adipose tissue lipolysis with subsequent augmentation of substrate oxidation.



3 — 8

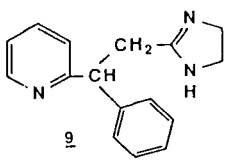
		R	R^1	R^2	R^3	n
3	BRL 26830	H	H	CH_3	CO_2CH_3	1
4	BRL 35135	3-Cl	H	CH_3	$OCH_2CO_2CH_3$	1
5	LY 79771	H	H	CH_3	OH	2
6	LY 104119	H	H	CH_3	$CONH_2$	2
7	RO 16-8714	H	$\phi CH(OH)CH_2$	CH_3	CO	2
8	TA 078	2-OH	H	H	CO_2CH_3	1

Independent of effects secondary to weight loss, compounds 3 and 4 display antihyperglycemic activity which is equipotent to second generation sulfonylureas in normal mice and slightly less potent in normal rats (44). BRL 26830 (3) stimulates insulin secretion in normal (44) and some diabetic models (45,48), but concurrently elevates glucose production (46,47).

The mechanism by which antidiabetic effects occur in diabetic rodent models is not well understood. Acute administration of 3 or 4 to insulin resistant diabetic rodents either exacerbates their glucose intolerance or has no effect (44,45,48). With multi-week administration of 3 or 4, however, glucose intolerance and other diabetic symptoms are improved or normalized in db/db mice (49,50), ob/ob mice (44,46,47,50) and Zucker rats (45,48) without significant reduction of body weights. Early clinical studies with 3 have shown variable effects on weight loss in obese subjects, with no antidiabetic trials yet reported (51,52).

In multi-week dosing studies, LY 79771 (5) decreased insulin or glucose levels of diabetic mice (38), while LY 104119 (6) reduced the hyperglycemia (39). Ro 16-8714 (7) reduced blood glucose and plasma insulin levels of ob/ob mice and Zucker rats after chronic dosing (40). TA-078 (8), an *in vitro* insulin secretagogue, was inactive in streptozotocin-diabetic rats and improved glucose tolerance of normal animals and diabetic KK mice (53).

Alpha$_2$ antagonists have also been used to increase insulin secretion. Midaglizole (DG 5128, 9), decreased fasting glucose levels and/or improved glucose tolerance in normal animals and diabetic A^{y}KK mice (53-56). Clinical studies with 9 have demonstrated similar activities in normal (57) and NIDDM subjects (58). Activity in both animals and man is apparently secondary to stimulation of insulin secretion (56-59), although effects on glucagon secretion (58) and peripheral tissue glucose utilization (60) may contribute. *In vitro*, the insulin secretory mechanism of 9 differs from that of tolbutamide (60). Imiloxan (RS 21361, 10), also stimulates insulin secretion in rats *via* an alpha$_2$-antagonistic mechanism (61-64).



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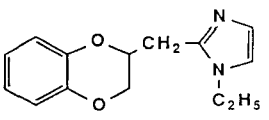

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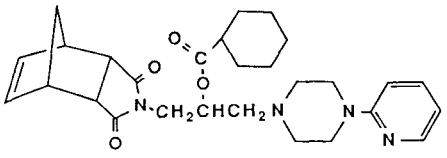


11

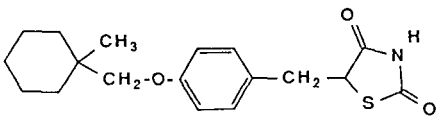
Other Insulin Secretagogues - Several nonsulfonylurea, insulin secretagogues have been reported recently. One of the most interesting is E-0713 (11), which suppressed hyperglycemia following glucose load in elderly rats, genetically and streptozocin-subdiabetic mice and rats and is in clinical trial (65).

Insulin Resistance - Target-tissue insulin resistance is characteristic of both IDDM (66) and NIDDM (67) patients. Therapeutic agents capable of reversing this resistance could significantly advance diabetes treatment. Insulin resistance can be separated into two components: a receptor-associated reduction in insulin "sensitivity" and post-receptor-mediated loss in insulin "responsiveness" (68); both components have been demonstrated in man (69). Insulin resistance is particularly important in NIDDM (70) and its reduction following exercise (71), sul-

fonylurea (72), biguanide (73), and insulin (72) therapy has been documented.

Ciglitazone (ADD-3878, 12) has shown activity in many normal and diabetic animal models (74,75). Treatment with 12 enhanced insulin action in muscle (76) and adipose tissue (77,78), and reduced hepatic gluconeogenesis (79). It also lowered plasma triglycerides and free fatty acids (74,75) which may explain some of its activity (79). In limited clinical testing, 12 lowered fasting blood glucose and improved glucose tolerance in NIDDM patients and reduced plasma insulin during a glucose-tolerance test (80). Recently, more potent analogs were disclosed (81).

Ascochlorin (AS-6, 13) has shown activity in two diabetic models (82). In addition, adipocytes from treated db/db mice exhibited increased insulin action and binding (78), altered lipid composition (84), and plasma membrane protein and phosphoprotein (85) composition more like nondiabetic controls.



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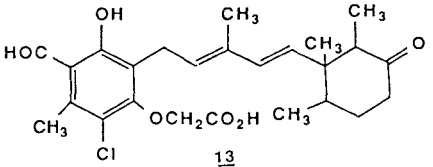
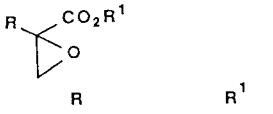


13

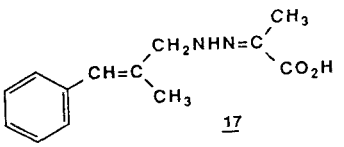
METABOLIC INTERVENTION TO RELIEVE HYPERGLYCEMIA

Fatty acid oxidation inhibition - The importance of the carnitine palmitoyl transferase (CPT) system in the physiological regulation of fatty acid oxidation (86) and the rationale for reduction of fatty acid oxidation in diabetes (87,88) have been discussed. Glycidates (14-16) are potent inhibitors of CPT. The synthesis, pharmacology, and mechanism of action of methyl palmoxirate (McN-3716, methyl TDGA, 14) has been reviewed (89), and the structure-activity of the analogs published (90). Its hypoglycemic and antiketonemic mechanism (91-93) and its inhibition of CPT-I (94) have been studied further. The active form in situ is the R-CoA ester corresponding to 14 which is proposed to act as an enantioselective, active-site-directed, irreversible inhibitor of CPT-I (94,95). While clinical testing is limited, the results are consistent with the animal data (96,97). The activity of clomoxir (POCA, 15) in vitro (98) and in vivo (99,100) is similar to 14. For etomoxir (16), only limited preclinical data is available (101), however, a phase I study has been reported (102).

The activity profile of MCHP (BM 42-304, 17) is similar to 14 and 15 but differs by its site of inhibition within the mitochondrial CPT system (103-105) and by its inhibition of intestinal glucose absorption (107). It is currently in clinical trial.



	R	R^1
14	$CH_3(CH_2)_{13}$	CH_3
15	4-ClC_6H_4-$(CH_2)_5$	Na
16	4-$ClC_6H_4O(CH_2)_6$	C_2H_5



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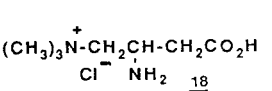


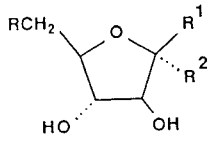
18

Aminocarnitine (emeriamine, 18) and its analogs are potent inhibitors of carnitine-utilizing enzymes and show in vivo hypoglycemic and antiketonemic activity in fasted and diabetic rodents (108-110).

Compounds 14, 15 and 17 all affect the heart CPT system (89,111,112). From studies with the cardioselective CPT inhibitor oxfenicine (p-hydroxyphenylglycine), the inhibition of heart fatty acid oxidation reduces "metabolic capacity" and imposes an increased energy demand which is compensated for by hyperemia in the short-term and by hypertrophy in the long-term (113). Consistent with these findings, both 14 and 15 produce myocardial weight increases in rodents after high-level chronic dosing (112,114).

Inhibition of liver gluconeogenesis - Renewed interest in this approach has been stimulated by two findings: the identification of fructose-2,6-bisphosphate (19) as a physiological regulator of gluconeogenesis/glycolysis (115) and the recognition that fasting hyperglycemia in NIDDM is a direct consequence of elevated liver glucose production (116).

In hepatocytes, the fructose-like compound 2,5-anhydromannitol (20) is phosphorylated and produces a pattern of enzyme activation and inhibition consistent with a fructose-1,6,-bisphosphate analog (117-119). This results in decreased gluconeogenesis and glycogenolysis (120). Oral hypoglycemic activity has been demonstrated in fasted and diabetic rodents (120).

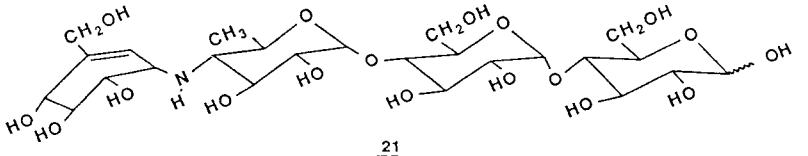


19 $R,R^2=H_2O_3PO$, $R^1=CH_2OH$

20 R=OH, R^1=H, $R^2=CH_2OH$

MODULATORS OF CARBOHYDRATE ABSORPTION

Studies in animals (121) and man (122) indicate that postprandial excursions of plasma insulin and glucose can be reduced by delaying intestinal carbohydrate digestion. Inhibiting the enzymes which cleave the terminal glucose units from starch and other dietary oligosaccharides is one way of achieving this and has led to the development of several α-glucosidase inhibitors. Flatulence and occasional diarrhea are common side effects, but can be ameliorated by lower doses, admixture with food (123) and individual accommodation over time (124).

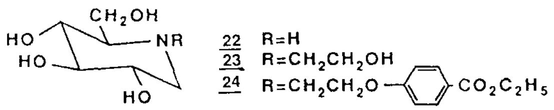


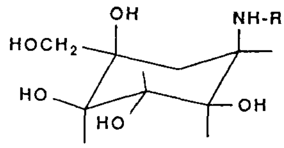
21

Acarbose, 21, the most widely studied glucosidase inhibitor, is hypoglycemic in streptozocin rats and ameliorates diabetic nephropathy in db/db mice (125). In IDDM patients, 21 reduced postprandial hyperglycemia and insulin requirements (122,124). Compound 21, used as adjuvant therapy to sulfonylureas or diet, improved glycemic control in NIDDM patients (126). The treatment of obesity through acarbose-induced malabsorption of carbohydrates was studied in animals and man with mixed results (127).

Derivatization of 1-desoxynojirimycin 22 gave rise to miglitol (Bay m 1099, 23) and emiglitate (Bay o 1248, 24) which have different dura-

tions of activity in man (128-130). In addition, 23 also exhibited moderate β-glucosidase inhibitory activity (131).





25 R=H, 26 R=CH(CH$_2$OH)$_2$

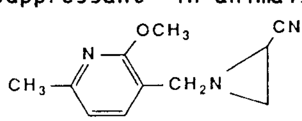
Other differences among 21, 23 and 24 are seen in their affinity for sucrase (21>23>>24) and in their effects on glucoamylase (127,131). AO-128 (26), a derivative of valiolamine (25), is suggested to be a more potent glucosidase inhibitor in vitro (121,127) and in vivo than acarbose and is currently in clinical trial (121). Two α-amylase inhibitors, trestatin complex and tendamist, have also been studied clinically. Trestatin complex (Ro9-0154), a water soluble mixture of complex oligosacaharides trestatin A, B and C, is active in vitro and in both normal (132) and diabetic humans (133). Tendamist (Hoe-467A), a 74-amino acid protein isolated from Streptococcus Tendae strain 4158, has been shown to bind tightly to mammalian α-amylase (134) and to lower blood glucose levels in normal volunteers following ingestion of a starch meal (135).

ALDOSE REDUCTASE INHIBITORS

The use of insulin and a variety of antihyperglycemic agents does not prevent the occurrence of the secondary complications of chronic diabetes, viz., neuropathy, nephropathy, retinopathy and cataracts. There is substantial experimental evidence in animals for the relationship between elevated aldose reductase (AR) activity, the resultant increased accumulation of sorbitol and the onset and maintenance of the pathology of secondary diabetic complications (136,137). Excellent reviews presenting the evidence for the involvement of AR (136,140), and the effects of drugs on this enzyme and on the course of experimentally-induced diabetes have been published. In vitro inhibition of AR has been observed for thousands of compounds, but the incidence of potent in vivo activity following oral administration is relatively rare (139). The structural, pharmacokinetic and tissue distribution requirements for uncovering compounds with greater potency/selectivity have yet to be defined.

IMMUNOMODULATORS IN DIABETES

The autoimmune etiology of IDDM is supported by the occurrence of cytotoxic T-lymphocytes specific to pancreatic B-cells, by the occurrence of islet antibodies (141,142) and by the clinical efficacy of cyclosporin in IDDM patients (143). Further studies on the expression of aberrant human leukocyte antigen (HLA) in the B-cell of the pancreas also support the hypothesis of autoimmune attack in IDDM (144). Unfortunately, the immunosuppressive agents, e.g., cyclosporin, are nonspecific in their action and widely suppress autoimmune reactions necessary for natural defenses of the subject (143). Ciamexone, (27), an immunosuppressant in animals and man (145), caused remissions in 8 out of 11 IDDM patients and appears to be better tolerated than cyclosporin (146). A large multi-center clinical trial of 27 in IDDM patients is planned (146).



27

Summary - Although numerous mechanistic approaches to achieving improved metabolic control in both IDDM and NIDDM exist, therapeutically useful antidiabetic agents have consisted mainly of insulin and the sulfonylurea insulin secretagogues for over a decade. The entry into clinical trial of several agents with novel mechanisms may expand the methods of treating diabetes. Among these are compounds 3, 4 and 9 which interact with pancreatic adrenoreceptors and compound 12 which decreases insulin resistance. In addition, fatty acid oxidation inhibitors (14, 16 and 17), proinsulin and somatostatin analogs all have shown antihyperglycemic activity in humans. Clinical trials of carbohydrate absorption modulators (e.g., 21) support their imminent entry into clinical use. It is not yet clear that the achievement of glycemic control, by either current or future agents, will significantly prevent the development of chronic complications. Aldose reductase inhibitors may provide new therapy for preventing the progression of some diabetic complications. Finally, the prospect of immunological intervention in IDDM is an exciting area for future research.

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Section V - Topics in Biology
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Chapter 22. Regulation of Phospholipase A_2

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Introduction - Phospholipases catalyze the hydrolysis of ester bonds in glycerophospholipids. Various ester bonds are attacked by distinct types of enzymatic activities (phospholipases A_1, A_2, C and D). Although these enzymes are intimately involved in cellular phospholipid metabolism, phospholipases A_2 and C have been the focus of much attention, because of their crucial role in cell activation. In a wide variety of cells, both phospholipases A_2 and C are rapidly activated upon specific stimulation. Several recent reviews have dealt with various aspects of phospholipases C and A_2 (1-6). Following a brief discussion of phospholipase C, this chapter will focus primarily on regulation of intracellular phospholipases A_2.

Phospholipase C- Activation of phospholipase C leads to the degradation of inositol phospholipids generating inositol phosphates and 1,2-diacylglycerols. Inositol-1,4,5-trisphosphate releases Ca^{2+} from intracellular stores (7) and 1,2-diacylglycerols activate protein kinase C, a novel phospholipid/Ca^{2+}-dependent enzyme (8). In addition, Ca^{2+} and 1,2-diacylglycerols function synergistically to promote various cell responses such as secretion (8). Although it has been generally accepted that mammalian phospholipase C exhibits strict specificity for inositol phospholipids (1,4), recent studies have demonstrated the existence in cardiac tissue of a phosphatidylcholine-specific phospholipase C (9). Cell-free preparations obtained from cultured murine smooth muscle cells hydrolyze both phosphatidylinositol and phosphatidylcholine in a phospholipase C-catalyzed reaction (10). In several cell types, this latter enzyme is activated upon specific stimulation (11,12).

Phospholipase A_2- Activation of phospholipase A_2 leads to the degradation of major glycerophospholipids generating free arachidonic acid (AA) and lysophospholipids (4). The AA is converted to prostaglandins, thromboxanes, leukotrienes and various hydroxyarachidonates via the cyclooxygenase and lipoxygenase (LO) enzyme systems (13). In addition, AA activates protein kinase C (14) and releases Ca^{2+} from intracellular stores such as endoplasmic reticulum (15). In certain inflammatory cells such as neutrophils, lysoplatelet-activating factor (1-O-alkyl-2-lyso-sn-glycero-3-phosphocholine) is a major product of phospholipase A_2 activation. This lysoderivative is rapidly acetylated by a specific acetyltransferase to form platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF) (16,17). AA metabolites as well as PAF are potent mediators of allergic and inflammatory reactions (13,16,17).

Recent attempts to purify phospholipase A_2 activity have revealed the existence of multiple forms of this enzyme (18,19). In human platelets, phosphatidylcholine and phosphatidylethanolamine are degraded by two distinct enzymatic activites (18). Sheep platelets possess at least two distinct families of phospholipases A_2, one of which exists in dimeric form (58-kDa) and exhibits remarkable specificity for ether-linked phospholipids (19). Yet another distinct enzyme may hydrolyze phosphatidic acid and phosphatidylinositol in horse platelets (20). Interestingly, AA is preferentially concentrated in the ether-linked phospholipids such as 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine and plasmalogen phosphatidylethanolamine (16,17).

Role of Ca^{2+} in phospholipase A_2 activation - In most cells, phospholipase A_2 activation is closely linked to the occupancy of Ca^{2+}-mobilizing receptors (4). The Ca^{2+}-ionophore A23187, which introduces Ca^{2+} into the cell interior without receptor activation, is a potent inducer of phospholipase A_2 (21-23). In cell-free preparations, phospholipase A_2 also requires Ca^{2+} for activity (18,19,23). Thus, a rise in intracellular Ca^{2+} concentration appears to be obligatory for phospholipase A_2 activation. Much of the Ca^{2+} regulation in eukaryotes is mediated by the ubiquitous intracellular calcium receptor calmodulin (24). In several cell types, calmodulin antagonists such as trifluoperazine (25) inhibit AA release (26,27). Because several cellular proteins in addition to calmodulin bind to calmodulin antagonists in a Ca^{2+}-dependent manner (28), criteria other than drug inhibition are needed to confirm the involvement of calmodulin in phospholipase A_2 activation. An earlier claim that calmodulin stimulates phospholipase A_2 in platelet homogenates (29) could not be confirmed (30).

Recent studies using human promyelocytic leukemia (HL60) cells indicate that a rise in intracellular Ca^{2+} concentration is necessary but not sufficient for phospholipase A_2 activation (23). HL60 leukemia cells possess limited capacity to release AA and to form PAF. However, upon differentiation to mature granulocytes, they acquire these capacities. This inability of undifferentiated HL60 cells is explained by neither the lack of appropriate phospholipase A_2 activities, substrate deficiencies nor enhanced acylating capacity. Apparently, factors that are essential for phospholipase A_2 activation develop during granulocytic differentiation (23).

The possibility that phospholipase A_2 activation might also involve Ca^{2+}-independent mechanisms has been raised by several recent studies (18,23). For example, a phosphatidylethanolamine-specific phospholipase A_2 purified from human platelets does not require Ca^{2+} for activity (18). Furthermore, in HL60 cell homogenates, a phospholipase A_2 degrades phosphatidylcholine in the absence of free Ca^{2+} (23). Whether these so-called Ca^{2+}-independent enzymes are involved in enhanced deacylation of phospholipids during cell activation remains to be seen. A role for such enzymes in basal phospholipid turnover deserves consideration.

Modulation of phospholipase A_2 by lipids - It is becoming increasingly clear that 1,2-diacylglycerols are important intracellular mediators (8). Although the plasma membrane is impermeable to these diacylglyerols, certain synthetic diglycerides such as 1-oleoyl-2-acetylglycerol (OAG) can readily permeate this membrane (8). OAG as well as

the tumor promoting phorbol esters such as phorbolmyristateacetate (PMA) mimic 1,2-diacylglycerols in activating protein kinase C (8). In several cell types including neutrophils (31,32), mast cells (33) and platelets (34,35), PMA augments phospholipase A_2 activation initiated by ionophore A23187. Similarly, OAG potentiates ionophore-induced AA release in human platelets (35). However, these agents by themself are ineffective at inducing phospholipase A_2 activity (31-35). In rabbit peritoneal neutrophils, cytochalasin B greatly enhances AA release induced by the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine, although cytochalasin B by itself is inactive (36). This enhancement of AA release may be mediated at least in part by diacylglycerol, because in human peripheral neutrophils stimulated with chemotactic peptides, cytochalasin B causes dramatic accummulation of diacylglycerols (37). Furthermore, in human neutrophils, cytochalasin B mobilizes intracellular Ca^{2+} and prolongs the increase in intracellular Ca^{2+} concentration induced by chemotactic peptides (38). Taken together, these results suggest a synergistic interaction between diacylglycerols and Ca^{2+} in the activation of phospholipase A_2. It is not clear whether PMA and diacylglycerols exert their effects on phospholipase A_2 directly or indirectly via a mechanism involving protein kinase C.

In human neutrophils, inhibitors of 5-LO suppress both AA release and PAF formation (39). These inhibitions are overcome by the addition of hydroperoxy- or hydroxy-AA (5-HPETE or 5-HETE). Furthermore, these metabolites greatly potentiate ionophore A23187-induced AA release and PAF formation, although these agents by themselves are ineffective (39). Hence, the 5-LO metabolites are capable of amplifying the phospholipase A_2 response initiated by Ca^{2+}. The mechanism of this amplification is presently unknown.

Crude cell-free preparations of platelets show little phospholipase A_2 activity (40). However, during protein purification, phospholipase A_2 activity is greatly enhanced (40). This enhancement has been attributed to the removal of cell-associated free fatty acids. Purified phospholipase A_2 preparations are inhibited by unsaturated free fatty acids at micromolar concentrations. Cells contain free fatty acids in amounts sufficient for the suppression of phospholipase A_2 (40). The physiological significance of these findings remains to be clarified.

Involvement of guanine nucleotide-binding regulatory proteins in phospholipase A_2 activation - A family of guanine nucleotide binding proteins (G proteins) functions in receptor-linked signal transduction, coupling receptor activation to modulation of effector systems such as adenylate cyclase, phospholipase C and cGMP phosphodiesterase (41,42). Cholera and pertussis toxins stoichiometrically ADP-ribosylate specific G proteins, altering their capacity to function in signal transduction (41,42). Pertussis toxin interacts with inhibitory G proteins, stabilizes their inactive forms and thereby blocks their functions (41,42). Cholera toxin, on the other hand, stabilizes the active state of stimulatory G proteins and perpetuates their functions (41,42).

In several cell types including mast cells (43), neutrophils (44), fibroblasts (45) and endothelial cells (46), pertussis toxin blocks AA release induced by specific receptor activation. Ionophore A23187-induced AA release is not affected by pertussis toxin (43-45).

In rod outer segments of bovine retina, light activates phospholipase A_2 activity, releasing AA from exogenous phosphatidylcholine (47). This AA release is inhibited by pertussis and cholera toxins and is mimicked by GTPγS, a highly potent nonhydrolyzable GTP analog (47). In addition, depletion of rod outer segment membranes of transducin, the relevant G protein, diminishes the light activation of phospholipase A_2 and the activity is partially restored when transducin is added to the depleted membranes (47). In a thyroid cell line (FRTL5), norepinephrine stimulates AA release from endogenous sources and this release is inhibited by pertussis toxin (48). Addition of GTPγS to permeabilized FRTL5 cells or to crude membrane preparations releases AA from exogenous phosphatidylcholine (48). This effect of GTPγS is potentiated by norepinephrine (48). Although in neutrophils both phospholipases A_2 and C are inhibited by pertussis toxin (44), in several other systems mentioned above (45,46,48), pertussis toxin inhibits AA release but not phosphoinositide-specific phospholipase C. This indicates the involvement of distinct G proteins in phospholipase A_2 activation. More significantly, these observations are suggestive that phospholipase A_2 activation is intimately linked to specific receptors and can occur independent of prior activation of phospholipase C.

Modulation of phospholipase A_2 by endogenous inhibitory proteins - Glucocorticoids are potent antiinflammatory drugs that suppress both acute and chronic inflammation by inhibiting virtually every step in the inflammatory process (49-52). All steroids including glucocorticoids mediate their effects by binding to specific intracellular receptors located in the cytoplasm of target cells (49-52). Activated steroid-receptor complexes then translocate to the nucleus of the cell, where they interact with chromatin. This interaction modulates the expression of specific genes, leading to the synthesis of mRNA and subsequently to the synthesis of specific enzymes or proteins that mediate the effects of the steroids (53). Whereas all active steroids are capable of binding to intracellular receptors, the affinity of binding correlates with the intrinsic potency of the steroid (54).

The antiinflammatory action of glucocorticoids has been attributed at least in part to the inhibition of phospholipase A_2 activities (55-57). Glucocorticoids inhibit synthesis of all products of AA metabolism by suppressing the release of AA from phospholipids (55,56). Recently, several groups of researchers have independently identified a family of phospholipase A_2 inhibitory proteins, which are induced and secreted when cells are treated with glucocorticoids (55-57). Related proteins have been detected in peritoneal exudates from rats (58) and rabbits (59), macrophages (60), renal medullary cells (61), fibroblasts (62), splenic lymphocytes (63) and thymocytes (64). These proteins (15-40kDa) were variously known as macrocortin (60), lipomodulin (59) and renocortin (61). With the realization that all these proteins are functionally identical and that all the active fragments derive from the same precursor, a unified nomenclature, lipocortin, has been adopted (65). The predominant active form of lipocortin has a molecular mass of 40-kDa (56,57). The assay routinely used to monitor lipocortin activity utilizes pancreatic phospholipase A_2 with autoclaved *E. coli* membranes or purified phosphatidylcholine as substrate. The effectiveness of lipocortin against hydrolysis of other phospholipids, phospholipid mixtures or phospholipids of intact membranes from mammalian cells has not been documented.

The partially purified preparations of lipocortin mimic the effects of glucocorticoids and mediate their antiinflammatory activity in various in vivo model systems such as carrageenan-induced paw edema in rats (55,61,66). In addition, lipocortin blocks neutrophil chemotaxis, cytotoxic action of peripheral blood lymphocytes (67) and immunoglobulin synthesis (68). Furthermore, glycosylation inhibitory factor, a 15-kDa fragment of lipocortin (69), upon repeated injections into antigen-primed mice, suppresses the formation of immunoglobulin E and G both prophylactically and therapeutically and facilitates the generation of antigen-specific suppressor T lymphocytes (70). Autoantibodies against lipocortin have also been detected in sera of patients with chronic inflammatory diseases such as systemic lupus erythematosus (71).

The amino acid sequence of rat lipocortin has recently been established (72). Using this sequence information, cDNA for human lipocortin has been cloned and expressed in E. coli (73). In addition, two 40-kDa proteins with in vitro phospholipase A_2 inhibitory activity have been purified from human placenta (74). These two proteins, designated as lipocortins I and II, are immunologically distinct. However, based on sequences deduced from cDNA clones they are structurally related, showing approximately 50% homology (74).

Sequence information from several laboratories (74-78) has revealed that lipocortins I and II are homologous to proteins p35 (79) and p36 (80), respectively. These proteins, also known as calpactins, are the predominant cellular substrates of transforming tyrosine-specific protein kinases and growth factor receptor protein kinases. They have many common features but can be distinguished structurally and immunologically (81). The p36 protein is phosphorylated on tyrosine in cells transformed by many oncogenic retroviruses that encode tyrosine-specific protein kinases (82). The p35 protein is also phosphorylated on tyrosine in cells stimulated with epidermal growth factor and platelet-derived growth factor (82). One rather interesting feature of p35 and p36 is that they bind to membranes and phospholipid vesicles as well as to actin and spectrin in a Ca^{2+} - dependent manner (83-85). It has been speculated that these proteins might function by linking membrane phospholipids to cytoskeletal elements such as actin and spectrin (81).

The homologous sequences in lipocortins can be aligned as four 70-80 amino acid repeats and these repeats contain Ca^{2+} -, phospholipid- and actin-binding sites (83-85). Within each repeat, there is a 17 amino acid consensus sequence (78), which appears to be essential for the calcium/phospholipid-binding properties of lipocortins. The tyrosine phosphorylation sites are located within the first 30 N-terminal amino acids (74-78). Interestingly, these N-terminal regions of lipocortins I and II show no sequence homology (74). The central repeat region contains the phospholipase A_2 inhibitory activity of lipocortins (74). An additional family of proteins (32-36kDa), which bind to phospholipid vesicles in a Ca^{2+} - dependent manner, has been isolated from diverse tissues including electroplax of Torpedo, adrenal medulla, liver and intestinal epithelium (86). These proteins possess the lipocortin consensus sequence with similar internal repeats (87).

Lipocortins are abundant cellular proteins widely distributed among tissues (74). However, the physiological significance of these proteins to the regulation of intracellular phospholipase A_2 is unclear. Because of their phospholipid-binding property, it is possible that they limit the accessibility of phospholipase A_2 to membrane lipids. Indeed, lipocortin inhibition of in vitro pancreatic phospholipase A_2 appears to involve substrate depletion by direct substrate/inhibitor complex formation and not specific interaction with the enzyme (88). Phosphorylation of intracellular lipocortin might alter the phospholipid-protein interactions, enhancing the availability of membrane phospholipids to phospholipase A_2. If this is true, then lipocortins may be envisioned as physiological regulators not only of phospholipase A_2 but also of other enzymes whose activities depend on the availability of lipid substrates and/or modulators.

The functional consequences of lipocortin phosphorylation on phospholipase A_2 inhibition have not been fully explored. Earlier studies have shown that phosphorylation of purified lipocortin results in the loss of its inhibitory activity (89). Furthermore, glycosylation inhibitory factor inhibits phospholipase A_2 but only after treatment with alkaline phosphatase (69). In mitogen-stimulated thymocytes (90) and in platelets treated with thrombin or PMA (91), enhanced phospholipase A_2 activity may correlate with lipocortin phosphorylation. In a variety of tumor cells and in epidermal growth factor-induced cells where tyrosine-specific protein kinase activity is enhanced, AA release occurs at elevated levels (92). In A431 cells, lipocortin is phosphorylated by tyrosine-specific protein kinase activity of the epidermal growth factor receptor (93). In thymocytes treated with mitogen, lipocortin is phosphorylated at a tyrosine residue (90). In stimulated platelets, 40-kDa protein, the presumed substrate for protein kinase C, is heavily phosphorylated (8,91). Protein kinase C phosphorylates lipocortin in vitro (94,95) and in vivo (94). Ca^{2+} enhances phosphorylation of lipocortin (calpactin) by a tyrosine-specific protein kinase (96). Thus, in stimulated cells lipocortin may be phosphorylated via Ca^{2+} regulated protein kinases with consequent loss of its inhibitory activity and enhanced expression of phospholipase A_2. Recent availability of recombinant lipocortin should facilitate the confirmation of this interesting hypothesis.

Endogenous phospholipase A_2 activating proteins - Peptidoleukotrienes stimulate AA release in cultured murine smooth muscle cells and bovine aortic endothelial cells (97,98). This effect is blocked by prior treatment of the cells with cycloheximide or actinomycin D (97,98), suggesting a requirement for protein and RNA synthesis. Leukotriene D_4 treatment of intact cells results in an increase in phospholipase A_2 activity as measured in cell homogenates with phosphatidylcholine as a substrate (10). Phospholipase C activity is unchanged (10). The persistent activation of phospholipase A_2 may be due in part to the proteolytic cleavage of a proenzyme. It is known that the inactive zymogen of pancreatic phospholipase A_2 is converted to the active form by tryptic cleavage of a heptapeptide moiety from the N-terminus (99). Isolation of a leukotriene D_4-inducible protein with phospholipase A_2 stimulatory activity has been noted (10).

Inhibition of phospholipase A_2 - Activation of phospholipase A_2 is an early, rate-limiting step in the cascade of reactions leading to the formation of prostaglandins, thromboxanes, leukotrienes and PAF.

Because these lipid mediators are involved in a variety of disorders, phospholipase A_2 is an attractive target for therapeutic intervention. However, phospholipase A_2 activities are also involved in phospholipid remodeling that is critical to cellular integrity. Thus, direct inhibition of phospholipase A_2 activities could lead to undesirable toxic effects. A more logical approach should, therefore, focus on preventing activation of phospholipase A_2 without affecting basal turnover of phospholipids. It is presently uncertain whether phospholipase A_2 activities involved in basal phospholipid turnover are distinct from those that are activated upon cell stimulation. However, recent studies have clearly indicated that intracellular phospholipase A_2 activity exists in multiple forms with distinct properties (18-20). Such diversity may be exploited to discover compounds capable of direct inhibition of specific phospholipase A_2 species.

Despite intensive research, there are, at present few compounds that directly inactivate phospholipase A_2 (4). More recently, it has been found that cyclosporin A, an immunosuppressive agent, inhibits pancreatic phospholipase A_2 *in vitro* (100). It also blocks prostaglandin production by zymosan-stimulated rat peritoneal macrophages, presumably by inhibiting phospholipase A_2 activity (100). Tiaramide, a nonsteroidal antiinflammatory drug, inhibits the activation of phospholipase A_2 in stimulated rabbit platelets without blocking Ca^{2+} mobilization or calmodulin effects (101). Manoalide, a sesterterpenoid originally isolated from the sponge *Luffariella variabilis*, inhibits phosphatidylcholine hydrolysis by venom phospholipase A_2 (102,103). This compound binds to the enzyme in a pH-dependent, irreversible manner (102,103). Manoalide prevents PMA-induced inflammation of the mouse ear, but has no effect on AA-induced inflammation (104) indicating a mechanism involving the inhibition of phospholipase A_2 activity. Furthermore, manoalide inhibits the release of AA in stimulated rabbit neutrophils (36). It should be stressed, however, that all of the currently available phospholipase A_2 inhibitors lack the desired specificity or potency.

Phospholipase A_2 activation is a complex multifactorial process (Fig. 1) offering multiple sites for intervention. An approach to phospholipase A_2 modulation with considerable therapeutic potential involves lipocortins. Although these proteins themselves may be of limited therapeutic value, the precise knowledge of their conformation and active sites should facilitate rational design of heterocyclic compounds with selective phospholipase A_2 inhibitory properties. If an essential role for protein kinases such as protein kinase C in the functioning of lipocortins is confirmed, then specific inhibition of such enzymes should constitute another practical approach. Initiation of phospholipase A_2 activity upon cell stimulation appears to be linked to such fundamental biochemical mechanisms as phospholipase C activation and G proteins. However, the amplification of phospholipase A_2 stimulation may be mediated by peripheral steps such as LO reactions that are readily inhibited.

Summary - Our understanding of the regulation of phospholipase A_2 enzymes is advancing rapidly. It has been recognized that 1,2-diacylglycerols and 5-LO metabolites can regulate phospholipase A_2 activation by a positive feedback control mechanism. Although an obligatory role for Ca^{2+} in phospholipase A_2 activation is emphasized, data indicate that additional factors are required for optimal

activity. One such requirement may be the involvement of guanine nucleotide binding proteins. Finally, lipocortins have been cloned and sequenced, revealing their identity with the major cellular substrates for tyrosine-specific protein kinases. Current views on the regulation of intracellular phospholipase A_2 activation are summarized schematically in Fig. 1.

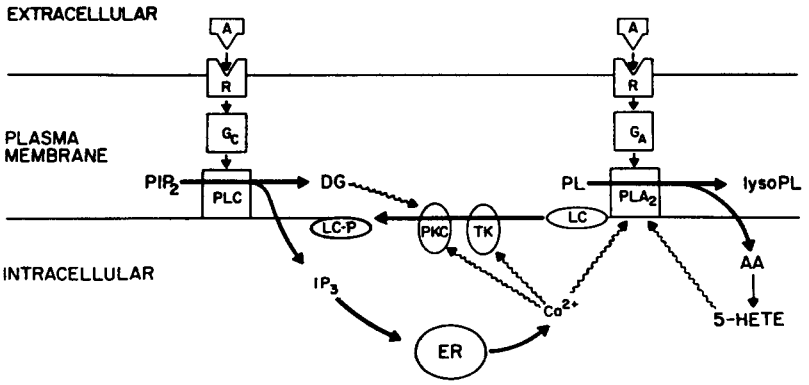

Fig. 1. Scheme showing the coordinate control of activated phospholipase A_2 reaction via various modulators. Interaction of an agonist (A) with a specific receptor (R) activates phospholipase C (PLC) via a specific GTP-binding regulatory protein (G_C) resulting in the degradation of phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce 1,2-diacylglycerol (DG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 releases Ca^{2+} from the endoplasmic reticulum (ER), raising the levels of intracellular free Ca^{2+}. Ca^{2+} activates tyrosine-specific protein kinases (TK), and DG in combination with Ca^{2+} activates protein kinase C (PKC). As a result of kinase activation, active lipocortin (LC) is phosphorylated to inactive lipocortin (LC-P) relieving the inhibitory constraint on phospholipase A_2 (PLA_2) exerted via LC interaction with PLA_2 and/or phospholipids (PL). Interaction of an agonist (A) with specific receptor (R) 'primes' PLA_2 via distinct GTP-binding regulatory protein (G_A). The reaction is then initiated by intracellular Ca^{2+} with consequent hydrolysis of PL, generating lysophospholipids (lysoPL) and free AA. AA is converted via the 5-LO activity to 5-HETE, which further potentiates PLA_2 through an unknown mechanism.

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Chapter 23. Response of the Endothelium to Tumor Necrosis Factor (Cachectin) and Interleukin-1

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Introduction. The response of the endothelium to inflammatory stimuli has recently received a great deal of attention due, at least in part, to the realization that it is not just a passive, vascular tissue, but an active cellular component of the inflammatory response. Inflammatory stimuli may directly affect the endothelium, causing increased vascular permeability, altered antigen presentation and enhanced adhesiveness of polymorphonuclear leukocytes and macrophages. In addition, the endothelium may become prothrombotic by increasing production of plasminogen activator inhibitor, and may also display immune functions such as expression of cell surface receptors for Fc and complement. The purpose of this review is to discuss the role of the endothelial response to tumor necrosis factor (cachectin) and interleukin-1, cytokines with proinflammatory properties, and the potential for pharmacologic modulations of these reponses. Numerous other proinflammatory factors such as bradykinin, complement and eicosanoids, although they are also important, will not be discussed.

Heterogeneity of the Endothelium

The endothelial lining is not a discrete homogenous layer, but is composed of continuous, fenistrated and sinusoidal tissues (for review see 1). The continuous endothelium found in muscle, brain and connective tissue, is characterized by an intact layer of endothelial cells that separates the lumen from the extravascular space. In contrast, fenistrated capillaries are composed of cells with numerous 80-100 nm pores that are usually covered by a thin membrane. This type of endothelium is found in renal glomeruli and endocrine glands. The sinusoidal endothelium in endocrine glands, spleen and liver is characterized by large and irregular spaces. In addition to the histological differences, the responses of the three types of endothelium to inflammatory stimuli varies as will be described for tumor necrosis factor. Most of the in vitro studies have been performed using endothelial cells derived from large bore arteries and veins, because they are easier to isolate. The response of the endothelium of individual capillary types to inflammatory stimuli is still largely unexplored. Investigations of inflammatory stimuli using one type of endothelium may, therefore, not be directly applicable to responses of other types.

The Response of the Endothelium to Inflammatory Stimuli. Increases in endothelial permeability in response to various factors is frequently measured in vivo by dye extravasation. However, studying this response in cell culture has been difficult, primarily because the

morphological response is difficult to quantitate. Disposable cell culture chambers have recently been developed that consist of an inner chamber, the bottom of which is a porous membrane, where the endothelial cells may be grown. This inner chamber is in turn placed in a dish containing media. The confluent endothelial cell monolayer prevents the diffusion of protein bound dye from the upper into the lower chamber (2). An inflammatory stimulus may be added to the upper chamber and the amount of dye diffusing into the lower chamber may be measured either spectrophotometrically or radiometrically. This procedure allows direct quantitation of endothelial cell retraction, facilitating a greater understanding of this important response.

A variety of inflammatory stimuli affect the endothelium and can induce the synthesis of several additional autocoids by the endothelium itself (for review see 3). At least some of these stimuli cause continuous endothelium to develop large gaps between adjacent cells, leading to plasma exudation and lymphocyte, macrophage and polymorphonuclear leukocyte (PMN) migration into extravascular spaces. Typically, the PMN are the first cells to attach to the endothelium. These cells produce additional chemotactic agents such as LTB_4 and release superoxide anions and specific granule contents that can kill not only the invasive organisms, but also the endothelial tissue. Macrophages also attach to the endothelium and, in turn, generate interleukin-1 and tumor necrosis factor, as well as prostaglandins and leukotrienes. These cells also secrete factors that induce endothelial cells to divide and produce new capillaries which permeate the site of inflammation and enhance the inflammatory response. The endothelium may also acquire some functions of macrophages such as the expression of receptors for complement and the Fc portion of antibodies, the presentation of antigen to B cells and an accessory role in generating antibodies.

Regulation of Endothelial Cell Function by Interleukin-1. The term interleukin-1 (IL-1) describes a family of proteins produced by a number of cell types in response to inflammatory stimuli. Although originally thought to be exclusively a product of activated monocytes and macrophages, it is now known that many other cell types including B cells, keratinocytes, epithelial cells, fibroblasts, microglia and endothelial cells can produce IL-1. Currently, two genes coding for two distinct proteins are known, but the possibility exists that additional IL-1 genes coding for other proteins are still undiscovered (4-6). IL-1 is synthesized as a 31 kD precursor molecule, which is subsequently cleaved to a mature molecule of 17.4 kD (7). The cellular (or extracellular) enzymes responsible for this processing step are currently unknown.

Our knowledge of the biological effects of IL-1 has expanded, since its original definition as a T-cell co-stimulator molecule. Both natural and recombinant IL-1 have endogenous pyrogen activity, stimulate neutrophil mobilization from the bone marrow, promote acute phase protein levels in the plasma, and stimulate bone loss and cartilage degradation. Thus, the targets of IL-1 include the hypothalamus, hepatocytes, osteoclasts, chondrocytes, synovial cells, epithelial cells, neutrophils, macrophages, B-cells, fibroblasts and endothelial cells (for review see 8,9). The scope of the biological effects of IL-1 on so many target tissues has strongly implicated this molecule as one of the key mediators of a number of physiological and pathological processes, including both acute and chronic events.

One of the earliest observed effects of IL-1 on endothelial cells (EC) in culture was the expression of a tissue factor-like procoagulant activity (PCA) by endothelial cells, implicating IL-1 in both physiological and pathological clotting processes (10). Peak activity was reached within 6 hours of exposing the cells to IL-1, and declined to baseline levels within 24 hours. The cells were refractory to restimulation with IL-1 at this time. The significance of these studies was greatly enhanced by the in vivo studies showing that intravenous infusion of recombinant IL-1 resulted in a 10-fold increase in tissue factor activity of the aortic endothelium, which reached a peak within 5 hours after the infusion (11). Furthermore, the expression of endothelial-mediated protein C activation, an important pathway of anticoagulant activity, was suppressed by more than 70%. The prothrombotic activities of IL-1 were extended when it was discovered that IL-1 also induces the production by EC of one or more inhibitors of plasminogen activator (PA). IL-1 induced a PA-inhibitor in vitro, which was effective in inhibiting both TPA and urokinase activities (12,13). IL-1 did not appear to affect TPA production by EC. These observations were extended to an in vivo setting, where it was observed that both IL-1 and lipopolysacrides (LPS) could increase the levels of a plasma inhibitor of a PA (14). Taken together, these results suggest an important role for IL-1 in the promotion of blood coagulation following the introduction of inflammatory agents.

Another of the observed effects of IL-1 on endothelial cells is potentiation of the adhesiveness of leukocytes to endothelial cell surfaces in vitro. A dose-dependent enhancement of the adhesion of human polymorphonuclear leukocytes (PMN) or a human promonocytic cell line U-937 resulted when human umbilical vein endothelial cells were preincubated with IL-1 (15). A two hour induction period between exposure of the EC to IL-1 and the increased adhesiveness was observed. A similar effect was produced by bacterial LPS. Inhibition of protein synthesis by cycloheximide or actinomycin D abrogated the adhesion. It was also observed that the tumor promoting phorbol diesters had similar activity on endothelial cells but that interleukin-2 and interferon-gamma did not enhance PMN adhesiveness (14). Pretreatment of the leukocytes with IL-1 did not enhance adhesiveness, strongly implicating EC as the target of the IL-1 effect (17). Treating other cell types such as fibroblasts with IL-1 does not enhance their ability to bind leukocytes, suggesting that this IL-1 effect is EC specific.

The adhesion of T and B cells to vascular endothelial cells is also enhanced by treatment of the EC by IL-1, and, as with PMN, the target of the IL-1 is the EC and not the T or B cell (18,19). In contrast, phorbol myristate acetate or phorbol dibutyrate-treated T cells show enhanced adhesiveness to human endothelial cells, but treating the EC with phorbol esters did not enhance adhesiveness (20). The possibility that the mechanism of T-cell adhesiveness to endothelial cells may be distinct from IL-1 induced adhesion was further supported by experiments designed to explore the role of a cell surface antigen, lymphocyte function-associated molecule (LFA-1), which is also found on neutrophils, in the adhesion of T cells to EC. Monoclonal antibodies to LFA-1 could inhibit phorbol-enhanced adhesion of T-cells, although the enhancement was not mediated by an increased expression of LFA-1 (21). In contrast, this same monoclonal antibody had no effect on the ability of IL-1 or LPS to enhance adhesion of

T-cells to EC. However, it is possible that IL-1 induces the expression of other EC-surface molecules involved in the adhesion of leukocytes. IL-1 and TNF also induce the expression of a cell surface antigen (defined by a monoclonal antibody, H4/18) that may be involved in leukocyte adherence (22).

These cytokines also increase the expression of another monoclonal-antibody-defined EC molecule, termed intercellular adhesion molecule 1 (ICAM1), that is also implicated in leukocyte adhesion. IL-1-mediated induction of ICAM1 on EC correlated with increased adhesion of T-cells (23). The exact relationship between LFA-1 and ICAM1, and their respective roles in T-cell and neutrophil adhesion to EC is still uncertain. One hypothesis is that the two molecules actually interact in the formation of the adhesive bond between the cells. Thus, the role of IL-1 would be to enhance the expression of the EC receptor (ICAM1) for the leukocyte adhesion molecule (LFA-1), or a more complex CDw18 antigen complex (24).

IL-1 has been shown to induce the production and release of a number of proinflammatory mediators from endothelial cells. These would include platelet activating factor (PAF) (25), prostacyclin and prostaglandin E2 (26), and superoxide anions (27). Each of these has its own pattern of effects on endothelial cell function and make the interpretation of the direct versus indirect effects of IL-1 more difficult. The observations that both EC themselves and those cells that interact with the endothelium respond to and produce IL-1 (see below) strongly suggests that it plays a key role in regulating endothelial cell function.

The Production of IL-1 by Endothelial Cells. The role of IL-1 in the physiology of vascular endothelium is not limited solely to effects of IL-1 on those cells, but includes the production of IL-1 by endothelial cells stimulated with inflammatory agents. LPS and thrombin can induce the release of IL-1 activity from cultured EC (28). The IL-1 production peaked at 12 hours, and was inhibited by cycloheximide. Furthermore, the IL-1 activity could be removed on an anti-IL-1 affinity column. In addition the IL-1 that was produced had biochemical characteristics similar to both alpha and beta IL-1 (29). TNF can also stimulate IL-1 production by endothelial cells (30). This ability of TNF to induce IL-1 correlated with the binding of TNF to endothelial cells. Furthermore, both TNF and LPS can induce specific mRNA for IL-1 in endothelial cells (31), and interferon gamma can enhance the production of IL-1 by LPS (32). This enhancement was demonstrated only when the cells were pretreated for 24 hours with interferon before the addition of LPS, although the IFN was not required in the cultures during the period of stimulation with LPS. IFN alone did not affect either intracellular or extracellular IL-1 levels.

Regulation of Endothelial Cell Function by Tumor Necrosis Factor. Tumor necrosis factor (TNF), also known as cachectin, is a soluble protein mediator produced by activated macrophages, that was initially identified by its ability to induce hemorrhagic necrosis of transplanted tumors in mice (33,34). Subsequently, TNF was also found to have direct cytotoxic effects on many human and murine tumor cell lines *in vitro* but not on normal mouse embryonic fibroblasts nor many non-transformed cell lines (34,35,36). The tumor-specific cytotoxicity of TNF motivated many laboratories to clone and express

its gene, hoping to develop and produce a "natural" therapeutic antitumor drug by recombinant DNA techniques (37,38,39). However, recent clinical trials revealed toxicity in man. TNF may, therefore, not materialize into an antitumor or antiviral drug in its own right. However, a diverse range of biological activities have recently been uncovered using recombinant TNF, including effects on the endothelial cells *in vitro*, suggesting an important *in vivo* pathophysiological role of TNF on the vascular system.

Invasive gram-negative infections often induce vascular collapse and shock in the host (40,41). The acute "shock" state frequently results in lethal multiple organ failure and is characterized by fever, and vascular leakage and hypotension. Multiple system organ failure remains a principle cause of death after major operative procedures and/or severe trauma despite impressive advances in antimicrobial drugs and supportive therapy (42). Bacterial endotoxin (lipopolysaccharide, LPS), a component of gram-negative bacterial cell walls, mediates septic "shock" by inducing the production of endogenous mediator(s) including TNF (43). Macrophages appear to be the principal host cell that mediates endotoxin toxicity (44,45). TNF is synthesized *in vivo* primarily by activated macrophages as these cells can be induced *in vitro* by endotoxin to produce TNF (46,47). TNF is now thought to be a major endogenous mediator of endotoxin-induced shock since passive immunization of mice with polyclonal and monoclonal antisera against TNF/cachectin can protect them against the lethal effect of endotoxin. Moreover, when administered in quantities similar to those produced endogenously by animals in response to endotoxin, TNF induces shock and tissue damage similar to those induced by endotoxin in animals; hypotension, fever, metabolic acidosis, hyperglycemia, hyperkalemia, and hemoconcentration can occur within minutes to hours, as does death due to respiratory arrest (47,48).

Lymphotoxin, synthesized primarily by mitogen-stimulated lymphocytes, is a different protein which shares only 35% identity and 50% similarity with TNF/cachectin at the protein level (49,50), yet the biological activities studied to date are identical to TNF. Natural lymphotoxin is a secreted glycosylated protein while TNF is not glycosylated. TNF is now designated as TNF-α and lymphotoxin as TNF-β, and in this review, TNF is used synonymously with TNF-α. The genes for TNF and lymphotoxin are arranged tandemly on chromosome 6 of human or on chromosome 17 of mouse, in close proximity to the major histocompatibility complex (MHC) (51,52,53). Moreover, the human TNF genes were shown to be included within the MHC, most likely telomeric of the class II region. TNF to enhances transcription of the class I major histocompatibility genes (54), and the genes for TNF in humans (37,38,39), mice (54) and rabbits appear to be highly conserved.

In addition to its roles in toxic shock and killing of tumor cells, TNF is also an endogenous pyrogen, an activity shared by interleukin-1 (IL-1) and interferons. *In vivo*, these agents act directly on the hypothalamus thermo-regulation center and indirectly by inducing IL-1 production (55). Using cultured human umbilical vein or adult human saphenous vein endothelial cells, TNF has been shown to induce synthesis and release IL-1 (31,56). IL-1 causes endothelial cells to express procoagulant activity leading to blood clotting (57), promote adhesion of leukocytes (58), increase prostanoid synthesis (59,60), and undergo shape changes (61). These alterations in

endothelial functions by IL-1 appear to play an important role in inflammation and wound healing as well as in the pathogenesis of vascular diseases (62). Many of these alterations have also been demonstrated with TNF which stimulates the adhesion of neutrophils to umbilical vein endothelium (63), induces procoagulant activity in cultured human vascular endothelium (64), modulates the hemostatic properties of bovine aortic and human umbilical vein endothelial cells by enhancing procoagulant activity tissue factor, and suppresses the protein C pathway, an antithrombotic mechanism that functions on the surface of quiescent endothelial cells (65). TNF also stimulates the expression of the same endothelial activation antigen found at the site of inflammation in vivo (65). TNF acts singly or in combination with immune interferon to reorganize human vascular endothelial cell monolayers; TNF treated human umbilical vein endothelial cells become elongated, overlap, rearrange their actin filaments and lose their stainable fibronectin matrix (66). TNF modulates the morphology of cultured vascular endothelial cells (67). Upon treatment with TNF, human umbilical vein endothelial cells became elongated, bovine endothelial cells exhibited reversible irregular shortened shapes and the typical cobblestone morphology was disturbed, while bovine capillary endothelial cells became spindle shaped. TNF also inhibited proliferation of bovine aortic endothelial cells and smooth muscle cells but was not cytotoxic to these cells (67). However, TNF is cytotoxic to bovine capillary endothelial cells. These studies exemplify the diverse effects of TNF on various endotheliums. The distinct morphological changes of endothelial cells induced by TNF may lead to increased vascular permeability and attachment of platelets to the vascular walls, while the cytostatic/cytotoxic effects of TNF on endothelial cells may result in suppression of neovascularization and tissue injury.

TNF binding sites have been found on several cells, including endothelial cells (68,69). Antagonists to TNF such as antibodies or receptor blockers may, therefore, have prophylactic and/or therapeutic value against cachexia and/or acute septic shock for which no satisfactory treatment regimen is currently available.

The Roles of IL-1 and TNF in Endothelial Cell Physiology. A number of investigations have demonstrated that many of the biological effects of IL-1 on endothelial cells are mimicked by TNF. With the recent availability of recombinant IL-1 and TNF, the relative concentrations of the two mediators necessary to produce the same effect can now be investigated with more certainty. Where both mediators have been compared side by side on a quantitative basis, IL-1 has been more potent than TNF. For example, in a recent study both TNF and IL-1 induced procoagulant activity (62). Similar levels of procoagulent activity were induced with either 500 units/ml TNF, or 5 units/ml IL-1. The specific activities of the two molecules were reported as approximately 1.9×10^7 units/mg protein and 1×10^8 units/mg protein for TNF and IL-1, respectively. Therefore, on a molar basis, approximately 500 times as much TNF was needed to produce the same effect as IL-1. Taken along with the observation that cultured monocytes make very little TNF compared to IL-1, caution should be taken in evaluating the respective roles of these two molecules, even though, on a qualitative basis, they appear to be quite similar. In certain situations such as endotoxin shock there is clear evidence that TNF is a key mediator in the development of intravascular coagulation and endothelial damage (61). Most likely, we will have to

wait for the development of specific IL-1 and TNF antagonists in order to fully evaluate the role of each of these molecules in the functioning of endothelial cells *in vivo*.

Phospholipase Stimulation by IL-1 and TNF. IL-1, TNF and other inflammatory stimuli increase the release of eicosanoids from endothelial cells. Eicosanoids produced by these stimuli may contribute significantly to the inflammatory process. The importance of the prostaglandin synthetic response may be to modulate the effects of other inflammatory stimuli. Clinically this aspect of the inflammatory response may be aborted with the use of nonsteroidal antiinflammatory drugs. Because the rate limiting step in eicosanoid biosynthesis appears to be the release of arachidonic acid from phospholipids by phospholipase enzymes (70), it would appear that IL-1 and TNF should activate phospholipases in the endothelial cells. Although IL-1 has been shown to activate PLA_2 in chondrocytes (71), it has not been demonstrated that either TNF or IL-1 activate phospholipase A_2 in endothelial cells. However, IL-1 causes platelet activating factor (PAF) to be produced by endothelial cells (72). PAF is presumed to be produced by phospholipase A_2, which hydrolyzes 1-O-alkyl phosphatidylcholine. It has been proposed that this event could give rise to two bioactive products, free arachidonic acid which could be converted into eicosanoids and PAF (for review see 73).

Summary. IL-1 and TNF affect the endothelium and can contribute to the pathophysiology of several disorders. For example, a stimulus such as a localized bacterial infection initiates the accumulation of PMNs through the generation and release of chemotactic peptides. The bacterial components (such as LPS) activate both the neutrophil and the EC, promoting their mutual interaction and adhesion, and inducing the production of IL-1 by the EC. This IL-1 then serves to enhance neutrophil binding even further, as well as stimulating the generation of other inflammatory mediators from EC, neutrophils, and infiltrating T and B cells. Monocytes drawn to the site are in turn stimulated to release larger quantities of IL-1 and TNF and as the circulating levels of IL-1 and TNF build a typical systemic response to infection develops, including mobilization of more neutrophils from the bone marrow (leukocytosis), fever, and the acute phase response. Vasculitis may develop due to the damaging enzymes released by the bound and activated neutrophils. Endothelial cell damage may then initiate the clotting cascade, which is promoted by IL-1 and TNF through its induction of procoagulant activity and a plasminogen activator-inhibitor. In the case of an overwhelming release of bacterial toxins, the above scenario may be played out on a systemic level, leading to disseminated intravascular coagulation and hemorrhagic necrosis. Because these mediators of inflammation induce the production of other proinflammatory mediators, the consequence of this sequela of events is frequently death of the organism. Thus it is apparent that the regulation of this complex cascade of mediators is crucial in determining whether the outcome is benefical or harmful. Unregulated production and action of both IL-1 and TNF as well as the eicosanoids can have severe effects on the host. The development of agents that regulate these cytokines or their signal transduction process may provide new targets for therapeutic intervention.

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Chapter 24. Mediators of the Pain of Inflammation

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Introduction - Recent advances in the understanding of the chemical mediators of the pain of inflammation offer new biochemical and pharmacological targets for the development of novel analgesics. This chapter will review some of these advances and highlight some of the major unresolved questions. Emphasis will be placed on the mediators produced at the site of the inflammation, but some discussion of mediators in the CNS is also included. A comprehensive survey of many aspects of pain has been published recently(1).

Characteristics of the Pain of Inflammation - The pain of inflammation requires time to develop after the application of the noxious stimulus(2). This distinguishes it from the "immediate" response to intense stimuli (heat, electroshock, pin prick) in non-inflamed tissues(2). The time of onset of pain can vary from between a few minutes, in the case of the writhing response to intraperitoneal injection of irritants(3), to several hours, in the case of the pain induced by subplantar injection of yeast in rats(4-6). There is no clear association in various models between the time course of the pain response and the time course of other parameters of inflammation, such as edema and cell emigration, suggesting that different mediators may be involved(3,7,8). Inflamed tissues exhibit tonic pain in the absence of other obvious stimuli. However, intense pain can be superimposed on this background of tonic pain by stimuli at levels below the pain threshold of non-inflamed tissue, *i.e.* inflammation induces hyperalgesia(1,2). Some of the pain that is perceived as tonic pain could, in fact, be due to hyperalgesia induced responsiveness to non-obvious stimuli. Possible non-obvious stimuli include hydrostatic tissue pressure (which could be elevated due to inflammatory edema) or tissue distortion induced by the pulsatile nature of blood flow (also elevated in inflamed tissues and responsible for "throbbing" pain).

Animal Models of Inflammatory Pain - A wide range of rating scales and models for the study of pain and analgesics in man have been evaluated and each has its uses and limitations(9-11). In animal models, a behavioral response to a noxious stimulus is measured that is more accurately described as a nociceptive response rather than a pain response. These techniques have been recently reviewed(12-14).

One of the most commonly used animal models is the writhing response induced in rats or mice by intraperitoneal injection of one of the many suitable irritants such as acetic acid, phenylquinone, zymosan, oxidized archidonic acid, acetylcholine(2,3,12-15). In addition to its simplicity, this procedure has the advantage of permitting convenient collection of inflammatory fluids by peritoneal lavage to enable the levels of putative mediators(3,15,16) and local concentrations of analgesics(17,18) to be determined. A variety of techniques have been developed from the original paw hyperalgesia model of Randall and Selitto(4). The original method involved injection of an irritant (yeast) into the hind paw of rats and quantification of the resulting hyperalgesia by measuring the reduction in the pressure required to elicit withdrawal of the paw or vocalization. Variations of this method include measurement of biting or licking of paws inflamed with formalin in cats(19), rats(19,20) or mice(21,22); decreased spontaneous locomotor activity in rats(23) or mice(24) with inflamed hind paws and alterations in gait(25). In several recent studies, the levels of mediators in inflamed, hyperalgesic rat paws have been determined(26-28). A variety of instrumental methods have been introduced to assess the load borne by an inflamed joint in dogs(29), cats(30) or rats(31). Adjuvant arthritis in rats remains the most widely accepted model of chronic inflammatory pain(32-34) and a wide variety of methods of evaluating pain intensity have been devised: respiratory pattern(35), spontaneous vocalization(36), vocalization elicited by manipulating the joints(37), locomotor activity(39,40) and paw pressure threshold(40).

All of the above methods require meticulous control of the environmental conditions and animal handling must be rigorously standardized in order to avoid the powerful influences of stress. For example, the stress involved in simply transferring a rat from its home cage to an experimental cage is reported to

produce an analgesic effect equivalent to 2 mg/kg of morphine(41). It has also been demonstrated that a second inflammatory lesion in the same animal exerts an analgesic action on the first(42). This phenomenon needs to be borne in mind when administering potentially irritant materials parenterally to animals used in such studies. Congressional modifications to the Animal Welfare Bill could impact on pain research(43). However, scientists in this area have already established guidelines similar to those mandated by the new laws(44-46).

Prostaglandins - Evidence for the involvement of prostaglandins (PGs) in inflammatory pain has accumulated over the last 25 years (reviewed in 2). The case for a major role of PGs has generally been strengthened by more recent studies(47-48). A number of significant uncertainties still exist, however, and these are discussed below. Several PGs - PGE_1, PGE_2, PGI_2 - produce hyperalgesia when injected into peripheral tissues(48). However, there are only a few circumstances in which the particular endogenous PG mediating the pain response in a given inflammatory lesion has been identified. PGE_1 and other PGs derived from dihomogammalinolenic acid are unlikely to be important because this fatty acid is normally a minor component of mammalian lipids(49). Consequently, only very low levels of these prostanoids can be expected in inflammatory lesions. The writhing response to i.p. injection of zymosan in mice has been clearly shown to be mediated by PGI_2, although small amounts of PGE_2 were also present(15). PGE_2 and $PGF_{2\alpha}$ were detected in the peritoneal fluids of rats in which writhing was induced by acetic acid; however, the possible presence of PGI_2 was not examined(16). Intraperitoneal administration of PGE_2 potentiated phenylquinone-induced writhing in mice and reversed the analgesic activity of indomethacin in this model; other PGs were not studied(50). Writhing models provide a convenient method to determine the levels of PGs in inflammatory fluids obtained from animals exhibiting a quantifiable pain response; however, the conclusions drawn from these studies may not be applicable to other models. For example, it was demonstrated that low doses of PGI_2 injected into rat paws or dog knee joints, induced a transient hyperalgesia with a rapid onset while PGE_2 induced persistent hyperalgesia of delayed onset(51). It has been suggested that PGI_2 may mediate inflammatory pain of relatively rapid onset, such as writhing, and PGE_2 may mediate persistent pain of delayed onset such as carrageenan and yeast-induced paw hyperalgesia(48). PGE_2 levels are, in fact, elevated in inflamed rat hind paws, but the possible presence of PGI_2 was not examined (26-28). Identification of the particular endogenous PG involved in mediating clinical inflammatory pain could permit the development of analgesics which act by reducing the level of the relevant PGs through inhibition of the appropriate PGH_2 isomerase rather than reducing the levels of all PGs through inhibition of the cyclo-oxygenase enzyme itself. Such information could also allow development of analgesics which are antagonists at the appropriate PG receptor subtype(52,53). Preliminary studies in this direction have been hampered by the limited potency and selectivity of the available antagonists(54,55).

The hyperalgesic activity of locally administered PGs has been demonstrated in numerous human(56) and animal(2,51) studies but direct pain-producing effects have been seen in only a few studies at relatively high doses(57,58). These latter observations could be the result of PG-induced hyperalgesia enabling non-obvious, normally subthreshold, stimuli to elicit pain. Of particular note is the induction of writhing in mice by intraperitoneal injection of carbacyclin(59), a stable PGI_2 analogue, or PGI_2 itself at the concentrations found in the inflamed peritoneal cavity(15). This raises the possibility that PGI_2 is a direct pain-producing mediator. Fatty acid hydroperoxides have pain-producing properties in man(56) and animals(58,60) and such fatty acid peroxides are generated by cyclo-oxygenase, and lipoxygenases, enzymes involved in the synthesis of PGs and leukotrienes(61). It, therefore, seems possible that these enzymes could be responsible for producing both hyperalgesic agents (PGE_2, PGI_2) and mediators that directly stimulate pain receptors (fatty acid hydroperoxides).

A further point of considerable interest is the possibility that PGs enhance elicitation of pain by facilitating transmission in the central nervous system, in addition to their hyperalgesic effects in the periphery. The earliest suggestions along these lines followed the observation that acetaminophen, an antipyretic analgesic with limited anti-inflammatory activity, did not inhibit cell-free PG synthetase *in vitro* at reasonable concentrations unless the enzyme was obtained from nervous tissue(62). Furthermore, oral administration of acetaminophen does not inhibit PG synthesis in a number of peripheral tissues(63) but does inhibit PG synthesis in the CNS *in vivo*(64). This activity is known to be the mechanism underlying its antipyretic effects(64). Administration of exogenous PG directly into the CNS of rodents increases sensitivity to pain(65-69) although very high doses may have hypoalgesic effects(67,68).

It is now known that release of interleukin 1 from inflamed tissues will induce synthesis of PGs in the central nervous system(70). Therefore, all the necessary components are in place to justify the

following hypotheses: inflammation increases synthesis of PGs in the CNS, these PGs facilitate pain perception, and acetaminophen and other cyclo-oxygenase inhibitors have a central component to their mechanism of action(71-73). However, data from other studies conflict with the above hypothesis: (a) local (intraperitoneal) injection of PGI_2 completely reversed the ability of indomethacin to inhibit zymosan-induced writhing in mice(15) indicating that there is no central mechanism involved; (b) injection into the cerebral ventricles of the acidic PG synthesis inhibitors, zomepirac(17) or tolmetin sodium(18) produced no inhibition of acetic acid-induced writhing in rats while intraperitoneal injection of the same dose was effective, indicating that the mechanism of action of these compounds is entirely peripheral. Some of the conflict in the literature could be due to the nature of the experimental models used. When the inflammatory trauma is sufficiently severe and prolonged, sufficient IL-1 may be produced to raise CNS PG levels and influence pain perception. For example, carrageenan paw edema in rats raises the concentrations of PGs in the CNS(74) and injection of PG synthesis inhibitors directly into the cerebral ventricles inhibits carrageenan-induced paw hyperalgesia(71). Other models, such as writhing, are of short duration and may not produce sufficient IL-1 release to elevate CNS PG levels. The non-acidic PG synthesis inhibitors appear to have a greater central component to their analgesic activity than the acidic agents(72).

Amines - It has not been possible to demonstrate a peripheral role for histamine in models of inflammatory pain, despite evidence that local injections of histamine do induce hyperalgesia or pain(75). The widely reported analgesic activity of certain histamine antagonists(24,75-77) cannot be considered evidence for a peripheral role for histamine since most of these compounds also have local anesthetic activity, interact with cholinergic, serotonin and dopamine receptors, and have marked CNS pharmacology(75).

5-Hydroxytryptamine (5-HT, serotonin) has long been known to induce hyperalgesia when administered peripherally(2). It has no direct pain producing activity(78) unless the tissue is already sensitized by inflammation(79). The neuronal 5-HT receptor mediating this response, and the flare response to intradermal injection of serotonin in man, has been classified as a 5-HT_3 (previously known as "M") receptor subtype(80-83). In contrast, the vascular receptors mediating 5-HT-induced edema have been classified as 5-HT_2(84), while those mediating vasomotor effects are of both 5-HT_2 and 5-HT_1 subtypes(80,81,85). The nomenclature of 5-HT receptor subtypes has recently been standardized(86). Release of serotonin from platelets is implicated in the pathogenesis of migraine(87) and 5-HT_3 subtype specific 5-HT receptor antagonists are being investigated as potential anti-migraine drugs(82,83,88). High concentrations of 5-HT are found in the mast cells of rodents and 5-HT plays a major role in immediate hypersensitivity reactions, such as passive cutaneous anaphylaxis, in these species(89). The low concentration of 5-HT in human mast cells(89) explains the absence of a role for 5-HT in human allergic reactions and it remains to be seen whether 5-HT plays any peripheral role in human inflammatory pain. However, 5-HT is the neurotransmitter acting at several points in the central nervous system and spinal cord involved in "pain appreciation" where it mediates both hyperalgesic and analgesic effects(90). The 5-HT receptor subtypes of these central neuronal sites also provide targets for the development of novel analgesics(86,90,91).

It has been reported that norepinephrine injected directly into the rat hind paw potentiates the hyperalgesia induced by injury (topical application of chloroform)(92). Norepinephrine did not affect the pain threshold in non-injured paws. The potentiating effect of norepinephrine required both intact postganglionic sympathetic innervation and PG synthesis, and was apparently mediated by α-adrenergic receptors(92). Other workers have also demonstrated a catecholamine-mediated mechanism underlying that component of carrageenan-induced hyperalgesia of rat paws that is not blocked by PG synthesis inhibitors(93). In this case, the use of receptor antagonists provided ambiguous data, implicating either the β-adrenergic receptor(94) and/or the dopamine D_1 receptor(93). Despite their differences, these reports support earlier evidence on the involvement of the sympathetic nervous system in inflammatory pain, particularly when damage to nerves is involved(95,96). Pharmacological studies in this area are complicated by the powerful analgesic effects of catecholamines mediated *via* central α-adrenergic receptors(97).

Chemotactic Agents and Lipoxygenase Products - It has recently been demonstrated that injection of the chemotactic agents LTB_4(98), C5a(99) and N-formyl-methionyl-leucyl-phenylalanine [FMLP](99) into the rat hind paw induces hyperalgesia. These authors reported that the hyperalgesia was not inhibited by indomethacin, indicating that the mediator(s) involved is not PG(s)(98,99). FMLP and LTB_4-induced hyperalgesia was absent in polymorphonuclear leukocyte (PMN) depleted rats, but was restored by

infusions of PMN from syngeneic donor rats. Supernatants from PMN stimulated *in vitro* with FMLP or LTB_4 contained a lipid factor which, when injected into the paw, produced hyperalgesia in normal, PMN-deficient and indomethacin-treated rats(98,99). (8R,15S)-diHETE, a product of the 15-lipoxygenase enzyme of stimulated neutrophils, was identified as the factor responsible for this activity, while (8S,15S)-diHETE, a stereoisomer of the hyperalgesic factor, exhibited hypoalgesic activity and behaved as a specific competitive antagonist of (8R,15S)-diHETE(100). The above data identify a new family of mediators involved in the regulation of inflammatory pain and offer exciting possibilities for the development of novel analgesics.

Other laboratories have provided supportive evidence for at least some components of the above hypothesis. Platelet-activating-factor (PAF), also a chemotactic factor for PMN (both neutrophils and eosinophils)(101), has been shown to induce hyperalgesia when injected into the hind paws of rats(102-106). PAF also activates lipoxygenase in PMN(107) and there is evidence that some of the effects of PAF are mediated through lipoxygenase products, although the point has been disputed(107,108). PAF-induced edema and hyperalgesia in rat paws and skin are relatively resistant to inhibition by indomethacin(102-106, 109-112), although the hyperalgesia is inhibited by a number of lipoxygenase inhibitors(105,106). PAF also elicits pain/hyperalgesia when injected intradermally in human volunteers(113). The available data on PAF are consistent with it being involved in inflammatory pain *via* induction of the synthesis by PMN of lipoxygenase metabolites of arachidonic acid; however, the case is far from proven.

The hyperalgesic effects of LTB_4 have been confirmed in man(114) and rats(6). LTB_4 also induces edema and this response, like the hyperalgesia response(98), is dependent on the presence of PMN, although the mechanism of this phenomenon is not known(115). In other studies, it was found the LTB_4 injected into the rat paw with Brewer's yeast produced minimal potentiation of the maximal yeast-induced hyperalgesia but did accelerate its onset and completely eliminated the early hypoalgesia which precedes hyperalgesia(6). The time course of yeast-induced paw hyperalgesia was found to correlate with the levels in the paws of LTB_4 and cyclo-oxygenase products(26,28), but not with levels of 5-HETE and LTC_4 or with edema(28). Furthermore, a PG synthesis inhibitor (indomethacin)(28), a lipoxygenase inhibitor (phenidone)(28) and a dual inhibitor (BW755C)(27,28) all inhibited hyperalgesia, implicating both cyclo-oxygenase and lipoxygenase products in the mediation of yeast-induced paw hyperalgesia.

Not all forms of inflammatory pain can be readily accommodated by the PMN/15-lipoxygenase model outlined above(98-100). The hyperalgesia induced in rat paws by irritants (carrageenan, yeast) is accompanied by PMN accumulation, but is readily inhibited by PG synthesis inhibitors, leaving only a relatively small component to be attributed to PMN lipoxygenase products(116). This may be explained by a synergistic interaction between PGs and PMN lipoxygenase products under these conditions. Writhing responses to irritants occur before PMNs appear in the peritoneal cavity and are, in fact, over before the first PMNs arrive(3,15). In such circumstances, it is possible that resident cells (macrophages, mast cells) could be the source of 15-lipoxygenase products(100). In contrast to data cited above, it was demonstrated that LTB_4 (and the peptidoleukotrienes, LTC_4 and LTD_4) antagonized the nociceptive response to intravenous bradykinin in the perfused rabbit ear(117). The absence of PMN from the perfusate used in the rabbit model could explain this apparently anomalous result.

Kinins - Bradykinin (BK) and its close relatives, Lys-BK (kallidin) and Met-Lys-BK, are potent pain-producing peptides that are liberated from precursor proteins (kininogens) in plasma by specific serine proteases kininogenase, kallikrein)(118-120). Kinins produce overt pain when applied to peripheral tissues, in marked contrast to most other mediators, whose major effects are to increase sensitivity to other stimuli (induce hyperalgesia)(118,120). The pain-producing effects of BK are markedly potentiated by PGs and BK, itself, induced cells to synthesize PGs. It is, therefore, no surprise that BK-induced pain can be inhibited by PG synthesis inhibitors(118-120). In addition to the "classical" kinins, noted above, "T-kinin" (Ile-Ser-BK) was recently identified in rats and has been found in rat inflammatory fluids(121-123). The plasma concentration of the precursor protein for T-kinin (T-kininogen, also known as major-acute-phase protein or MAP) increases up to 7-fold after injury, due to increased synthesis in the liver(121-123). T-kininogen also inhibits thiol proteases(124), complicating interpretation of its biological role. It is not clear if an equivalent to the rat T-kinin/kininogen system exists in other species. The bioassay techniques used for the assay of kinins in inflammatory fluids are being replaced by immunoassays(125) and HPLC(122). Such techniques are much less susceptible to interference from other biologically active materials and also allow the four naturally occurring kinins to be differentiated. Such techniques have yet to be applied to the study of inflammatory pain.

The pharmacology of kinins has been reviewed and the existence of two subtypes of bradykinin receptor, B_1 and B_2, has been proposed(120,126). The B_2 subtype mediates the inflammatory and pain-producing properties of BK and several B_2 specific antagonists are under investigation(126-128). The availability of such antagonists, or specific kallikrein inhibitors, would enable the contribution of kinins to inflammatory pain to be determined. The absence of such specific reagents had compelled earlier workers to use notoriously non-specific reagents to deplete kininogens (*e.g.* cellulose sulphate, saliva, ellagic acid, dextran sulphate) or inhibit kallikrein (*e.g.* soy-bean trypsin inhibitor, aprotinin, hexadimethrine bromide)(129). A substrain of the Brown Norway rat developed at the Catholic University in Leuven, Belgium, is deficient in the components of the "classical" kinin system(130). These rats have reduced inflammation responses to some irritants and provide a unique tool for the study of the role of kinins in pain(130). They remain in short supply(131) and no reports on inflammatory pain in these animals have been published. Kinins have also been found in the brain(132) and ICV injections of bradykinin produced a number of pharmacological effects, including analgesia(133).

Neuropeptides - The central role of opiate receptors in the modulation of pain perception is well established. In addition, it has been observed that injection of opiate agonists, opiate antagonists and enkephalins into the rat hind foot pad inhibited inflammation induced hyperalgesia in that paw(48,134). The doses used did not affect the contralateral paw, confirming the local site of action. Quaternary derivatives of "pure" opiate antagonists, methylnalorphinium and methylnaloxone, were analgesic when administered locally or systemically, despite the fact that they do not cross the blood brain barrier. The expected antagonist activity of these compounds was observed at some doses(48,134). Similar evidence for analgesia mediated by peripheral opiate receptors has been obtained by other workers(135-137) and there is also confirmation of the analgesic activity of opiate antagonists in animals with inflammation(136-139). It is clear that inflammation, and/or the resulting stress, activates the endogenous opiate system which attenuates the perception of inflammatory pain *via* both central and peripheral mechanisms(140-142). Interleukin 1 released from the inflamed tissues may play some role in the phenomena since it has been shown to interact with central opiate receptors, although it is not known if it behaves an an agonist or an antagonist(143).

Afferent nociceptive specific neurones enter the spinal cord *via* the dorsal horn where they form synapses at which substance P (SP) is the neurotransmitter(144,145). SP antagonists administered into the spinal cord have analgesic activity(145-147). SP synthesized in the cell bodies of the afferent nociceptive neurones is transported both to the terminals in the dorsal horn and peripherally towards the nociceptors themselves 148,149). Certain irritant chemicals (mustard oil, xylene, capsaicin) induce release of SP from these afferent terminals and the released SP is a major mediator of the resulting "neurogenic" inflammation(148,149). Neurogenic inflammation is accompanied by a pain response which can be blocked by depletion of SP with capsaicin pretreatment(149,150) or by administration of SP-receptor antagonists(149). Evidence is accumulating that there is a neurogenic component to many inflammatory responses(150) including adjuvant arthritis(151-153). In addition to its peripheral and spinal actions, SP in the CNS mediates other phenomema; paradoxically one of these is analgesia(154,155).

Summary - There is a large number, possibly an unreasonably large number, of putative mediators of inflammatory pain. Many of these mediators have dual sites of action, peripheral and central, sometimes with opposite effects. Conclusive evidence that a particular mediator plays a role in a given form of inflammatory pain is often hard to find. The abundance of mediators does, at least, offer a plentiful supply of targets for drug discovery. For example, there are mechanisms still be to exploited within the PG system: receptor antagonism and inhibition of PGH_2 isomerases, in both the periphery and the CNS. Antagonists of kinin and SP receptors, plus both agonists and antagonists at peripheral opiate receptors and 5-HT receptor subtypes, are being actively investigated as potential analgesics. Leukocyte-derived 15-lipoxygenase products may prove to be worthwhile targets.

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Chapter 25. Free Radicals and Reperfusion Injury

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Introduction - Ischemia-induced tissue injury plays a major role in many disease processes in a variety of organs including the heart, kidney, brain, and intestine. Ischemia can occur under a variety of pathological situations and during certain surgical procedures. Occlusion of blood vessels during myocardial infarctions and strokes, shock due to severe hemorrhage, and cessation of blood flow to tissues to be transplanted from an organ donor to a recipient are all conditions which result in varying degrees of ischemia-induced injury to the affected tissue.

Numerous explanations for ischemia-induced injury have been proposed including hypoxia *per se* (1), the activation of proteolytic enzymes (2), tissue acidosis (3), and the release of vasoactive compounds such as histamine, prostaglandins, and bradykinin (4). Recent studies, however, point to an important role for oxygen-derived free radicals in the tissue damage associated with ischemia (5,6). This component of damage does not occur under ischemic conditions, but instead during the reperfusion and consequential reoxygenation of the tissue (7,8). The phenomenon of reperfusion injury has been reported in many tissues including the heart, liver, and intestine, and reperfusion injury has been blamed, at least in part, for tissue damage associated with organ transplantation and circulatory shock.

Considerable evidence points to the production and action of univalently-reduced oxygen (superoxide radical, $O_2^{\cdot-}$) as the event triggering post-ischemic or reperfusion injury in the heart (9), kidney (10), intestine (5), liver (11), skin (12), muscle (13), and pancreas (14). Superoxide dismutase, an enzymatic scavenger of superoxide, provides substantial protection against reperfusion injury both *in vivo* and *in vitro* in the heart, intestine, kidney and brain models. In the heart, it provides protection not only against reperfusion-induced tissue damage and necrosis, but against reperfusion-induced arrhythmias as well (15).

Xanthine Oxidase as a Mediator of Reperfusion Injury - A major source of superoxide in many reperfused tissues appears to be the enzyme xanthine oxidase. This is suggested by the observation that the drug allopurinol, like superoxide dismutase, blocks reperfusion injury in the heart, kidney, and intestine (6), improves post-transplant renal function and graft survival (16,17), and increases survivability in response to circulatory shock (18). This protective effect of allopurinol provided the basis for the proposed mechanism of xanthine oxidase-mediated reperfusion injury represented in Fig. 1.

According to the hypothesis, two events occur within the tissue during the ischemic periods. One event is the breakdown of ATP to the free purine base hypoxanthine which serves as a substrate for xanthine oxidase. The second event is the conversion of tissue xanthine

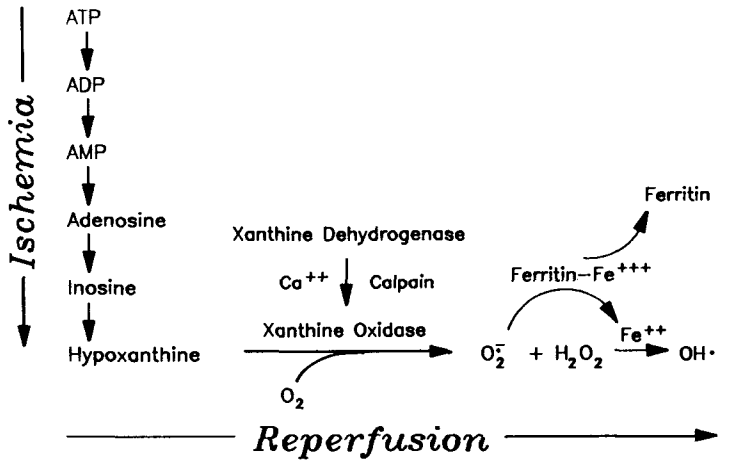

Figure 1: Proposed scheme for the production of active oxygen species during reperfusion. During ischemia, ATP is catabolized to hypoxanthine. The low energy state allows entry of calcium, which activates a cytosolic protease. The protease converts xanthine dehydrogenase to the oxidase. When molecular oxygen returns, a burst of superoxide production results. Dismutation provides hydrogen peroxide. Superoxide is capable of reductively liberating iron from tissue ferritin. The iron can catalyse the production of the strongly oxidizing hydroxyl radical.

dehydrogenase to xanthine oxidase. In normal, non-ischemic tissues the hypoxanthine-utilizing enzyme exists predominately (85-90%) as a xanthine dehydrogenase, for which NAD (rather than oxygen) serves as an electron acceptor. As a result, NADH is produced rather than superoxide:

$$\text{hypoxanthine} + H_2O + NAD^+ \xrightarrow{\text{xanthine dehydrogenase}} \text{xanthine} + NADH + H^+$$

In order for superoxide to be generated in large quantities the dehydrogenase or D-form of the enzyme must be converted to its oxidase or O-form. The oxidase form of the enzyme is capable of catalyzing the oxidation of hypoxanthine to uric acid with the concomitant production of superoxide:

$$\text{hypoxanthine} + H_2O + 2O_2 \xrightarrow{\text{xanthine oxidase}} \text{xanthine} + 2O_2^- + 2H^+$$

Upon reperfusion and the accompanying reoxygenation of an ischemic tissue, the accumulated hypoxanthine will be rapidly metabolized by the converted oxidase form of the enzyme, resulting in the production of a substantial burst of superoxide. The radical may be subsequently converted into hydrogen peroxide, hydroxyl radicals (HO·), and other active oxygen species capable of causing severe oxidative damage to the tissue.

Considerable evidence has accumulated that supports this hypothesis. The breakdown of AMP and accumulation of hypoxanthine in hypoxic and ischemic tissues is well-documented. Elevated concentrations of hypoxanthine are detected in perfusates of isolated hypoxic myocardia (19), in kidney perfusates during preservation for transplantation (20),

in the cerebrospinal fluid of dogs subjected to experimental hypoxia (21), and in the plasma following hemorrhagic shock (22).

It has also been established that the conversion of xanthine dehydrogenase to xanthine oxidase does occur during ischemia (23). In rat ileum, the process occurred quite rapidly, with nearly complete conversion to oxidase observed over a period of 10 seconds. Conversion in other tissues appeared to occur at a much slower rate with relatively low levels of conversion observed one hour following the onset of ischemia in the liver, spleen, lungs and kidneys. More recent work has confirmed that this conversion process proceeds to completion in the ischemic rat liver, heart, kidney, and lung with half-times of conversion at 37°C of 3.6, 7.0, 6.0, and 14 hours, respectively. In those same studies, the ischemia-generated oxidase was purified and demonstrated to be a product of the native dehydrogenase generated by limited proteolysis (24).

The length of time required for conversion in these ischemic tissues raises the question of whether conversion plays a significant role in reperfusion injury associated with shorter periods of ischemia. It is possible that the 10-15% xanthine oxidase that is present under even non-ischemic conditions is capable of producing sufficient quantities of superoxide to fuel the damage process when excess substrate is present and the action of the dehydrogenase is blocked by the inhibitory effects of NADH which accumulates during ischemia. However, another possibility under investigation is that conversion of the oxidase may also occur *via* sulfhydryl oxidation and that this conversion may precede the irreversible, proteolytic conversion examined in previous studies.

While a convincing case may be made for xanthine oxidase-mediated, superoxide-dependent reperfusion injury in a wide variety of tissues, some conflicting data has arisen. This is especially true in the heart. In canine heart, seven reports using similar, though not identical, models of coronary artery ligation for periods of 15 to 3 hours ischemia, followed by reperfusion periods of 2 hours to 4 days have given quite variable results. In one case allopurinol failed to limit infarct size (25); in two others significant protection was observed (26,27). Similarly, in one study superoxide dismutase failed to protect (28), while in four others superoxide dismutase and/or catalase decreased infarct size (29-32). From the results of these studies, it appears that superoxide dismutase and allopurinol may be particularly protective during the earlier hours of reperfusion, but that if damage is assessed at a much later time, the pharmacological intervention has little effect. Thus, these agents may delay, but not prevent the eventual development of the infarcted area. This suggests that the development of the infarct may be due to a multicomponent process with xanthine-oxidase mediated damage contributing at early times following reperfusion and a second event, possible inflammatory damage, occurring later. It might, therefore, be possible to greatly alleviate damage in the myocardium by early administration of allopurinol or superoxide dismutase followed in time by antiinflammatory agents.

A second problem in assessing the role of the xanthine oxidase system in the heart is that it is obvious that the human heart has considerably less xanthine oxidase than do other tissues (33). However, no one can say at this time how much xanthine oxidase would be necessary to make a contribution to the injury process. Furthermore, it has been reported that the enzyme in human heart is uniquely localized in the

capillary endothelium (34,35). Since these cells constitute only a small fraction of the total tissue mass, the xanthine oxidase of the heart may be very low. If however, the capillary endothelial cells are destroyed during reperfusion as a result of their complement of xanthine oxidase, then the entire tissue might be lost. Regardless of the outcome of studies in human heart, it does seem likely that xanthine-oxidase mediated reperfusion injury will prove to be important in many clinical problems including tissue transplantation, hemorrhagic shock, liver, renal, and cerebral ischemia.

The Role of the Neutrophil - A second generator of superoxide during reperfusion injury could be the neutrophil. The challenged neutrophil, at an inflammatory locus such as the site of an infarct, would be expected to produce superoxide through the activation of its NADPH oxidase. If neutrophils are activated at the site of an ischemic lesion, then superoxide and subsequently derived active oxygen species would be expected to damage the involved tissue.

Neutrophils adhere to the endothelium of the ischemic myocardium following one hour of occlusion of the coronary artery and then "stream toward the infarcted area" upon reperfusion (36). Antiinflammatory compounds such as BW755C (36) and ibuprofen (37) significantly limit infarct size and diminish the incidence of reperfusion-induced arrhythmias. A significant protective effect (43% decrease in infarct size) has been demonstrated when dogs are rendered neutropenic prior to one-hour ligation of their coronary arteries (38). Finally, canine neutrophils activated *in vivo* with tetradecanoyl phorbol acetate induce cardiovascular dysfunction that can be prevented by prior administration of superoxide dismutase or catalase or by neutrophil depletion (39). These studies indicate that neutrophils may serve as an important source of superoxide in some reperfused tissues. It is important to note that in some tissues such as liver, little infiltration by neutrophils occurs upon reperfusion, and that in the intestine damage occurs much too rapidly to allow for neutrophil influx.

Xanthine Oxidase-Neutrophil Interactions - One interesting possibility is that in some tissues the xanthine oxidase and neutrophil systems may interact to promote reperfusion injury. Superoxide is known to activate a latent chemotactic factor in the plasma attracting neutrophils to the source of its generation (40). As originally proposed, this was viewed as a mechanism by which neutrophils, producing superoxide, could elicit other neutrophils to the site of invading microorganisms. If superoxide were produced by xanthine oxidase in a reperfused tissue, then chemoattractant would be produced and neutrophils would flow to the site of the ischemic tissue. Once there, they would further damage the previously ischemic tissue. This combined attack on the tissue may explain why in some tissues, heart for example, allopurinol and treatments which effect neutrophil function both lead to decreases in tissue injury and also why allopurinol or superoxide dismutase given at early time periods may not alone protect against long-term damage. Even if the vast majority of xanthine-oxidase mediated superoxide production was blocked, that residual amount would be expected to begin the cascade of neutrophil recruitment through activation of the latent, superoxide-dependent chemotactic factor. Once a few neutrophils had reached the site, then the full-blown inflammatory process could proceed. Thus, while the initial tissue injury due to xanthine oxidase mediated superoxide might be ameliorated by inhibition of that enzyme, the long-term inflammatory response might not. This could explain the variability

between the observed efficacies of allopurinol and superoxide dismutase in the contradictory studies of myocardial ischemia described above.

Summary - Much recent evidence confirms a role for oxygen-derived free radicals in reperfusion injury, although the source of the radicals and the magnitude of the contribution to injury may vary considerably from tissue to tissue and species to species. The two principal sources appear to be the enzyme xanthine oxidase and activated neutrophils. During reperfusion injury to the myocardium in the dog and rat, both sources appear to make significant contributions to injury. In the rabbit and human, however, xanthine oxidase may make little or no contribution to injury in the heart.

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SECTION VI. TOPICS IN CHEMISTRY AND DRUG DESIGN

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Chapter 26. Approaches Toward the Design of Sequence-Specific Drugs for DNA

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Introduction - DNA is perhaps the best defined of all cellular targets for drug design, but because of its large size and partially redundant nature, discrimination between competing binding sites is a major problem. Nevertheless, many proteins are able to bind to DNA with sufficient sequence selectivity to very precisely "turn up" or "turn down" transcription of particular genes. The problem at hand for the medicinal chemist is how to design non-protein small molecular weight molecules that will bind to any sequence and site size of DNA. Design of sequence-specific DNA-binding molecules has been recently addressed by Dervan (1) and consequently we will concentrate on aspects that are of prime concern to the medicinal chemist. A report on the interaction of intercalating agents with DNA has previously appeared in this series (2).

As a consequence of the toxic nature of DNA reactive compounds, therapies utilizing these agents are usually reserved for life-threatening diseases such as cancer. Cancer chemotherapeutic agents react with DNA in a variety of ways including intercalation (Adriamycin, actinomycin D), and alkylation (mitomycin C, cyclophosphamide and cis-platinum) or cause extensive degradation of DNA (bleomycin) (3). Although some of these agents may exhibit some very limited sequence preference, this is not generally believed to be of sufficient magnitude to account for their selectivity against cancer vs. normal cells (4-6). If classes of agents could be designed that exhibit sufficient sequence specificity, then opportunities would be available to treat diseases which result from over-or under-expression of key genes or even genetic disorders in which genes are misplaced.

Within the last two to three years it has become a popular topic of debate whether it may be within the capabilities of medicinal chemists to evolve a strategy for design of a synthetic non-protein molecule, which through its interaction with a specific sequence in DNA will mediate a desirable biological response. Not surprisingly, this is a complex problem which will require a team of researchers spanning disciplines such as computational and biophysical chemistry, molecular pharmacology and biology and medicinal and bio-organic chemistry. The overall problem of design of non-protein DNA-effector molecules can be broken down into learning the rules of DNA sequence specific recognition; identification of key sequences to target for modulation of gene expression, amplification or recombination; design and synthesis of appropriate compounds and development of cell-specific delivery systems. This report will concentrate on the first three topics since the last topic is outside the scope of this article, even though its importance is recognized by the authors.

DNA as a Specific Receptor - If DNA sequence-specific binding is to be the basis for the selective effects of drugs, then agents must be designed which can effectively

read DNA in a manner analogous to proteins. The DNA molecule stores information in its sequence in a number of different ways. Perhaps the least subtle are the regional differences which exist between the structures of the different polymeric forms of DNA. While double-stranded DNA commonly adopts a right-handed helical conformation (B-DNA), it is also known that certain sequences of DNA have a propensity to undergo conformation transitions to other forms of DNA such as A, D and Z (7). One of these forms (Z-DNA) exists in a left-handed helix and can be readily distinguished from right-handed forms using chiral metal complexes (8). Barton and co-workers have also designed chiral probes that preferentially bind to A-form helices over B-DNA (9). Much less information is available on the structure of junctions between different polymeric forms of DNA, but presumably this is an alternative target for sequence-selective binding. Although chiral recognition probes for the different polymeric forms of DNA or even junction sites are extremely powerful tools in structural and molecular biology, they lack the characteristics of agents which are required to read a sequence of DNA.

The only known examples of unambiguous sequence recognition of DNA are by proteins. The primary sequence recognition occurs by complementary hydrogen bonding between the binding site on the protein and the major or minor groove of DNA (10). Amino acid functional groups on the protein interact with hydrogen bond acceptors or donors on the floor of one of the grooves. Most proteins use the major groove as the information output, probably because this groove contains more information in the hydrogen bonding donor and acceptor patterns than does the minor groove (11). The appropriate positioning of the functional groups of the active site of the protein is crucial for unambiguous sequence selection. X-ray crystallographic studies on various repressors (12-16) and EcoRI restriction endonuclease (17) reveal both the mechanism for primary sequence recognition, and how the secondary structure of the protein produces the correct positioning of the α-helices on the DNA. For repressors such as Cro and CI from coliphage, a "helix-turn-helix" motif is used. In this case, one of the α-helices carries the recognition probe, while the other helix positions the probe for precise alignment in the major groove. For EcoRI, two independent α-helices are positioned side by side to provide a concentrated recognition probe.

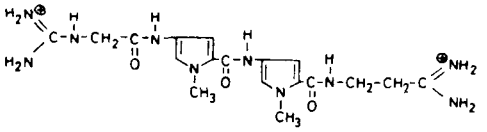
Based upon what is known about the sequence specific interactions involved in protein-DNA interactions, the major groove would be the preferred target for design of primary sequence specific probes for DNA. Consequently if we could learn the rules of α-helical sequence recognition, then synthetic non-protein molecules could be designed for virtually any sequence of DNA. Possible recognition schemes between functional groups of protein and nucleic acids have been suggested by Seeman (18), and Helene (19-20), and now can be critically evaluated in light of the recent structural determination of the DNA-EcoRI endonuclease recognition complex (17). However, a careful reading of this latter publication leads to the realization that a non-macromolecular drug design strategy based on major groove recognition by α-helices may be an extremely difficult proposition. For example, distortions of DNA are induced by the binding of portions of EcoRI not directly involved with sequence recognition and these result in increasing the separation of the DNA backbone across the major groove. As a direct consequence of these remote protein-DNA interactions, access is facilitated for the α-helices of the protein to read the base pair information on the floor of the major groove. In addition, covalent modification of DNA permits a higher degree of sequence specificity than simple protein binding through the kinetics of the catalytic events rather than just the selective recognition of base pair information. Consequently there are two levels of discrimination between cognate and noncognate sequences. One level of discrimination occurs at the sequence-specific recognition between base pairs and amino acids, and a second at the kinetic specificity of the covalent cleavage reaction. If either facilitated binding or kinetics of catalytic events are proven to be essential to obtain sufficiently high sequence-specific recognition of DNA, this will dramatically complicate the design and considerably increase the size of non-protein sequence specific probes for DNA.

Accepting the fact that a mimic of the primary sequence recognition of major groove base pair information by a non-protein low molecular weight molecule may not be a realistic objective at this time, what are the alternatives? Based on non-intercalating minor

groove binding agents, the use of planar aromatic recognition words for sequence recognition within the minor groove might be a more realistic design target at this time (1,21). It should, however, be borne in mind that the recognition mechanisms used by non-intercalating minor groove binding agents appear to be via secondary sequence mechanisms which do not rely on the unambiguous complementary hydrogen bonding interactions common to protein recognition of DNA in the major groove. There is ample reason to suspect that these secondary recognition mechanisms which read features such as groove geometry, secondary structure and electrostatic interactions give rise to a lower level of specificity and may therefore not be specific enough to adequately discriminate between target and non-target sequences. Only careful experimentation combined with computational methods will provide the insight needed to evaluate the potential of the minor groove as a suitable information source for unambiguous sequence-specific recognition.

If the human genome is the target for the sequence-selective agent, then it is important to consider the size of a unique target and by implication, the size of the sequence-specific probe to be designed. Using a conditional probability approach, a distinguishable sequence has a binding site size of 15 to 16 base pairs. This is a formidable objective when one takes into account the size of the potential drug (1900-2500 daltons) and the problems this may present in formulation and cellular uptake.

Strategy for Design of Sequence-Specific Probes for the Minor Groove of DNA - Dervan has spelled out a strategy for sequence-specific probes for the minor groove of DNA which involves design, synthesis, testing for sequence specificity and reanalysis of the design (1). A variety of natural and synthetic products including netropsin (1), Hoechst 33258 (2), SN6999 (3) and CC-1065 (4) provided the concept of utilizing planar, sometimes fused aromatic rings which can be sandwiched within the minor groove of DNA.



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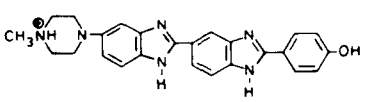

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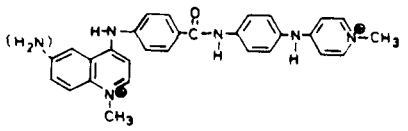

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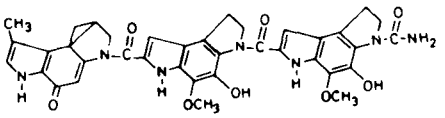

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The Minor Groove of DNA as a Drug Receptor - When considering the minor groove as a potential drug receptor, no single factor is by itself a determining element for sequence-specific binding (22-23). Hydrogen bonding, van der Waals contacts, electrostatic interactions, solvation and distortion energies may all play contributing roles. By analogy with sequence-specific protein-DNA recognition that occurs in the major groove, one would expect the different electron donating and acceptor roles of AT(TA) and GC(CG) pairs to play an important part. However, hydrogen bonding information in the minor groove is limited and redundant. Recent experiments show that the role of hydrogen bonding may not be in recognition of base sequence, but rather in stabilization of the drug-DNA complex (24). Therefore, hydrogen bonding is probably not of such importance for sequence recognition by non-intercalating agents in the minor groove. Base sequence information also provides DNA conformational variation which is important in sequence recognition (25-26). The steric protrusion of the exocyclic-2-amino group of guanine above the floor of the minor groove is a major contributing factor to the AT vs. GC specificity of many drugs. The energetics appear to play a crucial role

in generating a non-intercalative binding environment in the minor vs. the major groove of DNA (23). Solvation energies, the molecular electrostatic potential and in the case of covalent binders, distortion energies can all contribute to some degree to sequence specificity of the minor groove binding ligands (27). In many cases, the sequence specificity of non-intercalating agents is perhaps more appropriately termed regional binding specificity.

Design of the Minor Groove Binding Ligand - Based primarily on the known interactions of natural and synthetic non-intercalaters with DNA, several essential structural features of minor groove binding agents have been elucidated. In order for the ligand to fit isohelically in the minor groove, a planar aromatic moiety which must be "in-register" with the repeating unit of the minor groove appears to be the desirable repeating subunit. If multiple subunits are connected, the linker should contain a conformationally flexible bond such as an amide linkage. For non-alkylating species, hydrogen bonding stabilization appears to be important. Last and perhaps of prime significance for the design of sequence-specific probes, AT and GC recognition "words" need to be available that can be incorporated into the "sentence" that matches the DNA sequence to be read. While the literature is plentiful with examples of AT words contained in ligands such as netropsin, distamycin, benenil, stilbamidine, SN 6999 and Hoechst 33258, most of these agents bind poorly if at all to dG-dC base pairs (21). The preference of these drugs for AT over GC pairs is based primarily on the steric hinderance to non-intercalative minor groove binding by the exocyclic amino group of guanine, although the low molecular electrostatic potential of dA·dT sequences in the minor groove may also play an important role (28). Netropsin is the prototype compound for analyzing the molecular interactions between AT specific minor groove binding agents and DNA (21). A single-crystal X-ray structure analysis (24) as well as one- and two-dimensional ^{1}H-NMR studies (29) on netropsin bound to the self-complementary dodecamer sequence 5'CGCGAATTCGCG has provided considerable insight into the molecular interactions which give rise to sequence specificity. Figure 1A shows a schematic model of the bifurcated hydrogen

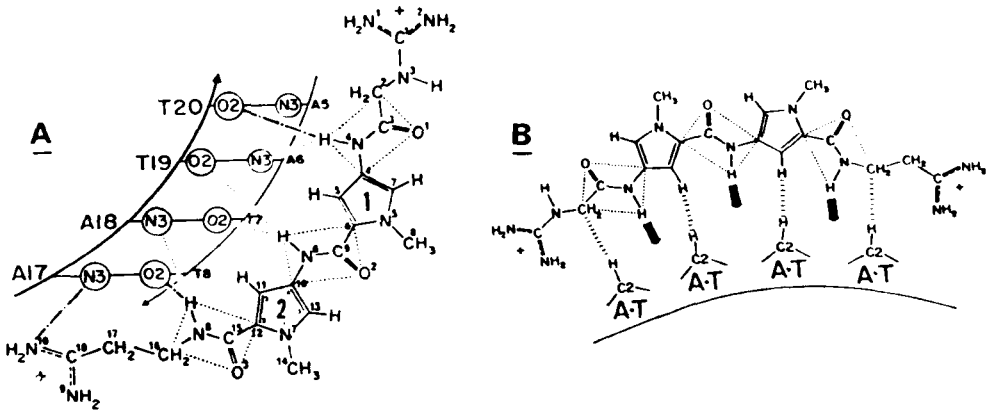


Figure 1. (**A**) Schematic model of the hydrogen bonding between the reactive sites on netropsin and adenine N3 and thymine 02 atoms on the floor of the central A-A-T-T region in the B-DNA dodecamer. (**B**) Schematic of binding of netropsin to the minor groove of DNA showing close van der Waals non-bonded contacts (barred lines). Heavy black arrows mark hydrogen bonds for NH donor to acceptor direction. (From reference 32 with permission.)

bonding between reactive sites of netropsin and adenine N3 and thymine 02 atoms in the floor of the central AATT region of the dodecamer. Importantly, the X-ray structure analysis shows that although these hydrogen bonding interactions may provide stabilization of the complex, the dA•dT base pair specificity originates from close van der Waals contacts between adenine H_2 hydrogens and the CH groups of the pyrrole rings of netropsin (24) (see figure 1B). Attempts to convert AT to GC specificity have been made and these will be discussed later (30).

Methods for Evaluation of Sequence Specificity - Several techniques have been developed to evaluate the ability of agents to discriminate between binding to target vs. non-target sequences. All depend upon single 3'-or 5'-^{32}P-end-labeled DNA molecules which are available through routine enzymatic procedures common in molecular biology (31). Typically, DNA fragments 100 to 300 base pairs in length are obtained single-^{32}P-end-labelled, and these are subjected to drug modification. Depending upon the particular drug to be tested for sequence specificity, the type of information required, and the expertise of the particular laboratory involved, footprinting with methidiumpropyl-EDTA•Fe(II) [MPE•Fe(II)] (32) or DNase I (33), affinity cleavage (34), exonuclease III digestions (35), or direct strand breakage (36) can be used.

(i) Footprinting Techniques (a) MPE•Fe(II) - This low molecular weight DNA-cleaving molecule is used widely for footprinting of DNA reactive drugs. Its design is based upon attaching an intercalator (methidium) via a linker to a metal chelator (EDTA), which in the presence of ferrous ion and reducing agents such as dithiothreitol and dioxygen, produces single strand breaks at 25° in double-helical DNA (37-38). MPE•Fe(II) cleaves DNA in a fairly random manner, but in the presence of DNA binding ligands it leaves protected sites (footprints) which provide direct information on binding locations and site size. Asymmetric cleavage protection to the 3' side of each binding site is a consequence of the generation of a diffusible reactive species in the minor groove of right-handed DNA (32). This reagent has been used to footprint a variety of natural products such as actinomycin D, distamycin, chromomycin, and echinomycin on DNA (39-41).
(b) DNase I - DNase I is a DNA cleaving enzyme whose structure with a cognate oligodeoxynucleotide sequence has recently been revealed by X-ray structure analysis (42). Based on the known interactions between the protein and DNA, a firm basis for the interpretation of DNase I drug footprinting results is possible. While MPE•Fe(II) provides exact site sizes for ligands, DNase I produces larger footprints and generally reveals more about drug-induced conformational changes in DNA (43-44). Therefore, when used side-by-side, DNase I and MPE•Fe(II) provide complementary information.

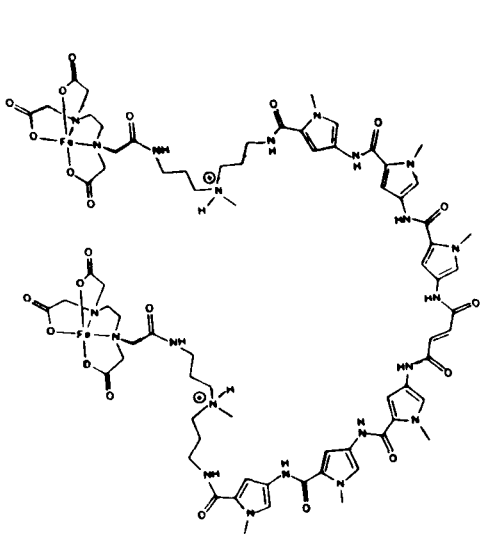
(ii) Affinity Cleavage - A natural extension of MPE•Fe(II) footprinting is the attachment of EDTA•Fe(II) to a DNA binding molecule such as distamycin to convert a sequence-specific DNA-binding agent to a sequence-specific DNA-cleaving molecule (34). This results in a positive image visualized on an autoradiogram rather than the negative image produced by footprinting. In addition, the orientation of the drug (3' or 5') in the minor groove is revealed by affinity cleavage to which footprinting is generally blind.

(iii) Direct DNA Strand Breakage - Under appropriate conditions, such as reduction in the presence of metals, alkali or thermal treatment, some DNA reactive compounds [e.g., bleomycin (36), aflatoxins (45), neocarzinostatin (46) , and CC-1065 (47)] produce breakage of the phosphodiester backbone. The sites of DNA breakage, and therefore the sites of DNA modification, can be determined by comparison of the electrophoretic mobility of the cleavage products created by treatment of the same non-drug modified DNA fragment with the products of the standard Maxam & Gilbert DNA sequencing reactions. While this method negates the need for the use of external reagents such as MPE•Fe(II) and DNase I, some caution is needed in the interpretation of the results, since the chemistry of DNA strand breakage can affect the gel electrophoretic mobility of the cleavage product, and in addition, not all drug binding sites may be revealed by this method (36,51).

(iv) Exonuclease III Digestion - Exonuclease III is a processive enzyme that degrades DNA from the 3' end of a double stranded DNA molecule (48). It has been demonstrated that covalent modifications of DNA by agents such as cis- and trans-Pt and cyclobutane pyrimidine dimers induced by ultraviolet light impede this degradation and produce identifiable stop sites that can be related to drug binding sites or lesions (35). This method is pertinent when the drug modification cannot be induced to produce direct strand breakage, and as a positive image method, is a more sensitive assay than footprinting techniques.

Redesign and Limitations - (i) Conversion of dA•dT to dG•dC Specificity. As described above, the available minor groove binding agents of both natural and synthetic origin are strongly biased towards dA•dT preference rather than dG•dC. Notable exceptions include the mithramycins (40), the pyrrolo(1,4)benzodiazepines (49), and mitomycin C (50). In the case of the pyrrolo(1,4)benzodiazepines and mitomycin C, the preference for dG•dC specificity is based upon covalent binding to the 2-amino group of guanine, while for the mithramycins, the molecular basis for the dG•dC preference is not clear. It has been proposed that the netropsin mandated binding sites consisting of $(AT)_4$ and $(AT)_5$ could be redesigned to accommodate GC-rich sequences (28,34). The proposal was based on the idea that substitution of an imidazole for a pyrrole ring should allow accommodation of one dG•dC base-pair. Experimentally, it was found that the average binding to native DNA with increased GC content is enhanced by the lexitropsins which contain this substitution. However, the DNase I footprinting data do not support the original proposed model for conversion of dA•dT to dG•dC preference. The relative electrostatic potential and solvation energies of AT- vs. GC-rich minor groove regions may be factors that have yet to be taken into account.

(ii) Upper limits of base-pair coverage - N-methylpyrrole tripeptide units attached by fumaramide linkers (5 and 6) have been designed to produce a crescent-shaped octamide (35), which binds to two AT-rich sequences: 5'-ATTTTTATA and 5'ATAATAAT (51). This is excellent proof for the idea that extended sequence specificity can be read by pure dA•dT requiring molecules. Results of interesting but less definitive experiments



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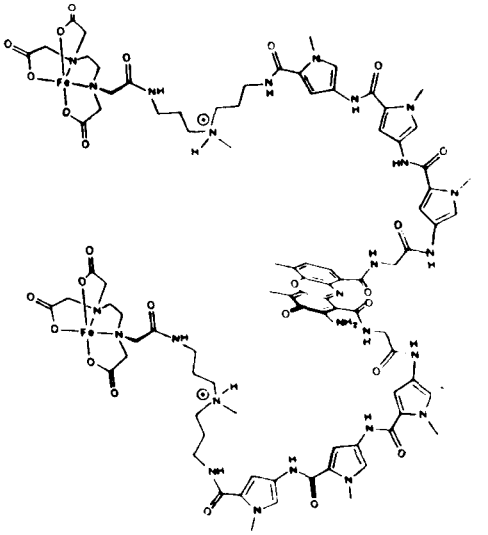

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show that the bis[Fe(II)•EDTA distamycin]phenoxazone molecule produces major cleavage sites flanking the sequence 5'TATAGGTTAA (52). This is precisely the sequence predicted for dA•dT (distamycin) dG•dC (phenoxazone) mixed specificity probe and is consistent with a groove binder mode. The presence of other single-cleavage loci, however, suggests that the tripeptides may bind in other modes also. For mixed dA•dT -

dG•dC specificity when intercalators are mixed with groove binding molecules, the linkers may be a critical design feature for which we still lack definite information.

Strategies for Determining the Structural, Biochemical and Biological Consequences of Sequence-Specific Modification of DNA - In addition to the design of sequence specific probes for DNA, a parallel effort to determine the structural and biological consequences of DNA modification is urgently required to identify appropriate types of DNA modification and sequences to be targeted. In this section we will first describe the strategy for preparation of suitable site-directed modifications of DNA for studies with oligomers, DNA restriction enzyme fragments, and plasmids. Our second objective will be to describe how these preparations can be used to probe structural, biochemical and biological consequences of DNA damage. Except for small oligomers, the use of site-directed modified DNA molecules is restricted to irreversible covalent adduct formation.

Construction of Site-Directed Duplex Complexes and Adducts - Where short duplex molecules of up to about 12 - 14 base pairs are used, it is often only necessary to choose the appropriate sequence to obtain an unambiguous complex or adduct. For example the Dickerson dodecamer 5'CGCGAATTCGCG duplex forms a 1:1 distamycin:duplex complex that has been examined by ^{1}H-NMR (29) and a single crystal X-ray structure has been solved (24). Likewise, triostin A forms a 1:1 drug:duplex adduct with the double-helical fragment having the sequence 5'CGTACG, the structure which has been solved by X-ray crystallography (53). However, in some cases even for small oligomers a combination of synthesis of a ligand-nucleotide adduct followed by incorporation into an oligomer by oligodeoxynucleotide synthesis is sometimes necessary (54).

The construction of longer oligodeoxynucleotide duplex adducts requires a strategy which combines procedures from organic synthesis and molecular biology, unless a duplex dependent construction strategy can be used. Essigmann has recently prepared a platinated DNA using a strategy which appears to be generally applicable for building other modified oligonucleotides into specific sites in DNA (55). In this experiment a chemically synthesized dodecanucleotide containing a unique site for cis-Pt modification was reacted with drug and then the modified oligodeoxynucleotide was ligated into a separately constructed gapped heteroduplex DNA molecule. In a different approach relying upon the known sequence specificity of CC-1065 and its selectivity to bind only duplex DNA, a 117 base pair duplex adduct with a single site of drug modification was prepared. (56).

Structural Characterization of Site-Specific Drug-Oligomer Complexes and Adducts - A wide array of physical methods can be used to probe the structures of oligomers that are drug modified including UV absorption, CD, ORD, IR, Raman spectroscopy, viscosity, electric dichromism, thermal melting, NMR and X-ray crystallography (21). Of these methods, NMR and X-ray crystallography are the most powerful and a few examples will be described here.

Modern methods in oligodeoxynucleotide synthesis (57) permit multimilligram amounts of short oligomers (up to about 15-mer size) to be prepared and purified by HPLC (58). These are suitable for NMR and X-ray crystallographic studies. One- and two-dimensional NMR experiments, principally the COSY and NOESY techniques, can be used to unambiguously assign many of the aromatic base and sugar protons in oligomer duplex drug complexes or adducts (59). Distance measurements between intra- or inter-nucleotide protons and between drug and nucleotide protons are made which provide information on the effect of drug binding on DNA conformation and drug positioning in the oligomer complex or adduct (59). Using these methods, the structure of oligomers modified with nonintercalating drugs such as distamycin (29), SN6999 (60), and anthramycin (61) have been probed. In a few cases, single crystal X-ray structures of drug modified oligomers have been obtained. These results have revealed a few surprises, such as the presence of a Hoogsteen base pair at the A•T base pair flanking the quinoxaline intercalating moiety in the triostin A-hexamer duplex complex (53) and the importance of van der Waals contacts for base sequence recognition by netropsin

(24). Other examples of X-ray structures of drug-oligomer complexes include daunomycin (62) and actinomycin D (63).

Computational methods as applied to drug-nucleic acid interactions have now reached a level of maturity at which they can be usefully applied to selected problems in this area. Where sufficient experimental evidence is available from X-ray crystallographic or NMR studies, meaningful information can be gleaned on the relative importance of molecular interactions and energetics on sequence specific binding to DNA. In a recent study, the experimentally determined sequence specificity of the pyrrolo(1,4)benzodiazepines has been rationalized on the basis of the energy required for deformation of DNA upon covalent adduct formation (27). For further information in this area, the reader is referred to a review article (64).

Site-Specific Degradation of DNA by DNA Reactive Drugs - As a consequence of DNA binding, some DNA reactive drugs such a bleomycin and neocarzinostatin carry out chemical reactions with DNA that lead to strand breakage or sugar degradation. One possible approach to drug induced deletion of undesirable genes or recombination in a site-specific manner is to attach a sequence-specific probe to a DNA reactive moiety. Such an agent could lead to sequence-specific *in vivo* degradation of DNA. Elegant studies on the bleomycin catalyzed chemical reactions on DNA have been carried out by a number of groups (65-67), but most pertinent to this report is the use of a synthetic self-complementary dodecamer ($5'CGCT_3A_3GCG$)-duplex to examine the products of DNA strand scission by bleomycin (68). The reaction of neocarzinostatin with DNA has been explored using single-end-labelled restriction enzyme fragments and careful analysis of the products of reaction using high resolution gel electrophoresis (46).

Exploring the Biochemical and Biological Effects of Sequence-Specific Modification of DNA - The results of recent studies designed to examine the effects of actinomycin D and a bisintercalator on transcription (69), gold compounds on regulation of metallothionein gene expression (70) and psoralen adducts on SV40 enhancer dependent transcription of the human ß-globulin gene (71) are indications of the types of studies that are now possible. In these cases, non-site-directed modification of the genome or DNA fragment was used, but, with the technology described above, it should be possible to construct unambiguously targeted adducts for such studies.

Conclusions - In this article we describe strategies which could lead to the development of drugs which are potentially useful as modulators of gene expression and directors of recombinogenic events in human cells. However, numerous problems need to be solved before this objective can be reached. Only scant information is available on the rules for sequence specificity and it is not clear that non-intercalating agents which read DNA by secondary sequence mechanisms may be capable of sufficient discrimination between target and non-target sequences. Information is unavailable on the appropriate DNA sequences to be targeted. The cell selective delivery of compounds in the 1900-2500 daltons molecular weight range remains a formidable hurdle.

Nevertheless, physical and biochemical techniques are now available to extract the type of sophisticated information needed to solve some of these problems. It is therefore with cautious optimism that medicinal chemists can now embark on research projects in this area. As molecular biologists unravel the mechanisms controlling genetically mediated events such as recombination, gene amplification and rearrangements, it may be possible to intervene or even direct these events using low molecular weight sequence-specific probes. These possibilities together with opportunities to modulate gene expression are exciting prospects for drug design in the next ten years.

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Chapter 27. Molecular Modeling as an Aid to Drug Design and Discovery

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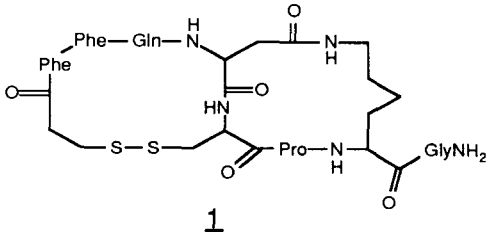
Introduction - It has been seven years since pharmacophoric pattern methodology was discussed in this series (1,2). Since then, interest in and use of this and other molecular modeling techniques in drug design has grown considerably. There have been a number of recent reviews of the field (3-14), proceedings of several meetings have been devoted to the subject (15-23), and more than one journal has emerged to cover this area (24,25). Every major pharmaceutical company has some molecular modeling capabilities, using software developed internally, at universities (26-28), and/or available commercially for mainframes (29-35) or for personal computers (36-40). This review will focus on significant recent applications of this methodology and some promising new developments. Recent bibliographies of molecular graphics (41) and of theoretical calculations in molecular pharmacology (42) contain more complete sets of references relevant to drug design and discovery.

We review here molecular modeling studies of bioactive conformations of drugs; of 3-D pharmacophore patterns, particularly for receptor agonists and antagonists; of inhibitor design, particularly when a 3-D enzyme structure is available; of receptor mapping; and, finally, of protein and genetic engineering.

Methods for Conformational Analysis of Bioactive Molecules - Many bioactive molecules may exist in numerous conformations in the solid state, in solution, in the gas phase, and when the biological response is elicited. Although proper treatment of solvation effects is still a research frontier (43,44), several theoretical methods for determining low-energy gas-phase conformations are routinely employed. While systematic torsion-angle search can, in theory, find all low-energy conformations, it is limited in the number of rotatable bonds which can be considered (45). Distance geometry (46) can be used for much larger structures, but the method generates random conformations, making it difficult - even for small systems - to be sure that all low-energy conformations have been found. Molecular dynamics (47), which simulates the vibratory motion of the molecule based on its physical properties, should be most realistic; but this method surveys conformational space rather slowly, and consumes much computer time. For each of these methods speed and efficiency is improved by inclusion of experimental data, usually in the form of nmr coupling constants and NOE close contacts determined by nmr spectroscopy (48).

Prediction of Bioactive Conformations - Particular attention has been paid to predicting conformations for bioactive peptides. A systematic search of conformations of cyclic hexapeptides, constrained by nmr coupling constant and NOE data, was developed and applied to somatostatin analogs (49). As a variant on systematic search, an

adaptive Monte-Carlo algorithm, which searches all of conformation space but concentrates its effort in regions of low free energy, was applied to Met-enkephalin (50). A multipronged strategy incorporating dynamic energy calculations to explore conformational space, synthesis of constrained analogs to test conformational hypotheses, and X-ray crystallography and nmr spectroscopy was used to confirm the postulated bioactive conformation of antagonists of gonadotropin-releasing hormone (GnRH) (47,51). In this work, a technique called "template forcing" (involving the use of artificial strain-energy terms to induce one molecule to superimpose on another) was used to show that a linear analog could assume the same proposed bioactive conformation as a constrained, cyclic GnRH antagonist at reasonable cost in energy (47). Similarly, derivation of a hypothetical bioactive conformation of lysine vasopressin, Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2 led to preparation of a bicyclic analog, 1, which acts as an antidiuretic antagonist (52). A combination of nmr and molecular dynamics studies on a cyclic enkephalin analog, 2, led to an active conformation model which is guiding the search for novel rigid peptide opiates (53). An example at the limit of current methods is the determination of the 3-D structure of the complex of ristocetin pseudo-aglycon, 3, with Ac_2-Lys-D-Ala-D-Ala employed as a model for binding of the antibiotic to bacterial cell-wall precursors (54). The 3-D solution-phase structure of the glycopeptide antibiotic aridicin A was solved in a similar way (55). These techniques are also being applied to larger peptides, often with



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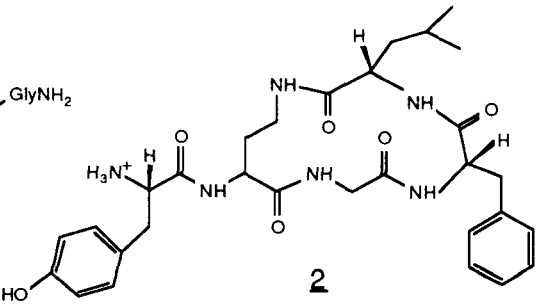

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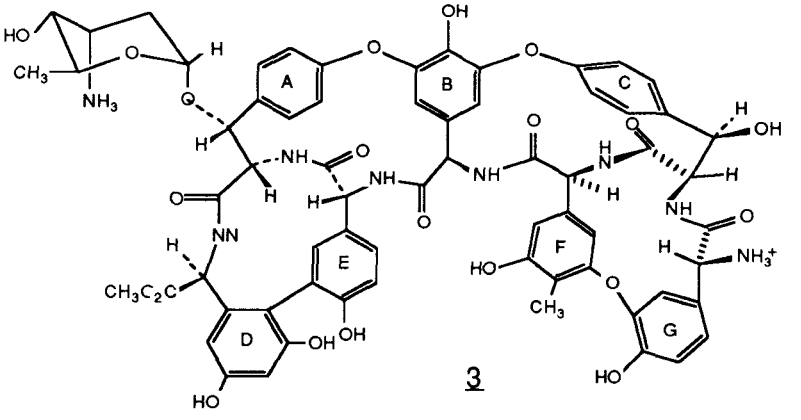

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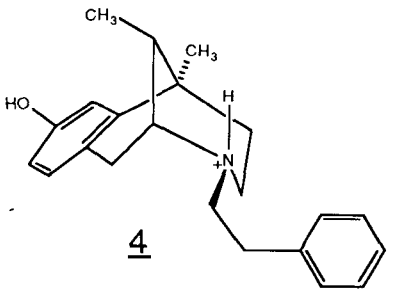
promising results (56-60).

Conformational predictions for several additional bioactive peptides, including tetragastrin, substance P, and angiotensin, have recently been reviewed (61). Such predictions often employ comparisons between calculated low-energy conformations and those computed for rigid or cyclic analogs. Calculated conformations for phenazocine, 4, were used to discriminate among previously proposed bioactive conformations of the



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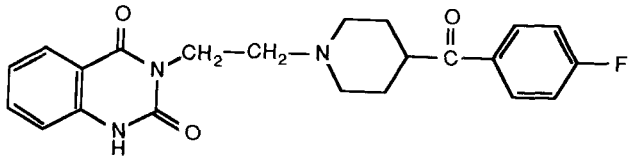
enkephalins (62). Conformational analysis has also been employed to eludicate the stereochemical requirements for ligand binding to ACE (angiotensin-converting enzyme) (12,63,64). Under favorable circumstances, conformational analysis on a single molecule can be utilized to identify a probable bioactive conformation, even in the absence of constraints imposed by experimental (e.g., nmr) data. For example, a steric-accessibility argument has been used to propose a receptor-preferred conformation for ketanserin, 5 (65), and the anti-depressive activity of midalcipran, 6, has been identified with the existence of a rigid "double-locked" lactamic pattern suitable for receptor recognition (66).



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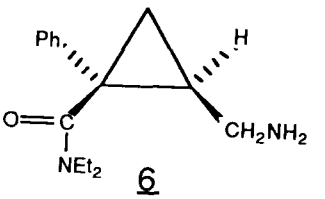

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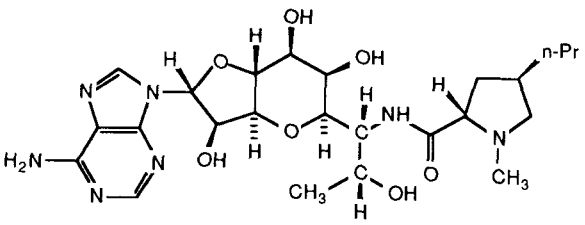
Identification and Utilization of Pharmacophoric Patterns - The pharmacophore may be envisaged as the essential geometrically arranged functionality possessed by a set of active compounds exerting a given biological effect by the same mechanism. Methods for identifying pharmacophores are frequently used in drug design, particularly for receptor agonists and antagonists, but also for enzyme inhibitors when the structure of the target enzyme is unknown. There are steric and electrostatic components to such pharmacophores (2). The techniques of excluded volume (2), shape analysis (67), and distance geometry (68,69), among others, have been used to derive pharmacophoric patterns. Thus, distance geometry has been used on an ensemble of nicotinic agonists to find a unique superpositon which defined the nicotinic pharmacophore (70). A combination of molecular superposition, systematic conformation generation and empirical energy calculations has been used to find a

common pharmacophore shared by many CNS-active drugs (71); the CNS differences between analgesics, hallucinogenics, anticonvulsives, stimulants, antidepressants, antipsychotics, etc., are suggested to be due to secondary interactions and steric fit to the different receptor types (72). A combination of parallel dose-response curves and conformational analysis of seven serotonin-uptake blockers was used to find a hypothetical serotonergic pharmacophore (73). Similarly, a correspondence of diverse functional groups in chemically unrelated classes of tumor promoters, including phorbol esters, teleocidin B, and aplysiatoxin, was proposed to explain their activities (74). For dopamine-receptor agonists and antagonists, empirical energy calculations and molecular superpositions were utilized to rationalize the activity and stereoselectivity of phenylpiperidines and aminotetralins (75,76). A similar approach was employed to develop a pharmacophoric pattern for antibiotic inhibitors of the enzyme peptidyl-transferase and to devise a novel, active inhibitor, quantamycin, 7 (77). Additional calculations on dopamine-receptor agonists (78) and antagonists (79) have been reported, and requirements for binding to the H_2-histamine receptor (80,81) and the morphine receptor (82-84), among others, have been studied. Recent vindication of a proposed pharmacophore model for analgesics (2,85), comes from the observation that 9-(m-hydroxyphenyl)-3,7-diazabicyclanes, 8, are active narcotic analgesics; previously, the inactivity of the unsubstituted phenyl analogs had seemed inconsistent



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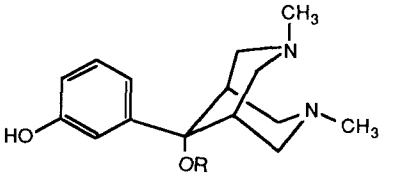

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with the hypothesis that 4-phenylpiperidines could have two different orientations of the nitrogen for interaction with the analgesic receptor (86). Ongoing investigations of computational methodology include use of interatomic distance screens pre-computed from a file of 3-D structures to locate structures displaying a specified pharmacophoric pattern (87), and the development of algorithms for finding 3-D substructures common to a set of compounds possessing similar activity (88,89).

Structure-Based Design of Enzyme Inhibitors - When 3-D structures of enzymes or other drug receptors are available from protein crystallography, more direct approaches than those discussed above come into play. Such approaches include methods for discovering new leads, for exploring enzyme mechanisms to better characterize enzyme function, for qualitatively assessing the requirements for inhibitor binding, and most recently for quantitatively predicting the effect of a structural modification on the free energy of binding. When the precise target enzyme structure is not available, these approaches may be applied to a homologous enzyme crystal structure or to a 3-D model constructed using sequence analogy to one or more homologous enzymes of known 3-D structure.

(i) New-Lead Discovery - A number of enzyme and other macromolecular structures are becoming available from X-ray crystallography (90). Logically, these structures continue to be exploited in design of potential enzyme inhibitors. A systematic computational method for "screening" rigid ligands (taken from a data base of 3-D structures) for complementarity of shape to an enzyme active site has been developed (91), as well as a method for allowing partial conformational flexibility in the "docking" process (92). Such methods ignore chemical (electrostatic, hydrophobic) complementarity, but nevertheless may prove effective in identifying frameworks for constructing effective enzyme inhibitors. Modeling has also been employed to suggest chemical modifications to existing leads. Superimposition of renin-based peptides on the X-ray conformation for the aspartyl proteinase inhibitor pepstatin (Ival-Val-Val-Sta-Ala-Sta) bound to rhizopuspepsin (93) (Figure 1) was used to develop the hypothesis that pepstatin's central statine residue could function as a dipeptide surrogate (94,95); the resultant replacement of Leu-10(P1)-Leu-11(P1') by Sta in sequences based on the minimal renin substrate has led to the development of a new class of potent inhibitors of human renin.

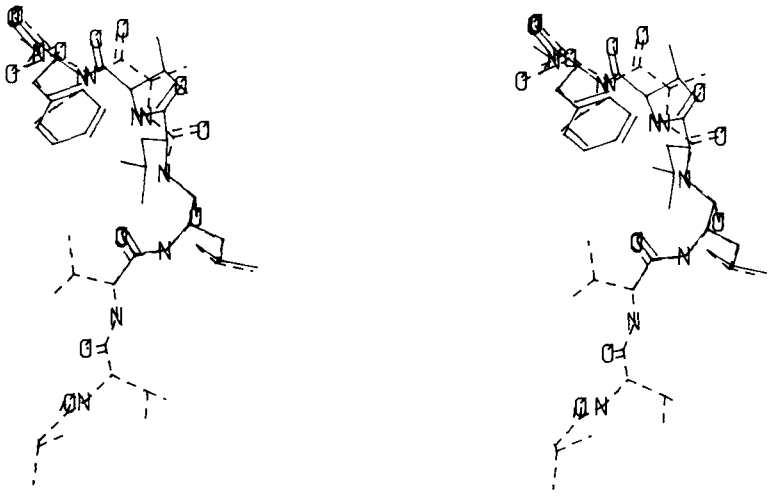


Fig. 1. Ac-Leu*Leu-Val-Phe-NH_2 (solid), where * denotes a tetrahedral carbonyl (-CHOH-NH-), matched onto Ival-Val-Val-Sta-Ala-Sta (dashed, pepstatin) as in the complex with rhizopuspepsin. Reprinted with permission from Ref. 95.

(ii) Generation of Model 3-D Structures for Target Enzymes - Despite much study of the "protein folding problem" (96), in the absence of a protein crystal structure it is not possible to properly generate that structure by folding the linear peptide primary sequence, though progress in this area continues to be made (97-100). However, a 3-D model of a protein can often be generated from the structure of a related protein (101,102). Thus, structural models of the complement proteins C4a and C5a were derived from the crystal structure of the related C3a (103). Similarly, a model 3-D structure for human renin was

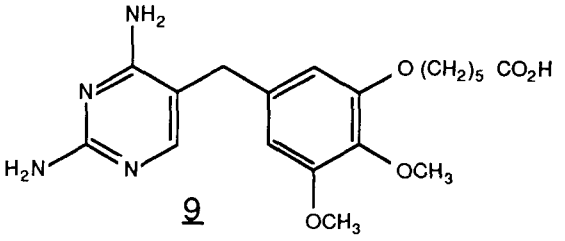
derived, based on sequence homology to other aspartyl proteinases of known structure, particularly endothiapepsin (104). Other renin 3-D models have been derived by similar techniques and employed for inhibitor design (105-107). These and other 3-D models of renin are being extensively utilized to help to design renin inhibitors.

(iii) Exploration of Enzyme Mechanisms - A better understanding of enzyme function should prove helpful in designing enzyme inhibitors. Combined X-ray and other physical information and computer-graphics model building have been used to formulate a proposed mode for productive substrate binding to the aspartyl proteinases rhizopuspepsin and penicillopepsin (108,109). Similarly, the mechanism of substrate cleavage by thermolysin, a zinc metalloproteinase sometimes used as a model for ACE, has been explored (17). Among several fully or partly quantum-mechanical studies are those involving the mechanism of substrate cleavage by serine proteinases (110-113), and the mechanism of action of carbonic anhydrase (114). Empirical energy calculations and systematic conformational search have been employed to model the interaction and subsequent reaction of haloenol lactone suicide substrates with alpha-chymotrypsin (115).

(iv) Assessment of Requirements for Inhibitor Binding - Recent theoretical studies have suggested that protein clefts (e.g., active sites) can "focus" electrostatic effects important for substrate binding (116), and that such effects might even help to "steer" substrate (and inhibitor) molecules into the active site (117-119). Several groups have employed calculated electrostatic potentials, obtained either quantum mechanically or empirically, to investigate and visualize the spatial and chemical requirements for such electrostatic complementarity. For several enzyme-ligand systems, strong mutual complementarities were found between the electrostatic potentials due to the enzyme and to the bound ligand expressed at the ligand's van der Waals surface (120). In a contrasting approach, the interaction with the enzyme of small probe fragments on a grid of points arrayed throughout the active-site accessible space was computed, and 3-D computer graphics used to visualize the results as contoured volumes depicting interaction regions favored for ligand fragments having various chemical properties (121). From a somewhat different perspective, combined molecular graphics and QSAR studies were used to validate the QSAR method and to account for the activity of inhibitors of such enzymes as alcohol dehydrogenase, dihydrofolate reductase, carbonic anhydrase, and trypsin (122).

(v) Qualitatively-Based Inhibitor Design - Most computational methods currently being applied to pharmaceutical problems are limited in accuracy. Nevertheless, qualitative modeling studies can help to formulate rational modifications to existing inhibitor designs. Methods of probing preferred regions of hydrophobic and hydrophilic interactions with an enzyme were considered above. Computer-graphics visualization of such favorable interaction regions can assist in developing a reasonable model for the enzyme-bound ligand conformation; energy-minimization or molecular dynamics calculations, used judiciously, can suggest structural modifications likely to achieve given design objectives. On an even more qualitative level, an X-ray structure of an inhibitor-enzyme complex can suggest fruitful design modifications, as exemplified by the use of computer-graphics model building (123) to design a series of alkylcarboxylate analogs of trimethoprim potentially capable of making a salt bridge to a positively charged dihydrofolate

reductase (DHFR) residue (Arg-57). The best of these analogs, **9**, proved to be 55 times more active than trimethoprim itself in inhibiting E. coli DHFR in vitro. Similar work on a related series of trimethoprim analogs has been described (12).



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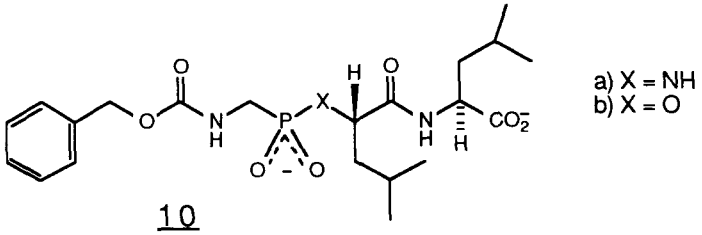
(vi) Quantitative Prediction of Relative Inhibitor Binding Affinity - Very recent work has shown that it is possible, for pairs of closely related inhibitors, to compute accurately the difference in free energy of binding to an enzyme of known 3-D structure. The free-energy perturbation method employs molecular-dynamics or Monte Carlo calculations to (unphysically) mutate one ligand into the other, the mutation being performed both in free solution and while bound to the enzyme. Application of a thermodynamic cycle yields the desired (physical) difference in binding affinity as the difference in the free energies computed for the free and enzyme-bound mutations. Using this approach, a preference for binding to trypsin of 0.9 kcal/mol for benzamidine relative to p-fluorobenzamidine was predicted, in good agreement with the experimental value of 0.5 kcal/mol; also calculated with reasonable accuracy was the preference for binding of benzamidine to trypsin relative to a mutant (Gly-216 --> Ala) trypsin (124). Similarly, a difference of 4.2 kcal/mol for binding to thermolysin of a pair of phosphonamidate and phosphonic ester (N-H --> O) inhibitors, **10a,b**, was calculated, in remarkable agreement with the experimentally



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determined difference of 4.1 kcal/mole (125). Further work is needed to determine the applicability of the method when a structural modification produces a significant conformational change, and to assess the adequacy of current force field models; the massive computations required by this approach may also limit applications in the near term.

Approaches for Modeling and Mapping Receptors - As discussed in the previous section, if a known drug acts by inhibiting an enzyme, there is the promise that a detailed high-resolution model of the drug-enzyme complex may become available from X-ray crystallography of the actual enzyme or be constructed from that of a related enzyme (126). For drugs which act by binding to DNA, it is often possible to derive a model for the binding complex (127-130). Other nonenzymic crystal structures

which have been used as drug receptor models (8) include hemoglobin (131) and prealbumin (132). However, receptors in the classical pharmacological sense do not chemically alter their substrate as enzymes do. For such membrane-bound receptors, detailed structural information is difficult to obtain. One of the more favorable cases is the nicotinic cholinergic receptor, which has been isolated and purified, and its primary sequence determined (133) (see also chapter 28). While studies based on the putative bioactive conformation of cholinergic agonists and antagonists continue to appear (70,134), structural models for the receptor itself are being derived (135-138) and may lead to new drug design approaches. Rough models or "maps" of other receptor sites have also been derived (76,79) on the basis of conformational analysis, testing of rigid analogs, and other pharmacophoric mapping studies of the types discussed earlier. Moreover, it is possible to develop higher resolution pharmacophoric maps, as shown in the uses of distance geometry to map the turkey erythrocyte beta receptor (139) and the DHFR active site (69).

Macromolecular Modeling and Genetic Engineering - Recent advances in such areas of molecular biology as cloning and site-directed mutagenesis have resulted in relatively facile preparation of large quantities of selectively modified polypeptides and proteins (140) (see also chapter 30). Many of the computational approaches discussed above are being pursued for modeling the structural effects of such mutation experiments (141), often in conjunction with protein nmr, protein crystallography, and other relevant experimental techniques. Such studies can help to interpret the experiments and guide further work (142). Given the rate at which genetic engineering research is growing, attention to molecular modeling techniques to support this field will also clearly grow -- despite the formidable algorithmic and computational challanges to treating such large systems by theoretical methods.

Conclusions - The computational aids to drug design surveyed here are growing in sophistication, in relevance to "real-world" medicinal chemical research problems, and in dissemination into the working pharmaceutical research environment. Major current and future trends include reduction of hardware and software cost for using these techniques, increased ease of use, and integration of these programs with other computational chemistry applications such as chemical and biological data handling.

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Chapter 28. MOLECULAR CLONING OF THE NICOTINIC ACETYLCHOLINE RECEPTOR: NEW OPPORTUNITIES IN DRUG DESIGN ?

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Introduction

The nicotinic acetylcholine receptor (nAchR) of vertebrate skeletal muscle and electric organ is one of the most intensively studied neurotransmitter receptors (1-8) and has served as the prototype ligand-gated ion channel. Several factors have contributed to the intense interest in this synaptic protein: the accessibility of the nAchR at the neuromuscular junction for electrophysiological and developmental studies; the availability from electric organ of electric rays and eels of milligram amounts of nAchR for biochemical and pharmacological studies; the discovery in Elapid and Hydrophid snake venom of α-neurotoxins (9), a group of 7-8 kDa polypeptides that bind with nM-pM affinity and exquisite specificity to nAchRs, allowing affinity-purification and sensitive detection; the crucial role of the nAchR in the human auto-immune disease myasthenia gravis; and the fact that the nAchR mediates the effects of cholinesterase inhibitors used as nerve gas or insecticides. Far less is known about nAchRs in the vertebrate nervous system or in invertebrates; this review will therefore not pertain to those, unless specified otherwise.

The molecular cloning of all nAchR subunits for several species over the past four years constitutes a watershed in nAchR research (10). Based on the deduced amino acid sequences, earlier research results have been re-interpreted, detailed structural models have been proposed, and new ways to test these hypotheses have become available. Sequence-specific antibodies can now be raised against synthetic peptides based on the deduced primary sequence, and amino-terminal sequencing of only a few residues now suffices to identify a nAchR fragment after labeling and proteolytic or chemical cleavage. In addition, recombinant DNA techniques allow (11,12): generation of (in principle) unlimited amounts of cloned protein or protein fragments for biochemical, pharmacologic or structural studies; examination of structure-function relationships using site-directed mutagenesis (13); specific and quantitative detection of mRNA or DNA complementary to the cloned sequence, either in situ or on blots; isolation of homologous genes or their transcripts and engineering of proteins to create new functional sites or chimeric forms (14).

This review discusses the relevance for medicinal chemistry of the molecular cloning of the nAchR. Being able to design from first principles a ligand for a receptor, given the structure of its binding site, has been an age-old dream of medicinal chemists. For the nAchR, elucidation of the binding sites for a variety of ligands now seems within reach. Moreover, other functional sites are being mapped onto the primary sequence, and the discovery of related coding sequences has ushered in a whole gene family which probably encodes a family of nAchR subtypes (15).

Structure of the nAchR

Most biochemical and structural studies of the nAchR have used electric organ nAchR from the marine ray Torpedo. It is generally assumed that conclusions from such studies are also valid for vertebrate skeletal muscle nAchR. The finding of strong sequence homology between electric organ and muscle nAchRs supports the assumption that their structure is highly similar. However, the recent demonstration in calf muscle of a second type of nAchR, differing in one subunit and in its single-channel properties from the type found in Torpedo (16), inspires caution against rash extrapolation from Torpedo data.

The nAchR is composed of four different, but homologous subunits in two known stoichiometries. Electric organ and probably adult denervated or embryonic muscle contain nAchRs with stoichiometry $\alpha_2\beta\gamma\delta$, whereas $\alpha_2\beta\epsilon\delta$ is the probable stoichiometry of nAchRs at the endplate of adult innervated muscle (except maybe chick)(17). All subunits are glycoproteins that span the cell surface membrane and whose approximate calculated molecular weights are: α: 50 kDA; β: 54 kDa; γ: 56 kDa; δ: 58 kDa; ϵ: 53 kDa. In Torpedo the α-,β-,γ- and δ-subunits contain 1, 1, 2 and 3 N-linked oligosaccharide chains, respectively, but no O-glycosidic chains (18). In each nAchR molecule the five constituent subunits are arranged symmetrically around a central pore, believed to be the cation-selective channel (19). Electron diffraction on pseudo-crystalline arrays led to a low resolution picture of the nAchR: a 13 nm long cylinder, with a diameter of 8 nm; its extracellular (synaptic) portion rises 7 nm above, and its cytoplasmic portion protrudes approximately 3 nm below the plane of the 3 nm thick lipid bilayer (20).

Between our complete knowledge of the primary structure and the information on the quaternary structure of the nAchR still lies a great void. Based on deduced amino acid sequences, however, several models have been proposed for the secondary structure (21) and transmembrane topology (22) of the nAchR (Fig. 1A). All models assume that the folding of each nAchR subunit will be highly similar, since inter-subunit amino acid sequence identity is 35-45% (23). Hydrophobicity analysis identified four hydrophobic regions (M1-M4, Fig. 1), each fulfilling the requirements for potential α-helical transmembrane domains (24): segments of 20-25 contiguous, mostly hydrophobic amino acids lacking charged residues entirely, but flanked by charged amino acids (model I, Fig. 1A). These 4 domains have been found in the same location for all nAchR subunit sequences determined to date, and the protein sequence of these segments is more conserved across species and subunits than any other region of the nAchR. Hydrophobicity spectral analysis revealed in each subunit the presence of a potential amphipathic α-helix (MA) preceding M4 (21,25)(Fig. 1). It was proposed that these five amphipathic helices (one from each subunit in the $\alpha_2\beta\gamma\delta$ pentamer) would form the wall of the ion channel with their charged faces, and close-pack with their hydrophobic faces against other hydrophobic transmembrane domains (model II) (21,25).

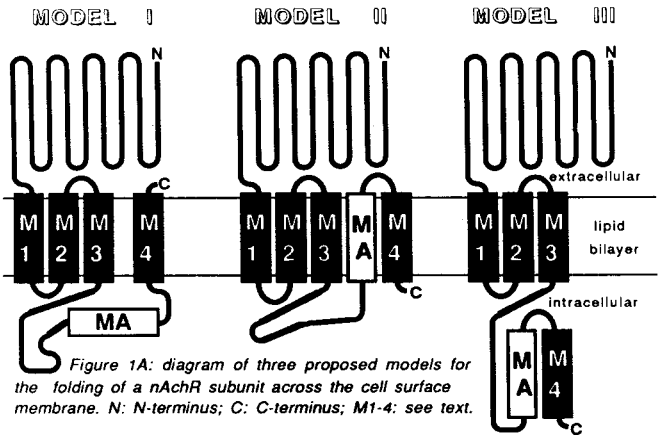

Figure 1A: diagram of three proposed models for the folding of a nAchR subunit across the cell surface membrane. N: N-terminus; C: C-terminus; M1-4: see text.

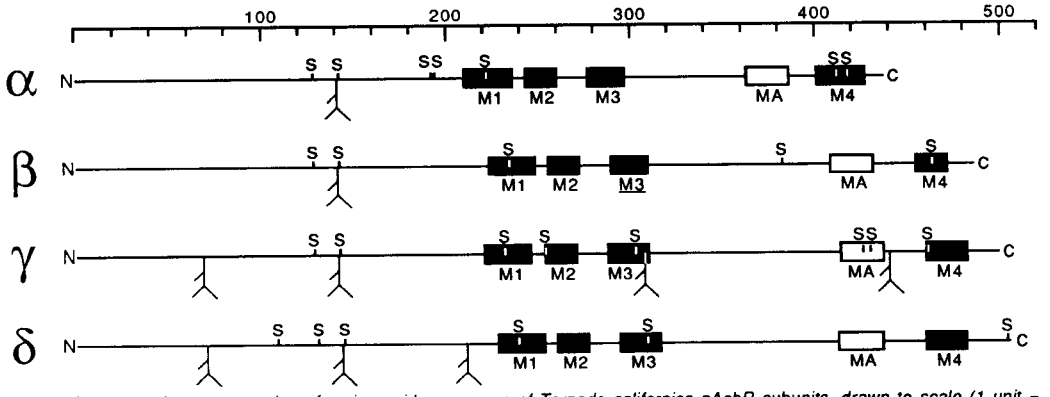

Figure 1B: schematic representation of amino acid sequences of Torpedo californica nAchR subunits, drawn to scale (1 unit = 1 amino acid). Putative transmembrane domains (M1-M4) and amphipathic helix (MA) are indicated by filled and open rectangles, respectively. N: N-terminus; C: C-terminus; S: cysteine residue; potential N-linked glycosylation site:

Both model I and II were consistent with most evidence available at the time they were proposed. The deduced amino acid sequence predicted, and in vitro translation experiments as well as N-terminal sequencing confirmed the existence of a cleaved signal peptide for all subunits, whose N-terminus is therefore extracellular (26). Torpedo sequences contain 1, 1, 4 and 3 potential glycosylation sites for α-, β-, γ- and δ-subunits, respectively (Fig 1B); only those located extracellularly, however, can get glycosylated. Both models predict the observed number of glycosylation sites for α-, β- and δ-subunits to be extracellular (Fig. 1). For the γ-subunit, model I correctly predicts 2, while model II predicts 3 extracellular glycosylation sites (Fig. 1). This does not rule out model II, since a potential glycosylation site may remain unused. Both models are consistent with the estimated mass distribution of the nAchR with respect to the membrane (2). Model I agrees better with secondary structure estimates based on circular dichroism measurements (27).

Experimental evidence obtained after the models were proposed has largely supported their common features. Extracellular affinity labels (28,29), α-neurotoxins and antibodies against extracellular antigenic determinants (30,31) all seem to react with predicted extracellular regions, except antibodies against synthetic peptide α152-167 (32). These monoclonal antibodies react better with vesicles containing nAchR in outside-out orientation after they are permeabilized, suggesting an intracellular epitope. However, the permeabilizing agents used could have unmasked an extracellular antigenic determinant by partially denaturing the nAchR. Verification by an independent technique (like immuno-electron-microscopy) is therefore necessary before this result, which predicts two additional and unusual transmembrane domains with multiple charged residues, can be accepted. Cytoplasmic antibodies (30,33-35) and a phosphorylation site (36) were mapped to predicted intracellular segments. However, monoclonal antibodies reacting with epitopes within MA (α360-370, α371-378) were also localized to the cytoplasm, inconsistent with the transmembrane topology of model II, but leading to the proposal of model III (Fig. 1A)(37). In addition, the proposal that MA formed the ion channel wall received no experimental support: affinity-labeling studies using nAchR channel-blocking drugs fail to label residues in MA, and site-directed mutagenesis studies show that segments of MA can be deleted without abolishing channel function (38). These data virtually rule out model II, but support models I and III. The prediction by model I of an extracellular C-terminus was contradicted by immuno-electron-microscopy studies (34,39). This is unexpected since a C-terminal 16 kDa fragment of the δ-subunit is thought to contain the half-cystine known to covalently link Torpedo nAchR monomers into dimers (40). The only Cys in this fragment is the penultimate δ-subunit residue (Fig. 1B). Not only are intracellular disulfide bonds exceedingly rare in general (41), but experimental evidence indicates that the disulfide bond linking Torpedo δ-subunits is extracellular (42,43). Furthermore, affinity labels that partition into the lipid bilayer label M4, supporting its transmembrane nature (44). In the face of these conflicting data, neither model I nor III can be rejected, although the former seems to us more likely. These models differ only, however, in the transmembrane topology of M4, which does not significantly affect our discussion here.

Ligand Binding Sites

Cooperativity in the dose-response curves of the nAchR has suggested that two sites on a nAchR monomer need to be occupied by acetylcholine or cholinergic agonists before the channel opens (45). Numerous competitive and non-competitive inhibitors of this ligand binding response are known (46), and the location of all these binding sites is of obvious interest for drug design (47).

Agonist Binding Site (ABS)-Exposure to agonists not only causes ion channel opening but within tens of milliseconds also allosterically shifts the nAchR into relatively stable states that do not conduct ions, but bind agonists with increased affinity. At least two such "desensitised" states have been described, with affinities for acetylcholine in the range of 1 μM and 10 nM, respectively, as opposed to 100 μM for the non-desensitised state (7,48). Some non-competitive antagonists also convert the nAchR into states that bind agonists with increased affinity, but competitive antagonists do not cause such conversion. Solubilization of membrane-bound nAchRs also converts them into a desensitised form.

Most biochemical experiments and agonist binding studies therefore study desensitised nAchRs, and affinity labeling occurs at the high affinity ABS. Most workers accept that the low affinity site where agonist binding induces channel opening and the high affinity ABSs of the desensitised states are interconvertible conformations of one and the same site. However, there is evidence for low affinity acetylcholine binding sites in addition to and separate from the high affinity ABSs (49-51). Their role is unclear, but agonist binding at one of these sites induces a conformational change similar to that accompanying channel opening (50). However, some ABS affinity labels can still induce channel opening after they have been covalently tethered to the nAchR (52,53). In what follows we will therefore assume that the high affinity ABS of the desensitised receptor is the physiological ABS.

Several proposals have been published for the location of the ABS (54), but experimental data are still conflicting. Agonists bound to the nAchR prevent the binding of α-neurotoxins such as α-bungarotoxin (α-Btx) and vice versa. Therefore, it has been concluded that the ABS is part of the more extensive area over which α-neurotoxins are known to interact with nAchRs. However, since several cholinergic ligands accelerate the dissociation of α-neurotoxins from the nAchR, the formation of ternary complexes has been postulated (55,56). Competition studies of cholinergic ligands with monoclonal antibodies directed against the ABS have also led to suggestions that classical competitive cholinergic antagonists may have only partly overlapping binding sites or may even form a network of coupled allosteric sites (57). Nevertheless, the same approaches have been used to map the α-neurotoxin or ABS onto the primary nAchR sequence:

Affinity Labeling-Because of its large size α-Btx can be covalently cross-linked to several nAchR subunits (58). Smaller cholinergic affinity labels, however, selectively label the α-subunit (59,60). It has been known for two decades that reducing agents can make sulfhydryl groups on the nAchR α-subunit accessible for subsequent reaction with cholinergic affinity alkylating agents (60). From studying a series of these affinity labels, some of which are competitive antagonists (e.g. 4-(N-maleimido)benzyltrimethylammonium (MBTA)), and others of which act as covalently bound agonists (e.g. bromoacetylcholine), it was concluded that the reactive sulfhydryl groups were located 0.9-1.2 nm from the binding site of the quaternary ammonium group of acetylcholine (61). Recently, αCys192 and 193 were identified as the residues reacting with MBTA (28). The low amount of radiolabeled MBTA recovered in the sequencing cycles that released these cysteines, and the low ratio of specific MBTA labeling versus aspecific alkylation of these cysteines raised the possibility that other residues might also be labeled, as was suggested by another group (62). Involvement in the ABS of residues around αCys192-193 has however been confirmed with two other ABS affinity labels which do not require free sulfhydryl groups. (+)-Tubocurarine photo-affinity-labeled a 20 kDA α-subunit V8 protease fragment with N-terminal Ser173 (63). p-(N,N-Dimethylamino)-benzenediazonium fluoroborate specifically labeled cyanogen bromide fragment α179-207, as well as two other fragments with a different but yet unpublished sequence (29).

Direct Ligand Binding to Purified Renatured nAchR Subunits or Proteolytic Fragments Thereof-Isolated nAchR α- (but not β-, γ- or δ-) subunits after renaturation reversibly bind α-Btx in solution (64), as well as on protein blots (65,66) with affinities between 5 and 200 nM. In contrast, α-Btx binds irreversibly to native nAchR (67). MBTA-labeled nAchR binds less α-Btx on blots (65,68). But since MBTA-labeling requires prior reduction, and reducing agents by themselves decrease α-Btx binding to α-subunit fragments on blots (69,70), the presence of the ABS cannot be inferred from these data. Several nicotinic cholinergic ligands inhibit the binding of α-Btx to α-subunits in solution, whereas on blots only (+)-tubocurarine seems effective (64-66). In either case however apparent affinities of the competing ligands are at least 100-fold lower than those for native nAchR. A 26 kDA α-subunit papain fragment exhibits ligand-binding properties very similar to those of isolated α-subunits in solution (64), but neither its exact N- nor C-termini are known (71). Other α-subunit proteolytic fragments have been shown to bind α-Btx on blots, but again competition with high concentrations of (+)-tubocurarine constitutes the only evidence that they contain the ABS (68). Based on those criteria, two V8 protease fragments, whose N-terminal sequence indicates that they are probably contiguous but do not overlap, have been claimed to contain the ABS (63,72,73). Since proteolytic fragments often comprise protein domains, the above studies suggest that most of the amino acids interacting with α-

Btx are contained on one or two domains of the α-subunit, although the boundaries of those domains cannot yet be outlined precisely. The low affinity of small cholinergic ligands for isolated α-subunits and reports of binding to non-overlapping α-subunit fragments preclude conclusions about localization of the ABS.

Direct Ligand Binding to nAchR Subunits or Fragments Thereof Obtained Through Recombinant DNA Technology-nAchR expression from cloned cDNAs has the advantage that conveniently tailored fragments with known sequence can be produced in large amounts and -in principle- with native folding. Individual nAchR subunits expressed in yeast, however, exhibit a non-native folding (74), and α-subunit fragments expressed in E. coli form aggregates that can only be solubilized by complete denaturation (69,75). The affinities for α-Btx and cholinergic ligands of these fragments or of the yeast α-subunit are in the same range as those of proteolytic α-subunit fragments described above.

Direct Ligand Binding to Synthetic Peptides Based on the Deduced Primary Sequence-Two sets of non-overlapping synthetic peptides have been proposed to specifically bind α-Btx: one set contains α128-142 (76,77); the other, amino acids surrounding α192-193 (70,72,77,78). Some authors detect only binding to the latter set. However, α185-196 does not bind α-Btx when its sequence is based on the human, rather than Torpedo sequence (70), whereas nAchRs from both species bind α-Btx. Affinities of α-Btx for these synthetic peptides are lower still (0.5-35 μM) than those measured on larger proteolytic fragments or whole α-subunit, suggesting that some binding residues that are present in the latter are lacking in the former. Binding properties for small cholinergic ligands are so different from those of native nAchR (70) that conclusions about the location of the ABS based on the peptide data seem unwarranted.

Site-Directed Mutagenesis-This technique has not yet yielded much information regarding the location of the ABS (38). None in a series of deletions C-terminal to α249 influenced α-Btx binding, and the decreased α-Btx binding seen after deleting α224-237 was interpreted as a consequence of altered folding in the extracellular domain. Replacement of αCys128 or 142 with Ser completely abolished α-Btx binding. Since both these cysteines are probably involved in a disulfide bond, these mutations have little localizing value since the breaking of a disulfide bond could have long-range effects. Converting αCys192 or 193 into Ser left α-Btx binding unchanged, although it decreased the apparent affinity for carbamoylcholine by approximately 10-fold.

Competition by Antibodies with Known Sequence-Specificity-The epitopes of antibodies known to compete with α-Btx or cholinergic drugs have not been mapped onto the primary sequence since most of these antibodies do not bind to α-subunit fragments on blots, except in one case where the antibody bound to the same 18 kDa V8 protease fragment recognized by α-Btx (78). Conversely, antibodies against synthetic peptides rarely bind well to native nAchR, so that their lack of competition with α-Btx is difficult to interpret (79).

In conclusion, it seems likely that an α-subunit domain carries most if not all of the ABS. Residues in the vicinity of αCys192-193 have clearly been implicated, although there are indications that other segments of the primary sequence contribute to the ABS as well. This information by itself is insufficient to aid in the design of new drugs, but will allow molecular modeling of the ABS once more detailed structural information becomes available.

Non-competitive Antagonist Binding Sites-Several nAchR antagonists are thought to act (at least in part) by lodging in the ion channel and thereby blocking it (80,81). Various lines of evidence support this mechanism of non-competitive inhibition, although not every drug in this chemically very diverse group exhibits all of the following properties: permeation through the open channel; increased antagonistic effect in the presence of increasing amounts of agonist; voltage-sensitivity of antagonism, thought to reflect the effect of transmembrane voltage on these charged compounds as they penetrate the channel; decrease of the mean channel open time because of brief interruptions in the current flowing through a single channel; occupation of a single site per nAchR monomer, in contact with all constituent subunits and trapping of the drug inside the nAchR when the channel closes before the drug dissociates. However, numerous drugs that qualify as channel blockers based on these criteria also allosterically shift the nAchR into desensitised states (82,83). Although open channel block or increased desensitisation is not the primary mechanism of action for most clinically used neuromuscular blockers (84),

it probably contributes to the muscle weakness seen as side effect of some antibiotics, anaesthetic agents, psychotropic drugs, procaine, chloroquine, and amantadine, mainly in the post-operative period or in myasthenia patients (85). The site of affinity labeling has been reported for four putative open channel blockers: chlorpromazine at α262 in M2 (86); triphenylmethylphosphonium at α248 + β254 + δ262 in M2 (87); quinacrine azide within M1 (α208-243) (88); and meproadifen mustard within a 20 kDa V8 protease fragment with N-terminal α173 (containing both M1 and M2) (63). Although it is unlikely that each subunit contributes more than one α-helical transmembrane domain to the channel wall, these studies are not necessarily contradictory because the drugs used may label different nAchR conformations: the open channel conducting state and a rapidly desensitising state (89). Site-directed mutagenesis indicates that M2 influences nAchR conductance, supporting its involvement in the ion channel (90). M1 on the other hand may contribute to an allosteric site close to the ABS, but distinct from the ion channel. Current data may provide enough constraints for profitable modeling of the channel, which was already attempted when MA was thought to form the channel wall (4,22,25,34).

Phosphorylation Sites

All Torpedo subunits can be phosphorylated by endogenous protein kinases: γ and δ by cAMP-dependent protein kinase; α and δ by protein kinase C; and β, γ and δ by a tyrosine protein kinase (91). The functional role of this post-translational modification is not clear (92). Recent experiments suggest that cAMP-dependent phosphorylation of γ- and δ-subunits increases channel open time (93) and the rate at which agonists shift the nAchR into non-conducting (desensitised) states (94-96). Based on the nAchR subunit sequences and the known sequence specificity of the involved kinases, their respective phosphorylation sites have been predicted (91). Synthetic peptide δ354-367 contains the proposed site of cAMP-dependent phosphorylation (Ser361), and can be phosphorylated in vitro by this kinase (36). Moreover, monoclonal antibodies directed against this peptide inhibit cAMP-dependent phosphorylation of the δ-subunit and some of these antibodies distinguish between the phosphorylated and non-phosphorylated δ-subunit on protein blots (36).

Main Immunogenic Region

The nAchR at the neuromuscular junction is the target of antibodies in the autoimmune disease myasthenia gravis (97). Through complement-induced lysis and increased nAchR turnover because of antibody cross-linking, the levels of nAchR at the muscle endplate decrease to the point where synaptic transmission is compromised, and muscle weakness becomes manifest. The majority of anti-nAchR antibodies in myasthenia patients are directed against a cluster of denaturation-sensitive epitopes: the main immunogenic region (MIR)(98). The physiological role of the MIR remains unknown, but it is strongly conserved across species, and it is confined to the extracellular portion of the α-subunit, although distinct from the α-Btx binding site. Determination of the residues involved in the MIR would have important diagnostic and potential therapeutic implications (99).

Based on hydrophilicity maxima in the α-subunit sequence, the major antigenic determinants were predicted to comprise residues 161-166, 330-340 and 387-392 (100). The last two regions are intracellular. Synthetic peptides containing α161-166 do not bind MIR antibodies and antibodies against α152-167 do not compete with MIR antibodies for binding to nAchR (39,101). Subsequently α125-147 was proposed as MIR because this synthetic peptide reportedly bound 25-55% of anti-nAchR antibodies (31). This number is probably an overestimate (30), and synthetic peptides from this region do not bind MIR antibodies (32). Indeed, none of a series of overlapping synthetic peptides covering the whole extracellular segment of the α-subunit binds MIR antibodies, demonstrating the limitations of this approach for mapping conformation-dependent epitopes (102). Probing of proteolytic α-subunit fragments with MIR antibodies mapped the MIR to the N-terminal side of α151-169 (71) and within α46-127 (30). Various segments of cDNAs encoding the Torpedo and chick α-subunit were used to produce fusion proteins containing large fragments of the extracellular portion of the α-subunit (69). From the reactivity of these proteins with MIR antibodies, it was concluded that the MIR was formed by at least 2 epitopes within α6-85.

nAchRs Related to the Electric Organ and Muscle nAchR

The abundance of nAchR mRNA in Torpedo electric organ, and the availability of N-terminal sequence data for all four nAchR subunits from this source allowed molecular cloning of the corresponding mRNAs (1). Subsequently, these clones were successfully used to isolate the homologous cDNAs from other vertebrate species (6,10). Radiolabeled single-stranded cloned cDNAs will form duplexes, not only with their complementary strand, but also with sequences that are up to 35% different. This permits detection of related sequences in cDNA or genomic libraries, and has been used to isolate clones related to the vertebrate muscle nAchR α-, β-, γ- and δ-subunits (15).

The nAchRs found on embryonic muscle or outside the endplate region on adult muscle were known to differ (96) from the nAchRs at the neuromuscular junction in their electrophysiologic (103) and biochemical (104)(except chick)(17,96), immunologic (105), pharmacologic (106), metabolic (107) and developmental (108) properties. These differences between junctional and extra-junctional receptors were thought to reflect differences in post-translational modifications of the same protein complex (109). Recently however, a clone most closely related to the γ-subunit was isolated from fetal or neonatal calf skeletal muscle and called ϵ-subunit (16). This ϵ-subunit, together with α-, β- and δ- subunits can form a functional receptor, whose properties most closely resemble those of junctional nAchRs (110). The electrophysiologic properties of the nAchR containing α-, β-, γ- and δ-subunits on the other hand are very similar to those of extra-junctional receptors. This represents the first case where the molecular basis of receptor subtypes has been elucidated.

Putative nAchRs have been described in several invertebrate phyla, including molluscs, worms and echinoderms (111), but most is known about nAchRs in arthropods (112), partly because they are potential targets for insecticides. The central nervous system of insects is rich in nAchRs which share some electrophysiological and pharmacological (113,114) as well as immunological (115) properties with vertebrate muscle nAchRs, but whose subunit composition seems considerably different (115). Nevertheless, using a chick nAchR muscle γ-subunit cDNA, a related clone was recently isolated from a Drosophila head cDNA library (116). This clone in turn detected the presence of other related sequences in the Drosophila genome, suggesting the presence of a gene family.

Vertebrate neural nAchRs are not well characterized due to their inaccessibility, the paucity of material available and the lack of suitable probes. Although neural nAchR subtypes have been postulated (117), the situation is complicated by the fact that some nicotine binding sites do not seem to bind acetylcholine (118), and some α-Btx binding components do not seem to form functional receptors (119), notwithstanding the fact that most nicotinic cholinergic ligands competitively inhibit this α-Btx binding (120). Only the ganglionic nAchR can at present be considered a defined nAchR subtype. Although the α-Btx binding component has been purified and exhibits sequence homology with muscle nAchRs (121), its acceptance as nAchR subtype in mammals awaits the demonstration that this protein complex has a receptor function. Recently, three new probes have become available for studying neural nAchRs. A snake venom polypeptide, κ-bungarotoxin, that has sequence homology to α-Btx, appears to specifically block ganglionic nAchRs (122). Secondly, MIR antibodies recognize putative nAchRs in chick brain and ciliary ganglia (123), and have been used to immuno-affinity-purify a high affinity nicotine binding site from chick brain which contains at least two subunits (124). Antibodies raised against these purified proteins have in turn allowed isolation of the homologous rat subunits (125). Finally, a mouse muscle nAchR α-subunit cDNA identified a clone with related sequence in a pheochromocytoma cell line, known to contain both a ganglionic nAchR and α-Btx binding component (126). This putative neural nAchR cDNA detected the presence of related genes on genomic DNA blots and was subsequently used to isolate clones coding for other members of what is becoming a rapidly growing gene family (15). Currently several approaches are used to correlate the physiologic, biochemical and recombinant DNA data. N-terminal sequences of the purified subunits can be compared with the translated cDNA sequences and antibodies to these subunits can be used to identify the clones encoding them in expression libraries. The cDNA clones and antibodies against synthetic

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peptides from the predicted sequence or purified subunits can be used to map the location of cells containing the corresponding nAchRs (127,128). Expression of cDNAs will allow determination of the physiologic and pharmacologic properties of the cloned sequences.

Conclusion

Through recombinant DNA techniques significant progress has been made towards determining the molecular structure and structure-function relationships of the nAchR. Mapping of functional sites onto the primary sequence is still coarse and the data are at times conflicting; increased accuracy and resolution of some ambiguities can be attained with existing techniques. Molecular modeling may fill some of the gap between primary structure and low resolution images, but the structural detail required for ligand design will probably have to await X-ray crystallography of the nAchR or a functional fragment thereof, which could be obtained by expression of cloned cDNAs (129,130). Meanwhile expressed receptors may provide a useful assay system for drug testing in cases where nAchRs from natural sources are difficult to obtain (e.g. human muscle, neural nAchRs). Comparison of the nAchR sequences from different subtypes or species and construction of chimeric nAchRs (131,132) or subunits (90) may yield clues about the location of binding sites and the reason for pharmacological differences. As demonstrated already for some enzymes (133-136), the combination of X-ray crystallography with site-directed mutagenesis will prove most powerful not only for mapping residues involved in a binding site, but also for assessing their role in binding and for elucidating the general principles of ligand-receptor interactions.

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Chapter 29. Progress Toward the Rational Study of Enzyme Structure-Function Relationships

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Introduction - In the past few years various techniques of *in vitro* mutagenesis have enormously enriched our ability to study the relationship between the structure and function of proteins. Though still in their infancy, these approaches are beginning to reveal, in ways hitherto inaccessible, some of the rules that relate the amino acid sequence of a protein to its three-dimensional structure and thence its function; nevertheless most mutations designed to cause a specific alteration in function still lead to surprising, often unanticipated consequences. In the sense that "engineering" implies that we can design *de novo* a protein for a particular purpose, the phrase "protein engineering" represents a considerable overstatement of our present abilities, though no overstatement of aspirations for the future.

Several years ago we reviewed the use of oligonucleotide mutagenesis as a new approach to enzyme structure-function studies (1). Several reviews have recently appeared summarizing the biochemical aspects of creating mutants (2-4), as well as a perceptive review of the strategic aspects of planning mutations (5). There is also a recent review of the subject, appropriately, in the first issue of *Protein Engineering* (6). The objective of this review, necessarily a snapshot of a very rapidly moving field, is to emphasize the generality of the approach by focusing on a few selected applications of various techniques of mutagenesis to studies of protein function.

A variety of systems has been studied and a wide range of questions about the structural requirements for protein function has been investigated. In general, target residues for mutagenic experiments are decided on the basis of existing structural or kinetic information; data from x-ray structures, chemical modification experiments, or from sequence comparisons may indicate sites that are of interest. Specific problems that have been addressed include determination of the roles of specific amino acids in catalysis and in substrate binding, in allosterism, in protein stability, in protein-protein interactions, and in protein-DNA interactions.

Catalysis and Substrate Binding - The role of an individual amino acid in catalysis has been assessed using oligonucleotide-directed mutagenesis on **triosephosphate isomerase** (TIM). TIM interconverts dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). Glu 165 was postulated on the basis of chemical modification experiments (7) and the crystal structure (8) to participate in catalysis as a general base, first accepting the pro-R hydrogen of DHAP and then protonating the resulting enediol at C-2 to form GAP (Figure 1). The mutant Glu 165 → Asp, which moves the general base by the distance of one methylene group, was constructed and found to have k_{cat} for the forward reaction reduced by a factor of 240, while K_M for the forward reaction was increased by a factor of 2. This confirmed that Glu 165 is important for catalysis, but involved only slightly in

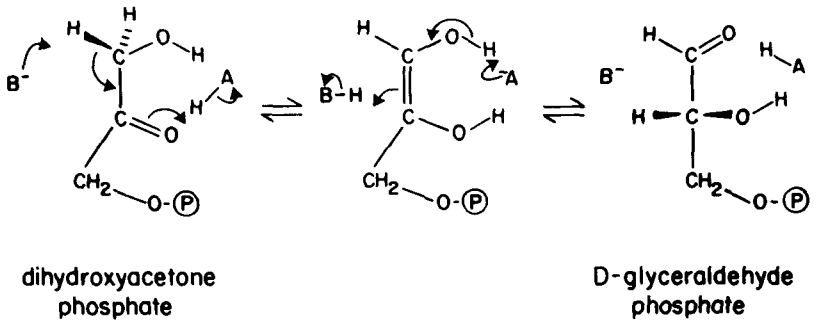

Figure 1. The reaction catalyzed by TIM. B^- represents the active site base, Glu 165 and AH is an electrophilic residue as yet not definitively identified. From ref. 9 with permission.

the binding of substrate (9). A more detailed study of the kinetics of catalysis by the Glu 165 → Asp mutant has been carried out by measuring the isotope effect on incorporation of solvent protons into the product. An increase relative to the wild-type enzyme of 4 kcal/mole in the energies of the transition states between the enediol intermediate and enzyme bound substrate and product was calculated (10). The increase in the energy of the transition states was great enough to cause a change in the rate-determining step from product dissociation for the wild-type enzyme, to conversion of the enediol to enzyme-bound substrate or product for the mutant in which the free energies of the transition states for the two enolization steps have been seriously affected such that each of the proton abstraction steps is slower by a factor of about 1000 in the mutant enzyme.

The energetics of substrate-active site interactions and how they affect catalytic efficiency has been investigated using a series of mutations of tyrosyl tRNA synthetase (TStyr, reviewed in ref. 11). TStyr from *B. stearothermophilus* is a dimer of 47.5 kdalton subunits whose crystal structure has been solved to 3.0 Å resolution with (12) and without (13) substrate bound to the active site. The enzyme catalyzes the transfer of tyrosine to tyrosyl tRNA, after forming a tyrosyl-AMP intermediate (Figure 2). Examination of the crystal structure implicated several residues as being involved in hydrogen bonds to the intermediate, tyrosyl adenylate. Thr 40 and His 45 are involved in hydrogen bonds to the γ-phosphate of the tyr-ATP transition state. The energetics of this interaction were determined by creating mutations which removed the hydrogen bonds, causing an increase in the energy of the transition state, but no significant change in the binding of substrates. Cys 35 was observed to contribute a good hydrogen bond to the C(3) hydroxyl of the ribose ring of ATP. Mutants Cys 35 → Ser and Cys 35 → Gly were constructed and shown to have reduced affinities for substrate ATP because of the complete removal of the hydrogen bond (Cys 35 → Gly) or its slight displacement (Cys 35 → Ser) (15). The Cys 35 → Ser mutant had a lower affinity for ATP than the Cys 35 → Gly, a difference that was attributed to the ability of Cys 35 → Ser to hydrogen bond to water in the absence of substrate. A similar effect was reported for mutations at Thr 51, which was thought from the crystal structure to be too far from the ribose O-1 ring oxygen to be involved in a strong hydrogen bond. The Thr 51 → Ala mutant showed a slightly raised affinity for ATP (16). Interestingly, a Thr 51 → Pro mutant showed a 50-fold increase for the efficiency of the formation of tyrosyl adenylate due mainly to a lower K_M for ATP (16, 17). This was explained by local distortions in the peptide backbone, which influenced the position of His

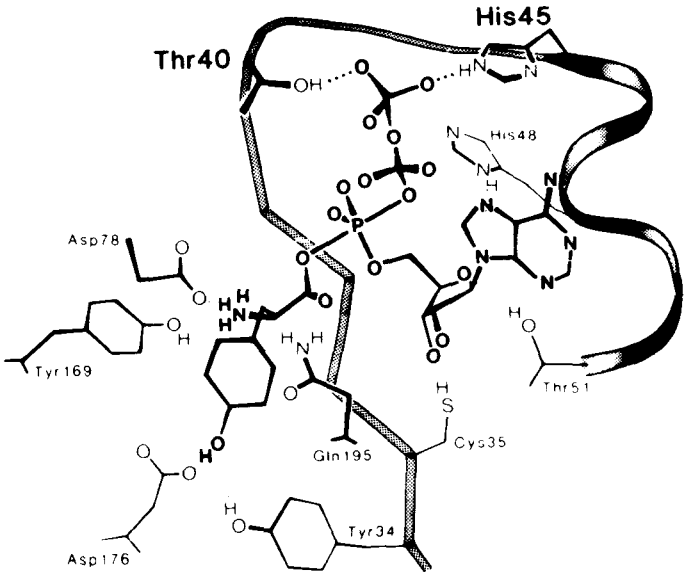

Figure 2. Representation of the bound intermediate involved in the formation of tyrosyl adenylate. From ref. 14 with permission.

48 so that it had an improved hydrogen bond to the O-1 of ribose. Investigations of the energetics of reactions catalyzed by these and other mutants of TStyr have led to the formulation of general rules about the energy of hydrogen bonds; those involving uncharged donors and acceptors are worth 0.5 to 1.5 kcal/mole of binding energy and, if either donor or acceptor is charged, the binding energy is increased by 3 kcal/mole (18). Such quantitative insights have only become possible through our recently acquired ability to generate essentially any structural variant of an enzyme.

The roles of conserved residues in the active site of **β-lactamase** have been investigated by site-directed mutagenesis. The conserved diad including the active site serine, Ser 70-Thr 71, was inverted to Thr 70-Ser 71; the resulting mutant was shown to be completely inactive as a β-lactamase (19). The mutant Ser 70 → Cys was constructed and shown to have 1-2% of the value of k_{cat} of the wild-type enzyme (20). Taken together with studies of the reversion of the double mutant Ser 70 → Thr, Thr 71 → Ser (21) these studies demonstrate the requirement for a primary nucleophile at residue 70 for any degree of catalytic activity. The conserved Thr adjacent to the active site serine was replaced with each of the other nineteen amino acids; though 14 of the 19 mutants were able to confer to cells a resistant phenotype, all nineteen mutants were less thermally stable than the wild-type enzyme (22) suggesting that the conserved Thr 71 plays an important role in the stable folding of the protein but is not directly involved in catalysis. The evolutionary relationship between **β-lactamase** and **D-ala-D-ala carboxypeptidases** was investigated by substituting a 30 amino acid sequence of a carboxypeptidase for a 29 amino acid sequence about the active site of β-lactamase. The resulting hybrid protein gained 2% of the carboxy-peptidase activity of the D-ala-D-ala carboxypeptidase (23).

Oligonucleotide-directed mutagenesis has also been used to study **serine proteases**. The binding of substrate to **trypsin** from rat pancreas has been investigated. Trypsin catalyzes the cleavage of peptide bonds with a specificity for peptides containing lysine or arginine. Mutations converting

Gly 216 and Gly 226, both located in the substrate binding site, to alanine, separately and as a double mutation affected the specificity (k_{cat}/K_M) of the enzyme for arginyl and lysine-containing substrates (24). The wild-type enzyme shows a specificity for arginyl substrates. The Gly 216 → Ala mutant shows an enhanced specificity for arginyl substrates, but the Gly 226 → Ala mutant has a higher specificity for lysine-containing substrates. The double mutant, Gly 216 → Ala/Gly 226 → Ala, had a lowered specificity for arginyl substrates.

For **subtilisin**, the variation in activity with pH was altered by introducing a mutation converting Asp 99 to serine. The mutant showed a reduced catalytic efficiency at alkaline pH, despite the location of the mutation 14-15 Å from the active site, probably due to long range electrostatic effects (25). The oxyanion of the tetrahedral intermediate generated during catalysis by subtilisin was postulated to be stabilized by hydrogen bonds from two sources, the amide protons of the side chain of Asn 155 and a proton from a peptide backbone amide. Removal of the amide side chain protons by converting Asn 155 to leucine caused a reduction in catalytic rate by 200-300, but did not affect the substrate binding constant, confirming the role of Asn 155 in stabilizing the transition state (26).

The roles of active site residues in the catalytic mechanism of **dihydrofolate reductase** (DHFR) have been investigated. DHFR catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. The carboxyl group of Asp 27 is believed to stabilize a protonated transition state. An Asp 27 → Asn mutant was constructed and found to have only 0.1% of the activity of wild-type enzyme (27). Structural requirements for catalysis were also studied by removal of a *cis* peptide bond between Gly 95 and Gly 196, by creating a Gly 95 → Ala mutant, resulting in the total loss of activity. A Pro 39 → Cys mutant was able to form a disulfide bond with Cys 85 which caused a reduction in activity (27). The role of Leu 54 was investigated by creating a mutant converting it to glycine. The mutant had an increased rate of substrate release and a decrease of 10^4 in the rate of hydride transfer. This indicated a change in the rate-determining step from product release for the wild-type to hydride transfer for the mutant, despite the location of Leu 54 some 10 Å from the active site (28).

One of the most striking examples of the effects of point mutations on biological activity is the activation of **proto-oncogenes**. The cellular analog of the transforming gene of Harvey murine sarcoma virus (c-Ha-*ras*) is able to transform cells when mutations in codon 12, normally coding for glycine, are present (29). All 19 mutations at residue 12 were constructed by oligonucleotide-directed mutagenesis and tested for their ability to transform NIH 3T3 cells. All but Gly 12 (which is encoded by normal cellular *ras* genes) and Gly 12 → Pro were able to transform cells. The authors suggested that these two amino acids (Gly and Pro) affect an α-helical structure in that region of the protein necessary for the protein's ability to cause transformation (30).

Investigations of the importance of phosphorylation of tyrosines on the transforming properties of oncogene products have utilized oligonucleotide-directed mutagenesis. The **middle-T antigen** of polyoma virus can transform cultured rat cells (31). Middle-T antigen maintains a tyrosine-kinase activity probably due to its association with $pp60^{c\text{-}src}$, a cellular **proto-oncogene** product (32). The association causes an increase in the specific activity of the kinase (33). Middle-T antigen is itself a substrate for the kinase activity. The effect of phosphorylation of several tyrosines on its transforming ability has been determined by the use of site-directed mutagenesis to convert known tyrosine phosphate acceptors to phenylalanine. Tyrosines 315, 250, 322 and

297 are known to be phosphate acceptor sites. Deletion of Tyr 322 or Tyr 297 had no apparent effect on transforming activity (34). A mutation converting Tyr 250 to Phe caused a reduction in transforming ability (35) as judged by a focus formation assay. Mutations converting Tyr 315, the major site of phosphorylation, to Phe have produced conflicting results, either causing a drastic reduction (35) or little change (34, 36) in transforming ability. Differences in the assays and cell lines used probably account for the different results. Similar experiments on the oncogene of Rous sarcoma virus, $pp60^{v\text{-}src}$, have shown that phosphorylation of Tyr 416 is not essential for transformation, since the mutant Tyr 416 → Phe could cause transformation (37). An autophosphorylation site, Tyr 1073, of $P130^{gag\text{-}fps}$, the oncogene product of Fujinami sarcoma virus, was mutated to serine and threonine. Neither Ser nor Thr were autophosphorylated and the mutants had lowered enzymatic and oncogenic activities (38). The ability of the c-*ras*-encoded protein, p21 (the cellular analog of the transforming gene of Harvey murine sarcoma virus) to bind GTP was abolished by conversion of Asn 116 to either lysine or tyrosine. The mutants also lost their ability to autophosphorylate and to transform NIH3T3 cells (39).

Allosterism - Oligonucleotide-directed mutagenesis has proven useful in the study of the roles of individual amino acids in the allosteric regulation of activity. Mutants of **aspartate transcarbamoylase** (ATCase) have been constructed. ATCase catalyzes the conversion of carbamoyl phosphate and aspartate to carbamoylaspartate and inorganic phosphate, the first committed step in pyrimidine biosynthesis. The enzyme consists of six 34 kdalton catalytic subunits and six 17 kdalton regulatory subunits. Homotropic cooperativity is exhibited by both substrates, and feedback inhibition is accomplished by the heterotropic inhibitor, CTP. ATP acts as a heterotropic activator to ensure the equal production of purines and pyrimidines. Biophysical techniques and x-ray structures of the enzyme alone and in the presence of N-phosphonoacetyl aspartate (PALA, a competitive inhibitor) indicate a substantial restructuring of the holoenzyme on binding of the allosteric effectors (40). The roles of several individual amino acids in mediating the conformational changes of the enzyme have been investigated. Target residues were identified by examining data from chemical modification studies in addition to the x-ray data. A mutant converting Gln 133, known to reside near the contact region between the catalytic and regulatory subunits, to Ala caused a marked increase in cooperativity without affecting activity (41). From the x-ray crystal structure, His 134 was shown to be near the phosphate binding site and was suggested as a possible proton donor to the transition state. Mutation of His 134 to Ala resulted in a mutant with 5% of the catalytic activity of the wild-type, considerable cooperativity and increased substrate binding constants (41). Mutation of Tyr 165, which was thought to be in the substrate binding site, caused an increase in binding constants for both aspartate and carbamoyl phosphate. V_{max} decreased to about 30% of the wild-type value, and cooperativity was also decreased (42). Lys 84 was suggested on the basis of inactivation by-pyridoxyl phosphate and $NaBH_4$ to be essential for catalysis (43). Substitution of Arg or Gln for Lys 84 gave a mutant possessing only 0.05 or 0.01% of the activity of the wild-type enzyme, respectively. Chemical modification of Lys 83 also caused considerable inactivation. However, substitution of Gln for Lys 83 caused little change in activity (41). A loop of the protein containing residues 230-245 was shown to undergo a large movement on changing from the T to the R state, and a Tyr 240 → Phe mutant showed altered homotropic interactions and a different reactivity towards p-hydroxymercuribenzoate in the unligated state, suggesting an altered conformation (44).

Stability - The contribution of disulfide bonds to the thermal stability and conformation of proteins has been studied. Successful attempts to introduce disulfides into proteins to increase the thermal stability have taken into consideration not only the proximity in the three-dimensional structure of the protein of the two sites for potential cysteines, but also their relative orientations, since not all dihedral angles are conducive to disulfide formation. An intramolecular disulfide bond was "engineered" into **T4 lysozyme** by replacing Ile 3 with Cys which could then form a bond with Cys 97 (45). The disulfide-containing mutant had a substantially increased thermal stability. An intermolecular thiol-disulfide exchange reaction was shown to occur with an unpaired Cys 54, leading to production of inactive oligomers of the Ile 3 → Cys mutant. This reaction was prevented by converting Cys 54 to Thr or Val (46). The introduction of a disulfide into **dihydrofolate reductase** also increased the thermal stability of the enzyme (47). Human **interleukin-2** contains a disulfide bridge between Cys 58 and Cys 105 and a free Cys at position 128. Mutant Cys 58 → Ala showed 250 times less activity than wild-type while mutant Cys 105 → Ala showed 8-10 times less activity. Cys 128 → Ser showed comparable activity to wild-type (48). Removal of the disulfide bond (Cys 77-Cys 123) of **RTEM-1 β-lactamase** by conversion of Cys 77 to serine caused a decrease in the thermal stability and stability at alkaline pH of the enzyme, though the catalytic parameters at lower temperatures and neutral pH were unchanged from those of the parent enzyme (49). Enzyme with the disulfide thusly removed (Cys 77 → Ser) was, however, considerably less able to accommodate changes at Thr 71 than the native **RTEM-1 β-lactamase** (50).

Single amino acid substitutions have been found that dramatically affect the stability of enzymes. The **lac permease** of *E. coli* catalyzes the H^+/β-galactoside symport. A secondary structural model of the single polypeptide chain of the protein based on hydropathic analysis and circular dichroic measurements proposes that 12 α-helical domains span the membrane. A mutation converting Gln 60, which resides in the middle of the second proposed α-helix from the N-terminus, to Glu caused a decrease in the thermal stability. The mutant lost activity at 45°C with a half-life of 20 minutes, while the wild-type had a half-life of 50 minutes (51). General methods for increasing the thermal stability of proteins by single amino acid replacements have been proposed (52). Comparison of the primary sequences of the **neutral protease** of thermophilic and mesophilic bacteria suggested the construction of a mutation containing the substitution Gly 144 → Ala, which is of higher thermal stability than the wild-type enzyme (53). Individual mutants of **kanamycin nucleotidyl transferase** that enhance the thermostability of the enzyme have been identified (54). The combination of two separate mutations into a double mutant resulted in a cumulative enhancement of stability (55). The stability of **subtilisin** to oxidative inactivation has been improved by mutagenesis (56). Mutation of Met 222 to non-oxidizable amino acids, such as Ser, Ala or Leu, prevented inactivation by peroxide. A Met 350 → Val mutant of **α_1-antitrypsin**, a protease inhibitor, was more resistant to oxidative inactivation by N-chlorosuccinimide than the wild-type enzyme (57).

Protein-Protein Interactions - Alterations in the interactions at the interface of protein dimers have been made. Phe 164 of **tyrosyl tRNA synthetase** interacts with its partner Phe 164 in the active dimer. A mutant converting Phe 164 to Asp showed a shift in the pH dependence of activity. At pH 6, where dimerization is favored, the mutant was fully active. At pH 7.8, dissociation of the dimer was favored and the activity was lowered (58). Mutagenesis of **λcI repressor** converted Tyr 88, known to interact with its partner Tyr 88 in the dimer, to Cys. The mutant could then spontaneously

form a disulfide bridge in the dimer without perturbing the structure of the dimer. The disulfide-crosslinked dimer was shown to have an increased binding affinity for its operator sequence. In contrast, a mutant converting Tyr 85, which was known to be distant from its partner Tyr 85 in the dimer, to Cys created a mutant which could not spontaneously form a disulfide bond. Reaction with dithio-2-nitro benzoic acid caused dimer formation, and the dimers had a 10-fold reduced affinity for the operator sequence (59).

Protein-DNA Interactions - The mechanisms of recognition and binding interactions between sequence-specific DNA-binding proteins and their DNA substrates have been explored using oligonucleotide-directed mutagenesis. A synthetic gene for the **λ cro protein** was constructed by ligation of a series of oligonucleotides, including conveniently located sites for restriction endonucleases (60). Excision of segments of the wild-type sequence and replacement with a duplex strand of synthetic oligonucleotides allowed the rapid production of several single amino acid replacements. Several mutations in the α-3 helix, one of the helices thought to determine DNA sequence specificity, altered the affinity of *cro* for its operator sequence (see Figure 3 for a stereo drawing of the presumed binding of *cro* to O_R3). Tyr 26

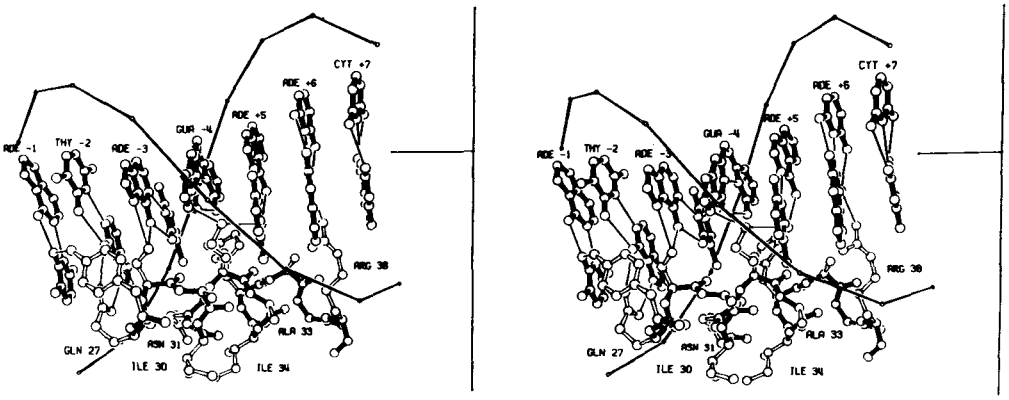

Figure 3. Stereo drawing showing the presumed hydrogen bonding of *cro* to base pairs in the major groove of O_R3, following model building and energy refinement. From ref. 61. Reprinted by permission from *Nature*, Vol. 298, No. 5876, pp. 718-723, Copyright (c) 1982 Macmillan Magazines Limited.

was suggested on the basis of the x-ray structure to donate a hydrogen bond to O-4 of a thymine (61). Conversion of Tyr 26 to Asp caused a substantial reduction in affinity for the O_R3 operator sequence, while conversion of Tyr 26 to Phe caused an intermediate level of affinity. Interestingly, converting Tyr 26 to lysine had little effect on the binding affinity, results explained by an ionic interaction between the lysine and the phosphate backbone substituting for the lost hydrogen bond. Gln 27 was thought to be involved in two hydrogen bonds as both a donor and acceptor with an adenine. Substitution of Leu, Cys, or Arg for Gln 27 resulted in a large decrease in binding affinity. Serine 28 was thought to be involved in two hydrogen bonds to an adenine. Removal of the hydroxyl of serine by conversion to alanine caused a pronounced decrease in binding affinity (60). The DNA-binding specificity of **bacteriophage 434 repressor** was changed to the specificity of the repressor from bacteriophage P22 by altering five amino acids on one side of an α-helix, the side thought to form specific contacts with the operator sequence (62).

Conclusions - Although still in its infancy, the use of mutagenic techniques to create new proteins has allowed us, as illustrated in the foregoing discussion, to gain many insights into the rules that relate amino acid sequence to three-

dimensional structure and function. Further applications and refinements of these approaches will eventually lead to an ability for rational design of proteins for applications in medicine, industry and commerce with a broad range of specifically tailored, novel and useful properties.

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Chapter 30. Prodrugs and Site-Specific Chemical Delivery Systems

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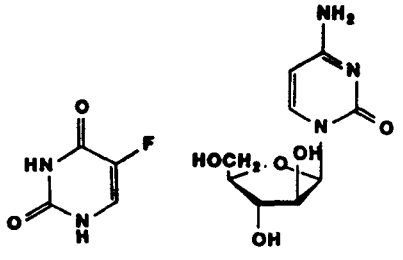
Introduction - A number of recent review articles and books address various aspects of prodrugs (1,2). The confusion between the terms prodrug and soft drug was clarified (3). These terms represent opposite concepts: a prodrug is inactive by design and it is activated predictably in vivo, while soft drugs are active species designed to undergo a predictable and controllable metabolic deactivation (4). Simple prodrug design cannot solve the variety of transport-delivery problems, particularly when site-specific or site-enhanced delivery is the objective. Chemical Delivery Systems (CDS) have been proposed as an approach to this problem (5,6). Previous reviews in Annual Reports have dealt with selected aspects of work in these fields (7, 8); this review presents a broader view of the subject, concentrating on the past 3-4 years.

Antiviral/Anticancer Agents - The pharmacology of prodrugs of 5-fluorouracil (5-FU, **1**) and ara-C (**2**) was reviewed (9). The L-isomer **3** of the ara-C phospholipid conjugate was found to be more active than the D and D, L-isomers, or than 5'-O-palmitoyl- and N^4-acyl-ara-C (10). The prodrug **3** is water soluble, and it is a substrate for the synthesis of phosphatidylinositol and releases ara-C as a by-product. Diacyl glycerol esters of ara-C diphosphate, like **3**, are readily taken up into both HDL and LDL, which is believed to be transporting them (11). The cyclic prodrug ancitabine (**4**) was demonstrated to undergo chemical, nonenzymatic hydrolysis to yield ara-C (12,13). Ftorafur (**5**), was shown to be converted to 5-FU both oxidatively by P-450 (14) and by hydrolytic cleavage catalyzed by soluble enzymes (15). The prodrug **6** was well absorbed orally and produced sustained 5-FU levels (16). Various "potential" prodrugs of 5-FU were described which are N_1 or N_2 mono- or N_1 N_3 - disubstituted derivatives, with alkoxycarbonyl (17, 18) or acyloxymethyl (19) groups. Novel peptide delivery systems have been investigated as prodrug forms of 5-FU (20). Diester prodrugs of 5-fluorodeoxyuridine (FUDR, **7**) were studied; long-chain (C_{10}-C_{14}) esters were more potent due to their sustained release properties (21). Dicarboxylic acid [-CO(CH_2)n-COOH; n = 2-6] esters of FUDR were made and studied for their hydrolysis rates with porcine liver esterase (22). Enzymatic cleavage rates were 100 times higher at pH5 than at 7, supporting a concept that negatively charged molecules are not good esterase



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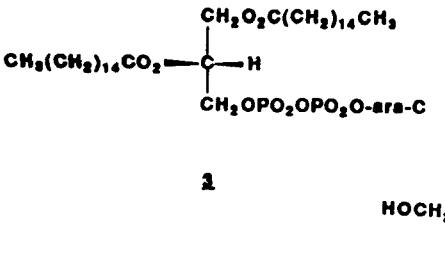

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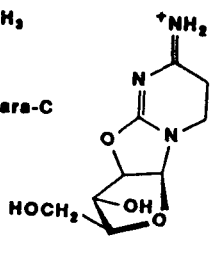

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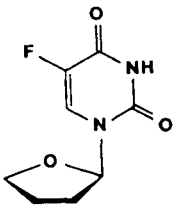

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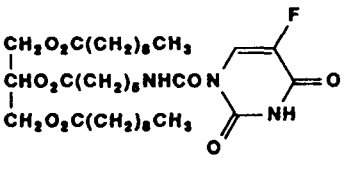

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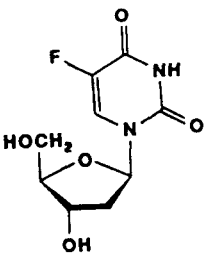

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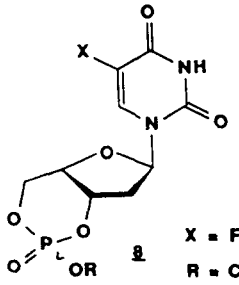
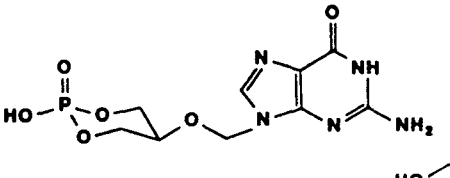
substrates. Neutral cyclic monophosphate triesters **8** were made for improved transport properties (23). The much lower activity of **8** suggests that they either do not readily enter the infected cells or are not converted easily to 5-FUDR-phosphate intracellularly. The cyclic monophosphate (**8**, R=H) was also much less active. In contrast the cyclic monophosphate **9** of DHPG (**10**) (but not the mono- or diphosphates) was active against thymidine kinase negative HSV-1 virus (24). A bibliography (25) of acyclic nucleosides other than acyclovir (AC, **11**) includes some prodrugs of AC.



8 X = F,I,Cl,CF_3; R = CH_3,CH_2Ph



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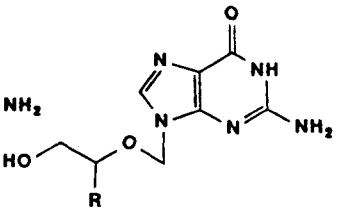
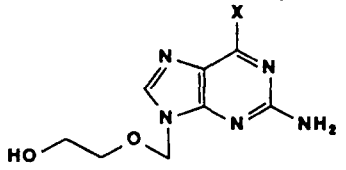


10 R = CH_2OH

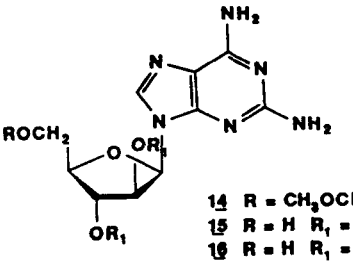
11 R = H

The 6-deoxyacyclovir (A515U, **12**) a xanthine oxidase-activated prodrug (26) was found to give high **11** plasma levels (27). The 6-amino analog **13** (A134U) has also resulted in better oral bioavailability (28) of **11**, both in dogs and rats (29), and in humans (30), via deamination catalyzed by adenosine deaminase. Optimal topical antiherpes activity of the methoxyacetate prodrug (**14**) of cycladine (**15**) was found (31). Among the other ester-type prodrugs of **15**, the 2',3'-diacetate (ara-ADA, **16**) was the most effective for topical treatment of HSV-2 infections (32). A large number (>50) of carboxyalkyl derivatives **17** of adenine and other purine bases was examined for antiviral activity (33). Ester prodrugs **18** were found much more potent than the parent compounds. The amino analog **19** and its prodrugs (R = C_1 - C_7 alkyl) showed potent antiviral activity (34). The dihydropyridine/pyridinium redox CDS was successfully applied for brain-selective delivery of trifluorothymidine (TFT, **20**). While **20** is ineffective, **21** resulted in significant reduction in Herpes Simplex Virus (HSV) titer in a rat encephalitis model (35). A similar CDS for acyclovir resulted in significant and sustained (over 2 days after one injection) brain delivery (36). The antitumor imidazotetrazine **22**, a prodrug of the alkylating agent **23** (NSC 157949) exhibited pronounced activity against P388, B16 melanoma, colon 38 tumor, Lewis lung carcinoma, and the LX-1 lump tumor xenograft (37).



12 X = H

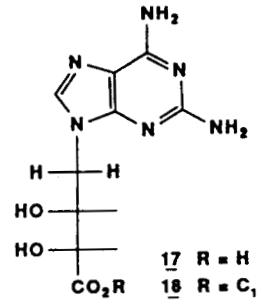
13 X = NH_2



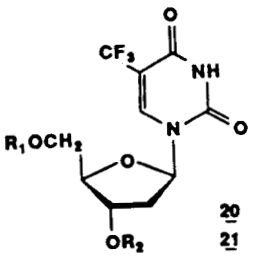
14 R = CH_3OCH_2CO R_1 = H

15 R = H R_1 = H

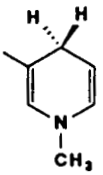
16 R = H R_1 = CH_3CO

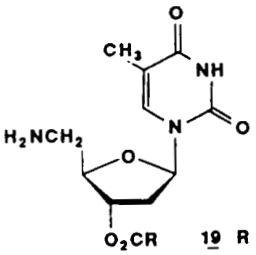


17 R = H
18 R = C_1 - C_7 alkyl

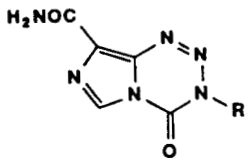


20 $R_1 = R_2 = H$
21 R_1 = COBu^t R_2 = CO

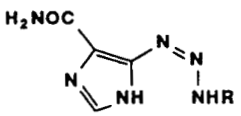




19 R = H



22 R = CH_2CH_2Cl



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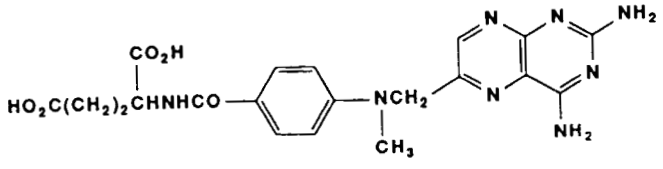
The greater cytotoxicity against tyrosinase-containing melanotic cells of 2,4-dihydroxyphenylalanine was explained by its conversion to the cellular toxin 6-OH-dopa (38). It was suggested that methotrexate (MTX, **24**) is a prodrug, as polyglutamation in the cells enhances its cytotoxic action (39). Nitrogen mustard prodrugs were reported with potential organ specificity, like the phosphoramide esters of diethylstilbestrol (breast), psoralen (**25**, skin), and propranolol (**26**, lung) (40). Selectivity was not observed, and the compounds have only marginal activity. Various alkyl and acyloxycarbonyl prodrugs of mitomycin C (MCC, **27**, R=H) were reported (41). The more lipophilic ones (**28**) were also incorporated in liposomes, which provided a slow, sustained release for **27** (42). Mitomycin was also coupled via an ε-amino-caproic acid spacer with dextran for sustained release after intratumoral injection (43). Dramatic brain-selective delivery was achieved using the redox CDS of hydroxy-CCNU **29** (44).



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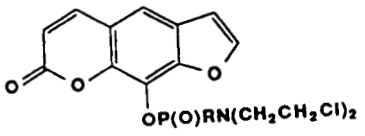

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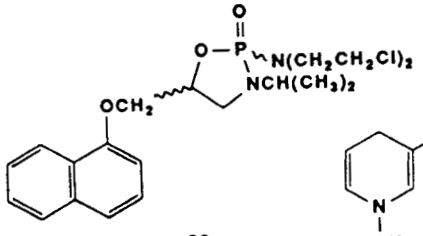

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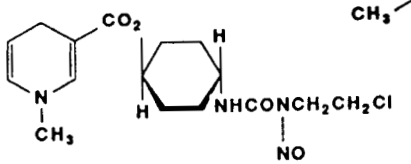

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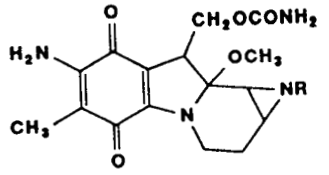
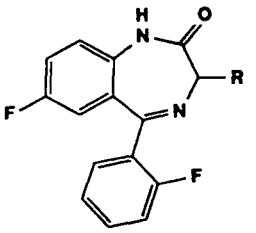
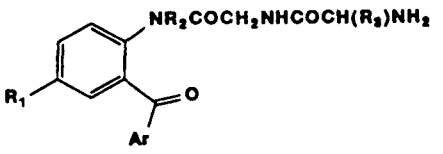


27 R = H
28 R = $CO_2C_9H_{19}$

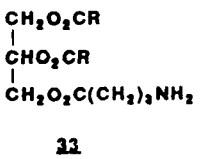
CNS Agents - Brief reviews on the delivery of drugs across the blood brain barrier (8) and on the interrelationship of prodrugs and directly-acting compounds in the field of "classical" and "annelated" benzodiazepines have appeared (45). Ethyl loflazepate (**30**) is a prodrug of the potent descarboxyloplazepate (**31**), which due to distribution-pharmacokinetic reasons has improved anxiolytic/sedative ratio (46). A review describes the water-soluble, bioequivalent peptide prodrugs (**32**) of the benzodiazepines (47). Esters of 3-OH-1,4-benzodiazepines (like oxazepam, or lorazepam) serve as prodrugs with increased brain penetration (48). Various prodrug attempts were reported to allow brain delivery of neurotransmitters and related agonists, antagonists, and precursors. The N-isonicotinoyl GABA was reported to produce increases in the brain GABA levels (49). Lipid esters like **33** and **34** were reported (50). While the "brain uptake index" obtained is impressive, the actual brain levels do not support the claims for increased GABA delivery. A redox CDS (**35**) for GABA was reported to give brain-selective "lock-in" of the oxidized form and to produce marked and sustained anxiolytic response with mimimal sedation (51). Analogs and prodrugs (**36**) of the powerful GABA agonist, muscimol were reported (52). Monocyclophosphates (**37**) ("probicyclophosphates") for highly potent, toxic bicyclic GABA antagonists were described (53). The pivalyloxymethylester prodrug of (+)-nipecotic acid showed significant anticonvulsant activity (54). Increasing both lipid and water solubility of L-DOPA by esterification did not produce increase in activity (55). A "lymphotropic" prodrug **38**, however, produced prolonged brain dopamine levels due to the lymphatic absorption of **38**, followed by slow release of L-DOPA (56). Direct evidence for the brain-specific delivery of dopamine from a redox CDS (**39**) was reported (57). Ethoxycarbonyl prodrugs of phenylethylamine, amphetamine and tranylcypromine have produced modified brain levels of these amines in the brain (58). Marginal improvement in the oral bioavailability of methyldopa was only achieved, using the esters **40** (59). Tryptamine was, however, successfully delivered



30 R = $CO_2CH_2CH_3$
31 R = H



32 R_3 = residue of gly-leu-lys



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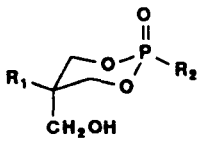
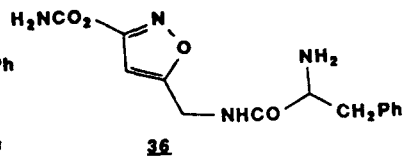


37 R_1 = Bu^t,cyclohexyl



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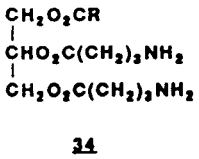

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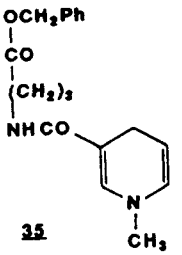

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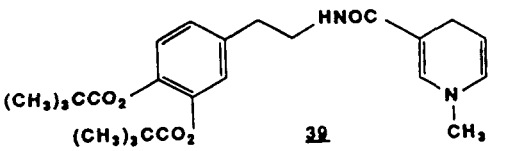

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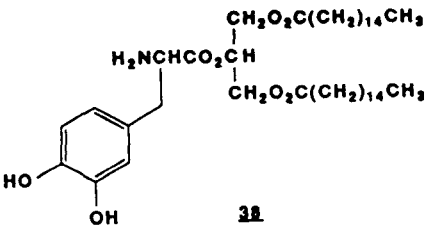

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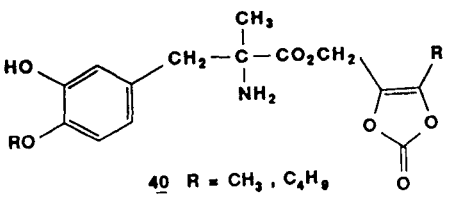
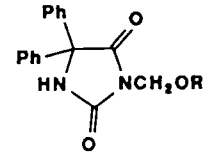
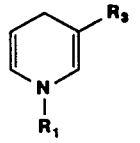


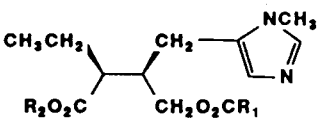
40 R = CH_3, C_4H_9

to the brain by the dihydropyridine redox CDS (60). Among the various water-soluble prodrugs of phenytoin, the phosphate **41** was found equipotent, while the N_1N-diethylglycinate **42** was most useful as an orally available precursor in dogs (61). A 7-fold decrease in the ED_{50} of electroshock therapy was achieved with the redox CDS **43** (62). The dihydropyridine ⇄ pyridinium salt redox CDS was further extended by developing alternate carriers, like the acids **44** (63) and the alcohols **45** and **46** (64). Clean separation of the central and peripheral effects in the case of estradiol was achieved using the redox CDS (65). The specific central effects lasted for 35 days after one single dose. The dihydropyridine-pyridinium salt redox delivery system was used for the specific delivery and sustained release of testosterone in the brain (66).

Ophthalmic Drugs - Sequentially labile prodrugs **47** of pilocarpine were reported recently (67). The "pro-prodrug" requires enzymatic hydrolysis to cleave the R_1COO-ester. The obtained monoester reverts in water to pilocarpine via specific base-catalyzed lactonization (68). A highly lipid soluble prodrug (**48**, L-645,151) of the carbonic anhydrase inhibitor (**49**, L-643,799) with improved corneal transport was developed (69). A CDS for site-specific intraocular delivery of epinephrine was developed. Adrenalone esters (**50**) are converted to epinephrine in vivo only in the iris-ciliary body by a reduction - hydrolysis sequence (70). Hydrolysis of the esters **50** anywhere in the body produces the inactive **51**. It was recently found that many of the β-blockers can also be produced specifically in the iris-ciliary body from a ketoxime CDS like **52** for propranolol (71).





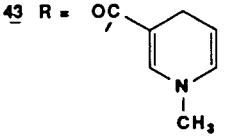


47

41 R = PO_3Na_2

42 R = $COCH_2N(CH_2CH_3)_2$

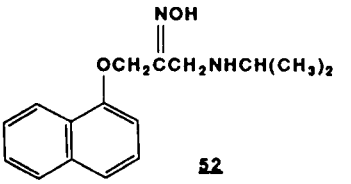
43 R =



44 $R_1 = (CH_2)_nCO_2H$ $R_3 = CONH_2$

45 $R_1 = R$ $R_3 = CONH(CH_2)_nOH$

46 $R_1 = (CH_2)_nOH$ $R_3 = CONH_2$



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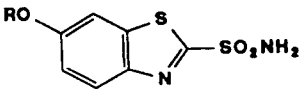
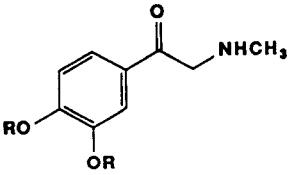


48 R = $OCBu^t$

49 R = H

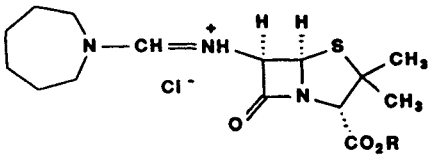


50 $R = COR_1$ C_2 -C_8 alkyl,aralkyl

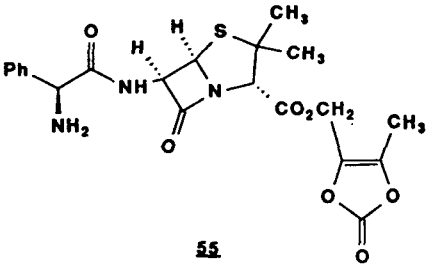
51 R = H

Antiinfectives - Improvement of the pharmacodynamic properties of antibacterial agents via transient chemical modification continues to be an area of active interest. The design of prodrugs of β-lactam antibiotics has recently been reviewed (72). The relationship between the physical-chemical properties and oral bioavailability in mice of prodrugs of parenteral cephalosporins was studied to obtain fundamental information for the rational design of oral prodrugs (73,74). Bacmecillinam **53** is a new orally well-absorbed prodrug of the amidinopenicillin mecillinam **54** (75). Acyloxyallyl esters of ampicillin were investigated as novel ester prodrugs. KBT-1585 (**55**) was identified and is currently in clinical trials (76-

78). The bifunctional prodrug (KY-109, **56**) was synthesized to improve the oral absorption of the parent drug (KY-087, **57**), a cephalosporin similar in activity to cefamandole (79). Mutual prodrugs of the olivanic acids and an inhibitor of the renal dipeptidase enzyme have been investigated with the preparation of the double ester formaldehyde hydrate prodrugs (MM22382 **58**) and (MM13902, **59**), respectively. Administration of these linked esters in mice resulted in improved urinary recovery of the anitbiotics (80). The pharmacology and pharmacokinetics in man of cefuroxime axetil, the acetoxyethyl ester prodrug of cefuroxime has recently been discussed (81-83). The preparation of amino acid esters as potential water soluble prodrugs has been reported for metronidazole (84-86) and several cephalosporins (87). The kinetics and mechanism of hydrolysis of a novel prodrug (**60**) of cycloserine has been described (88). Alafonsfalin (**61**) is an antibacterial phosphonopeptide which requires peptide transport for activity. The dipeptide prodrug is cleaved to aminoethylphosphonic acid which inhibits alanine racemase and uridine 5'-diphosphate-N-acetylmuramoylalanine synthetase (89). Novel peptide delivery systems have been investigated as prodrug forms of polyoxins (90). Benzophenone (**62**) has been synthesized as a prodrug of the anthelmentic, mebendazole (**63**) (91).



53 R = $CH(CH_3)OCO_2CH_2CH_3$
54 R = H



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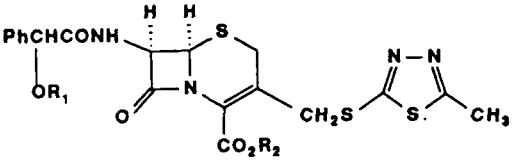
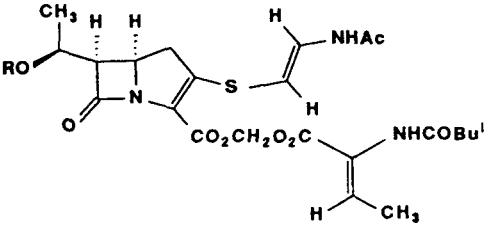
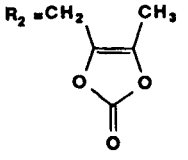
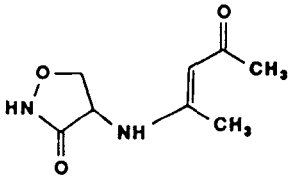


56 R_1 = $COCH(CH_3)NH_2$ HCl
57 R_1 = R_2 = H





58 R = H
59 R = SO_3H



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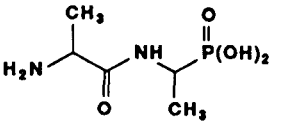

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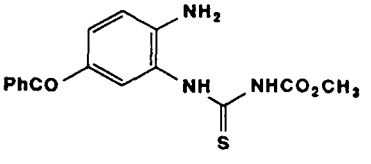

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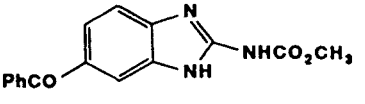

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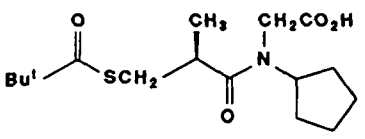
Cardiovascular Agents - The absorption and metabolism of the disulfide dimer conjugate of captopril has been investigated in the rat following both oral and intravenous dosing and compared to captopril (92). Several prodrugs of other sulhydryl-containing ACE inhibitors have been reported. Pivopril, (REV-3659, **64**) was one of the more active members in a series that lowered blood pressure in sodium-deficient spontaneously hypertensive rats (93). Related structures that also exhibited significant *in vivo* antihypertensive activity include compounds **65-68** (94-95).



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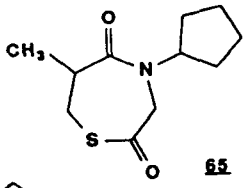

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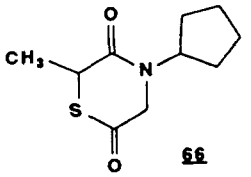

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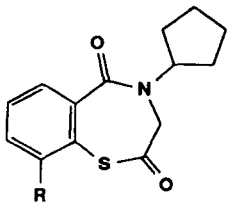
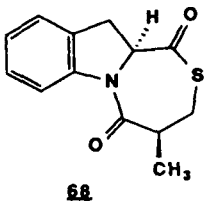
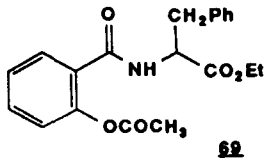


67 R = H, Cl, OCH_3



68

Antiinflammatory Agents - A review on prodrugs of the acidic nonsteroidal antiinflammatory drugs (NSAID's) has recently appeared (96). Salicylamide O- and N- derivatized analogs continue to be examined as prodrugs to improve both oral and rectal delivery of salicylamide (97, 98). Transient chemical modification of salicylamide at molecular sites not involved in presystemic conjugative metabolism has been reported also. This investigation has been confusingly referred to as an "alternative prodrug approach" (99). Aspirin phenylalanine ethyl ester (**69**) was reported as a mechanism-based, designed prodrug of aspirin (100-102). However, careful re-examination of this work demonstrated that **69** is enzymatically converted to aspirinphenylalanine and salicylphenylalanine, but not aspirin (103,104). *In vivo* testing of aspirin prodrug **70** produced measurable plasma levels of aspirin rather than salicylic acid (105). In several animal models, the prodrug morniflumate (**71**) was approximately equipotent to niflumic acid in antiinflammatory, analgesic and antipyretic activity (106). The gastrointestinal ulcerogenicity of **72** (CS-600, loxoprofen) is also relatively weak. The available evidence suggests that **72** is a prodrug and exerts its antiinflammatory activity after conversion to the active metabolite (**73**) (107). Ro 03-6037, **74** undergoes species-dependent metabolism to the propionic acid Ro 03-9341 (**75**). All species are capable of the initial metabolic step, reduction of the carbon-to-carbon double bond. However, only rat, dog, rabbit and marmoset are capable of removing the one-carbon fragment; the higher primates, including man, seem unable to achieve this cleavage to any great extent (108). Improved delivery of indomethacin through the skin has been investigated with the preparation of an N,N-diethylhydroxyl amine derivative. In animal models, this prodrug was minimally more effective than indomethacin in inhibiting thermal inflammation, but it showed no advantage over indomethacin in inhibiting UV-B radation erythema in human volunteers (109). From consideration of the influence of molecular structure on ester reactivity, a number of potential steroid prodrugs were prepared and evaluated *in vitro* and *in vivo* (110-112). The effect of incorporating biphasic solubilizing groups into prodrugs of steroids has been examined to improve delivery of the drug through biological membranes such as the skin. Prodrugs **76** and **77** were the most interesting candidates identified (113). Steroid glycosides and the unique glycosidase activity of the colonic microflora form the basis for a novel colon-specific drug delivery system (114, 115).



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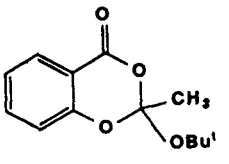

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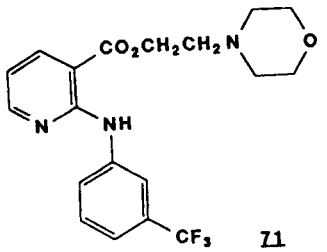

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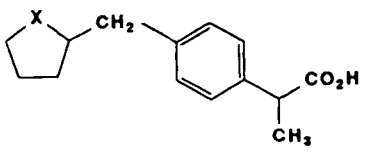
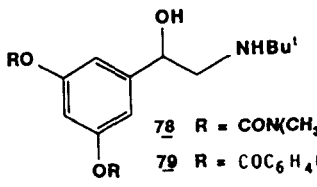


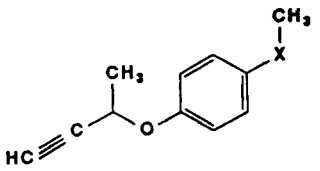
72 X = CO

73 X = 



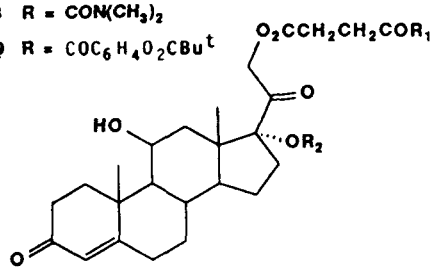
78 R = $CON(CH_3)_2$

79 R = $COC_6H_4O_2CBu^t$



74 X = $C{=}CHCO_2CH_2CH_3$

75 X = $CHCO_2H$

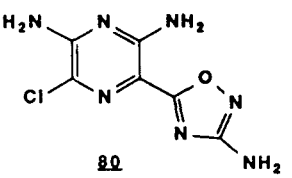


76 $R_1 = N(CH_2CH_3)_2$ $R_2 = H$

77 $R_1 = N(CH_2CH_3)_2$ $R_2 = COC_4H_9^n$

Antiallergy Agents - Prodrugs of selective bronchodilator β-adrenoceptor agonists have recently been reviewed with special emphasis on the development of agents with increased specificity for targeting to the lung (116-117). A comparative study of bitolterol with other β_2 - agonists has been reported (118) and duration studies in animal models of two new tertbutaline prodrugs, bambuterol (**78**) and the cascade ester (D 2438, **79**) have also been described (119). A number of potential prodrugs of theophylline analog dyphilline were prepared to prolong its duration of action (120).

Miscellaneous - Systematic alteration of the oxyacetic side chain in a new class of (aryloxy) acetic acid diuretics has shown that the carboxylic acid is the active species in vivo and that the ethyl ester serves as a prodrug to enhance oral absorption (121). The facile conversion of prodrug (**80**) to amiloride was demonstrated chemically and in vivo in rats and dogs (122). Esters and lactones of phenolic amino carboxylic acids were examined as prodrugs for iron chelators. The prodrug (**81**) exhibited the highest therapetic index and in vitro measurements show the rate of ester hydrolysis at pH 7.5 is increased by a factor of 10^4 in the presence of 5×10^{-4} M ferric ion (123). Several acyl derivatives were synthesized specifically as prodrugs of the alcohol deterrent agent cyanamide (124). Several 2-substituted thiazoldine-4(R)-carboxylic acids were synthesized as prodrugs of l-cysteine and their ability to protect mice against acetominophen-induced hepatoxicity was examined (125, 126).



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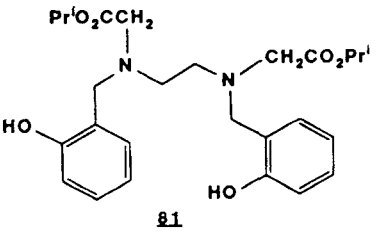

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Section VII. Special Topics

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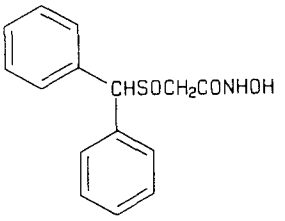
Chapter 31. To Market, To Market - 1986

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 in 1986 exceeded yearly launches during the past two years by about 25% (1,2). More than 40% of these first time worldwide NCE launches occurred in Japan, with West Germany, the United States, France, Italy and the United Kingdom, in aggregate, accounting for an equal percentage. Japan also topped the list of originators of the NCEs launched in 1986, followed by the United States, West Germany and France. Interestingly, all of the NCEs that originated in Japan also had their first launches in that country.

A record number of NCEs were marketed in the United States during 1986 (3). Four of these, butoconazole nitrate, dronabinol, muromonab-CD3 and trientine hydrochloride, were first time worldwide launches and are included in the following compilation.

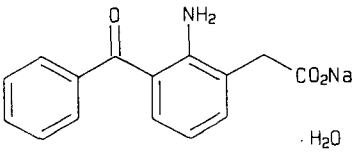
Adrafinil (psychostimulant) (4-6)



Country of Origin: **France**
Originator: **Labs. L. Lafon**
First Introduction: **France**
Introduced by: **Labs. L. Lafon**
Trade Name: **OLMIFON**

Adrafinil is a centrally-acting, α_1-adrenergic agonist useful in the management of vigillance disturbances and depression in the elderly.

Amfenac Sodium (antiinflammatory)(7-9)

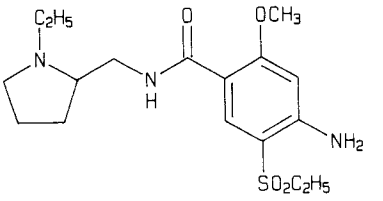


Country of Origin: **USA**
Originator: **A.H. Robins**
First Introduction: **Japan**
Introduced by: **Meiji Seika**
Trade Name: **FENAZOX**

Amfenac sodium is a non-steroidal antiinflammatory agent structurally related to ketoprofen, suprofen (10) and tiaprofenic acid. It is reported to be effective in the short term treatment of rheumatoid arthritis, osteoarthritis and pain associated with minor surgical procedures.

Amisulpride (antipsychotic)(11,12)

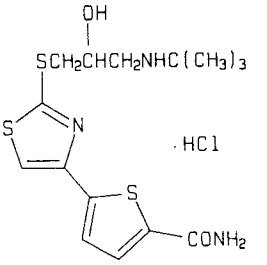
Country of Origin: **France**
Originator: **SESIF (Delagrange)**
First Introduction: **Portugal**
Introduced by: **INFAR**
Trade Name: **SOCIAN**



Amisulpride is an antipsychotic agent structurally related to sulpiride and sultopride. Useful in the treatment of schizophrenia, it is not, however, without EPS liability.

Arotinolol Hydrochloride (antihypertensive)(13-15)

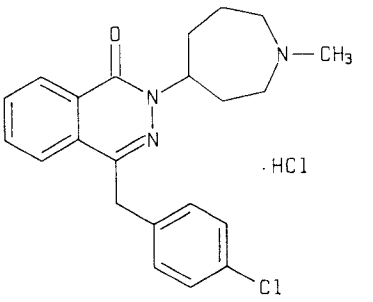
Country of Origin: **Japan**
Originator: **Sumitomo**
First Introduction: **Japan**
Introduced by: **Sumitomo**
Trade Name: **ALMARL**



Arotinolol hydrochloride is a β-adrenergic blocker with weak α-antagonistic properties, useful in the treatment of hypertension.

Azelastine Hydrochloride (antihistamine)(16-18)

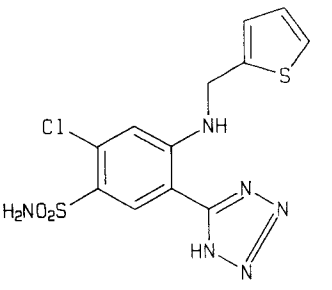
Country of Origin: **W. Germany**
Originator: **Asta-Werke (Degussa)**
First Introduction: **Japan**
Introduced by: **Eisai**
Trade Name: **AZEPTIN**



Azelastine hydrochloride is an orally effective antihistamine useful in the treatment of asthma and nasal allergy. It appears to inhibit release of histamine, in addition to antagonizing its action.

Azosemide (diuretic)(19-21)

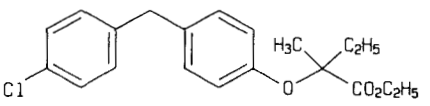
Country of Origin: **W. Germany**
Originator: **Boehringer Manheim**
First Introduction: **W. Germany**
Introduced by: **Boehringer Manheim**
Trade Name: **LURET**



Azosemide is a high-ceiling diuretic similiar in structure and profile to furosemide, albeit somewhat less potent and less bioavailable. It is useful in the treatment of congestive heart failure and similar edematous conditions.

Beclobrate (hypolipidemic)(22-24)

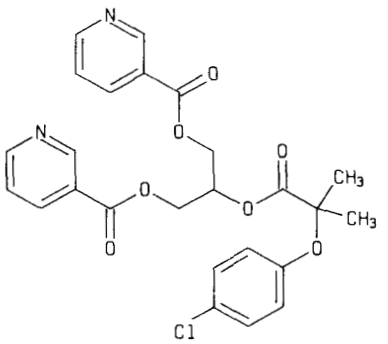
Country of Origin: **Switzerland**
Originator: **Siegfried AG**
First Introduction: **Switzerland**
Introduced by: **Siegfried AG**
Trade Name: **BECLOSCLERIN**



Beclobrate is a hypolipidemic agent related structurally to clofibrate. It is reportedly useful in types IIa-V hyperlipoproteinemias, promoting normalization of the atherogenic index.

Binifibrate (hypolipidemic)(25, 26)

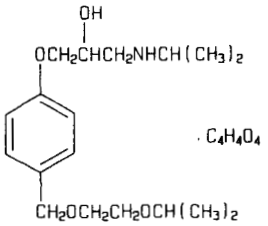
Country of Origin: **Spain**
Originator: **Labs. Wassermann**
First Introduction: **Spain**
Introduced by: **Labs. Wassermann**
Trade Name: **BINIWAS**



Binifibrate is a triglyceridic, mutual prodrug of the hypolipidemics, nicotinic acid and clofibrate. In addition to its usefulness in the treatment of types II-IV hyperlipoproteinemias, it is reported to possess beneficial microhemorheological properties.

Bisoprolol Fumarate (antihypertensive)(27-29)

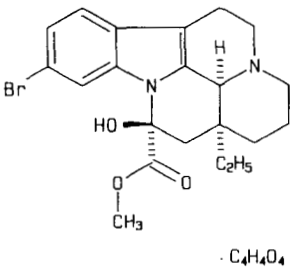
Country of Origin: **W. Germany**
Originator: **E. Merck**
First Introduction: **W. Germany**
Introduced by: **E. Merck**
Trade Name: **CONCOR**



Bisoprolol fumarate is a β_1-selective adrenergic blocker useful in the treatment of essential hypertension.

Brovincamine Fumarate (cerebral vasodilator)(30-32)

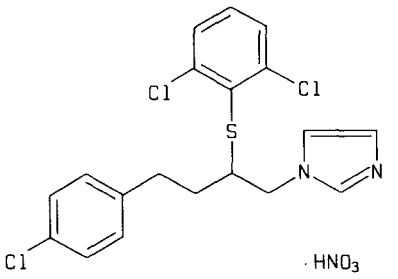
Country of Origin: **Switzerland**
Originator: **Sandoz**
First Introduction: **Japan**
Introduced by: **Sankyo**
Trade Name: **SABROMIN**



Brovincamine fumarate is a derivative of vincamine which selectively increases cranial and coronary blood flow. It is reported to be useful in the treatment of multi-infarct dementia.

Butoconazole Nitrate (topical antifungal)(33, 34)

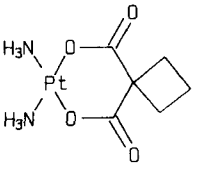
Country of Origin: **USA**
Originator: **Syntex**
First Introduction: **USA**
Introduced by: **Syntex**
Trade Name: **FEMSTAT**



Butoconazole nitrate is an antifungal agent related to miconazole and other imidazole antimycotics. It is useful in the topical treatment of vulvovaginal candidiasis, being similar in effectiveness to miconazole and clotrimazole.

Carboplatin (antineoplastic)(35-38)

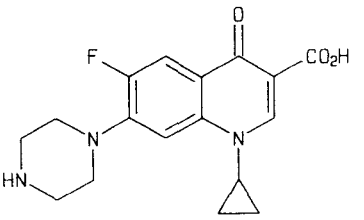
Country of Origin: **United Kingdom**
Originator: **Johnson Matthey**
First Introduction: **United Kingdom**
Introduced by: **Bristol**
Trade Name: **PARAPLATIN**



Carboplatin is a second generation, platinum-containing antineoplastic agent with significantly reduced nephro-, neuro-, and ototoxicity in comparison to cisplatin. It is effective in the treatment of advanced ovarian carcinoma of epithelial origin and small cell carcinoma of the lung.

Ciprofloxacin (antibacterial)(39,40)

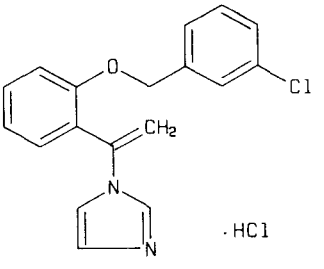
Country of Origin: **W. Germany**
Originator: **Bayer**
First Introduction: **Philippines**
Introduced by: **Bayer**
Trade Name: **CIPROBAY**



Ciprofloxacin is a quinolone antibacterial related to recently marketed norfloxacin (10), ofloxacin (2), pefloxacin (2) and enoxacin. It has a broad spectrum of activity against gram-positive and gram-negative bacteria, and is useful in the treatment of urinary and upper respiratory tract infections.

Cloconazole Hydrochloride (topical antifungal)(41,42)

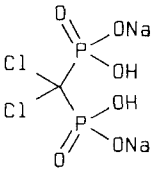
Country of Origin: **Japan**
Originator: **Shionogi**
First Introduction: **Japan**
Introduced by: **Shionogi**
Trade Name: **PILZCIN**



Cloconazole hydrochloride is a novel imidazole antifungal agent, useful in the topical treatment of dermatomycoses due to Tinea and Candidia spp.

Clodronate Disodium (calcium metabolism regulator)(43-45)

Country of Origin: **USA**
Originator: **Proctor & Gamble**
First Introduction: **Finland**
Introduced by: **Oy Star**
Trade Name: **BONEFOS**



Clodronate disodium is a bone resorption inhibitor, useful as adjunct therapy in the treatment of osteolytic bone metastases and malignant hypercalcemia.

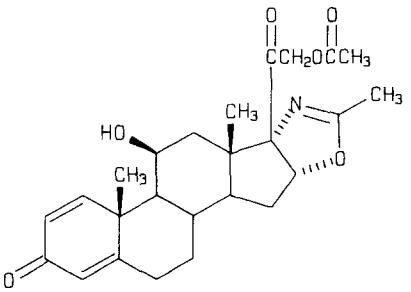
Defibrotide (antithrombotic)(46-49)

Country of Origin: **Italy**
First Introduction: **Italy**
Trade Names: **NORAVID; PROCICLIDE**
Originator: **Crinos**
Introduced by: **Roussel Maestretti; Crinos**

Defibrotide is an antithrombotic, partially-depolymerized polydeoxyribonucleotide obtained from bovine lungs. It is reported to be useful in the treatment of myocardial and severe lower limb ischemia, renal failure due to thrombotic microangiopathy, and thrombophlebitis, and in the prophylaxis of deep vein thrombosis, possibly via PGI_2 stimulation.

Deflazacort (antiinflammatory)(50-53)

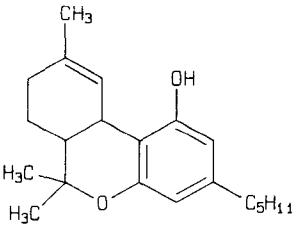
Country of Origin: **Italy**
Originator: **Dow Lepetit**
First Introduction: **Italy**
Introduced by: **Lepetit/Merrell; Guidotti**
Trade Names: **LANTADIN; DEFLAN**



Deflazacort is an antiinflammatory glucocorticoid useful in the systemic treatment of rheumatoid arthritis. Its antirheumatic potency is similar to that of prednisone, while its hyperglycemic and calcium depleting effects are reportedly less.

Dronabinol (antinauseant)(54-56)

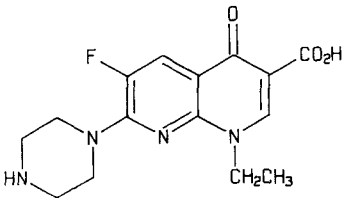
Country of Origin: **USA**
Originator: **Unimed**
First Introduction: **USA**
Introduced by: **Roxane**
Trade Name: **MARINOL**



Dronabinol (synthetic Δ^9-THC) is an antinauseant approved for the treatment of nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional antiemetics. A related cannabinoid, nabilone, was introduced in Canada for this indication in 1982.

Enoxacin (antibacterial)(57-60)

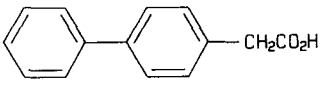
Country of Origin: **Japan**
Originator: **Dainippon**
First Introduction: **Japan**
Introduced by: **Dainippon**
Trade Name: **FLUMARK**



Enoxacin is a broad spectrum, quinolone-class, antibacterial agent closely related structurally to nalidixic acid. The serum half-life (6.2 hrs.) and urinary recovery (70%) are considerably greater than for other newer agents of this class, such as norfloxacin (10) and earlier mentioned ciprofloxacin.

Felbinac (topical antiinflammatory)(61)

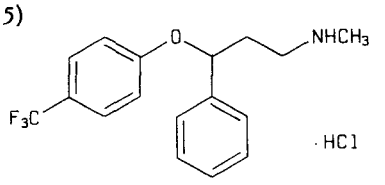
Country of Origin: **USA**
Originator: **Lederle**
First Introduction: **Japan**
Introduced by: **Lederle**
Trade Name: **NAPAGELN**



Felbinac is the active metabolite of the non-steroidal antiinflammatory agent, fenbufen. Applied topically as a gel to joints, it is useful in the symptomatic relief of articular inflammation and pain.

Fluoxetine Hydrochloride (antidepressant)(62-65)

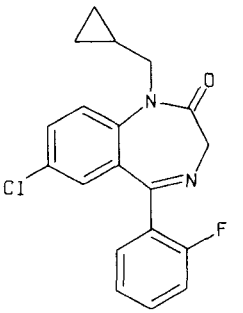
Country of Origin: **USA**
Originator: **Lilly**
First Introduction: **Belgium**
Introduced by: **Lilly**
Trade Name: **PROZAC**



Fluoxetine hydrochloride is a serotonin-selective antidepressant approved for the treatment of major depressive disorders, including those with concomitant anxiety. It also appears effective in the treatment of obesity.

Flutoprazepam (anxiolytic)(66-68)

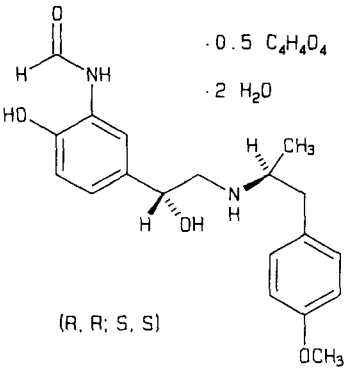
Country of Origin: **Japan**
Originator: **Sumitomo**
First Introduction: **Japan**
Introduced by: **Kanebo; Banyu**
Trade Name: **RESTAS**



Flutoprazepam is a benzodiazepine anti-anxiety agent, somewhat longer acting and more potent than diazepam.

Formoterol Fumarate (bronchodilator)(69, 70)

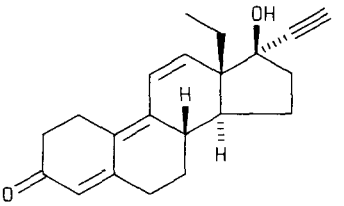
Country of Origin: **Japan**
Originator: **Yamanouchi**
First Introduction: **Japan**
Introduced by: **Yamanouchi**
Trade Name: **ATOCK**



Formoterol fumarate is a β_2-selective adrenergic agonist useful in the treatment of bronchial asthma. Its potency and duration of action are reported to exceed those of salbutamol, as is its ability to inhibit allergic and non-allergic histamine release.

Gestrinone (antiprogestogen)(71-73)

Country of Origin: **France**
Originator: **Roussel UCLAF**
First Introduction: **Brazil**
Introduced by: **Sarsa**
Trade Name: **DIMETROSE**



Gestrinone is a synthetic steroid with antiprogesterone and antiestrogenic activities. It is reported to be useful in the treatment of endometriosis and uterine leiomyomas.

Gonadoreline-6-D-Trp Acetate (hormone)(74, 75)

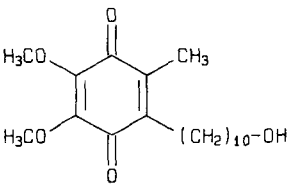
Country of Origin: **USA**
Originator: **Tulane Univ.**
First Introduction: **W. Germany**
Introduced by: **Ferring**
Trade Name: **DECAPEPTYL**

pyro-Glu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-$NH_2 \cdot CH_3CO_2H$

Decapeptyl is a modified (D-Trp^6) LH-RH. Like recently marketed buserelin and leuprolide (I), it is useful in achieving medical castration in the treatment of advanced prostate cancer.

Idebenone (nootropic)(76-79)

Country of Origin: **Japan**
Originator: **Takeda**
First Introduction: **Japan**
Introduced by: **Takeda**
Trade Name: **AVAN**



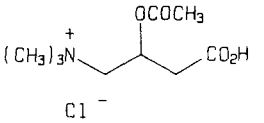
Idebenone is a structural relative of ubiquinone (Coenzyme Q_{10}) that protects the CNS from the effects of ischemia via improving brain metabolism. It is reported to be of potential use in the management of patients with cerebral apoplexy, cerebral arteriosclerosis and related disorders.

Lentinan (immunostimulant)(80-83)

Country of Origin: **Japan**
First Introduction: **Japan**
Trade Name: **LENTINAN**
Originator: **Ajinomoto**
Introduced by: **Morishita; Yamanouchi; Ajinomoto**

Lentinan is an immunostimulant β-1,6; β-1,3-D-glucan isolated from the edible mushroom, Lentinus edodes. It is currently approved, in combination with the cytostatic tegafur, for the treatment of gastric cancer.

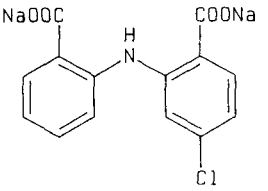
Levacecarnine Hydrochloride (nootropic)(84-87)



Country of Origin: **Italy**
Originator: **Sigma-Tau**
First Introduction: **Italy**
Introduced by: **Sigma-Tau; Glaxo**
Trade Name: **NICETILE; BRANIGEN**

Levacecarnine hydrochloride is a nootropic agent structurally related to the natural substance L-carnitine. It is reported to be useful in the treatment of cognition disorders in the elderly, perhaps due to its weak cholinergic properties.

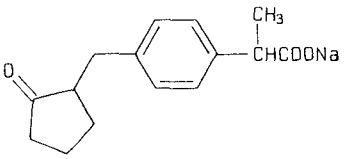
Lobenzarit Sodium (antiinflammatory) (88-91)



Country of Origin: **Japan**
Originator: **Chugai**
First Introduction: **Japan**
Introduced by: **Chugai**
Trade Name: **CARFENIL**

Lobenzarit sodium is an antiinflammatory agent useful in the treatment of rheumatoid arthritis, especially that of recent onset. Although structurally related to meclofenamic and mefenamic acids, its antiinflammatory profile is reported to be more immunomodulating in character.

Loxoprofen Sodium (antiinflammatory)(92-94)

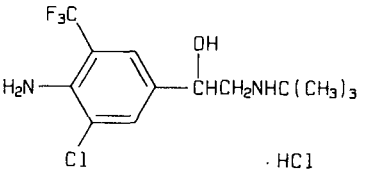


Country of Origin: **Japan**
Originator: **Sankyo**
First Introduction: **Japan**
Introduced by: **Sankyo**
Trade Name: **LOXONIN**

Loxoprofen sodium is an antiinflammatory/analgesic agent useful in the management of rheumatoid arthritis and related disorders. It is converted in vivo to the corresponding trans-hydroxycyclopentane analog, through which it appears to exert its activity.

Mabuterol Hydrochloride (bronchodilator)(95-97)

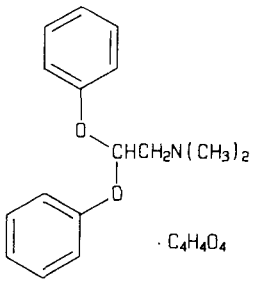
Country of Origin: **W. Germany**
Originator: **Dr. Karl Thomae**
First Introduction: **Japan**
Introduced by: **Kaken**
Trade Name: **BRONCHOLIN**



Mabuterol hydrochloride is a β_2-selective adrenergic agonist closely related to clenbuterol, and useful in the treatment of chronic bronchial asthma, bronchitis, and emphysema. Its oral potency and duration of action are reported to exceed those of salbutamol.

Medifoxamine Fumarate (antidepressant)(98-100)

Country of Origin: **France**
Originator: **Anphar Rolland (Lipha)**
First Introduction: **France**
Introduced by: **Anphar Rolland (Lipha)**
Trade Name: **CLEDIAL**



Medifoxamine fumarate is reported to be an imipramine-like antidepressant with anxiolytic, antispasmodic, and antiserotonin properties. It is devoid of anticholinergic activity.

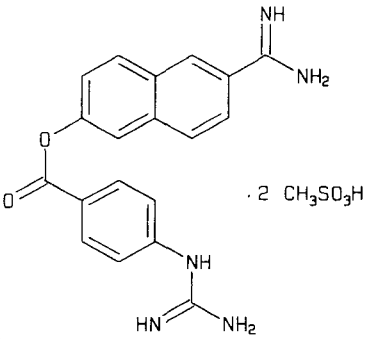
Muromonab-CD3 (immunosuppressant)(101, 102)

Country of Origin: **USA**
First Introduction: **USA**
Trade Name: **ORTHOCLONE OKT3**
Originator: **Ortho**
Introduced by: **Ortho**

Muromonab-CD3 is a murine monoclonal antibody to the T3 antigen of human T-cells, which blocks their killing ability. It is useful in the treatment of acute allograft rejection in renal transplant patients.

Nafamostat Mesylate (protease inhibitor)(103-105)

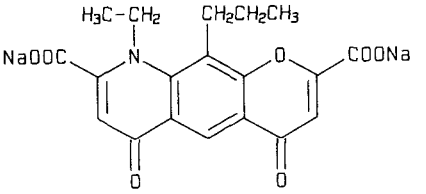
Country of Origin: **Japan**
Originator: **Torii**
First Introduction: **Japan**
Introduced by: **Banyu**
Trade Name: **FUTHAN**



Nafamostat mesylate is a protease inhibitor useful in the treatment of acute pancreatitis. It has anticomplement, anticoagulant, antikallikrein and other activities, and thus may have additional utility in the treatment of autoimmune diseases and disseminated intravascular coagulation.

Nedocromil Sodium (antiallergic)(106-109)

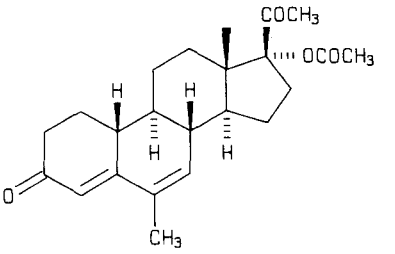
Country of Origin: **United Kingdom**
Originator: **Fisons**
First Introduction: **United Kingdom**
Introduced by: **Fisons**
Trade Name: **TILADE**



Nedocromil sodium is an antiallergic agent with broader mast cell stabilizing properties than related cromolyn sodium. Administered as an aerosol, it is useful in the treatment of bronchial asthma and related diseases.

Nomegestrol Acetate (progestogen)(110)

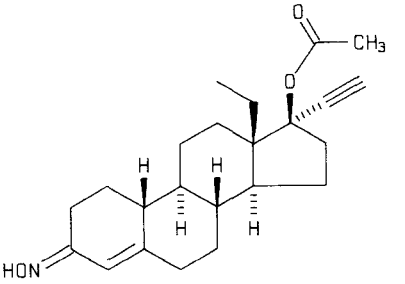
Country of Origin: **Monaco**
Originator: **Theramex**
First Introduction: **France**
Introduced by: **Theramex**
Trade Name: **LUTENYL**



Nomegestrol acetate is the 19-nor derivative of megestrol acetate, useful in the treatment of gynecological conditions associated with luteal deficiency.

Norgestimate (progestogen)(111-113)

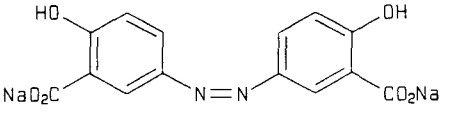
Country of Origin: **USA**
Originator: **Ortho**
First Introduction: **W. Germany**
Introduced by: **Cilag**
Trade Name: **CILEST**



Norgestimate is an orally-effective progestogen, recently launched in combination with ethinyl estradiol as an oral contraceptive.

Osalazine Sodium (intestinal antiinflammatory)(114-116)

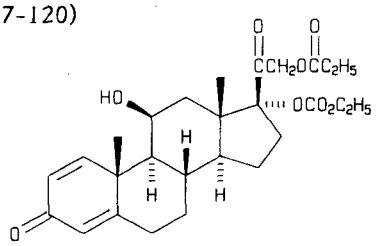
Country of Origin: **Sweden**
Originator: **Pharmacia AB**
First Introduction: **Netherlands; Sweden**
Introduced by: **Pharmacia AB**
Trade Name: **DIPENTUM**



Osalazine sodium is a prodrug of fisalamine (5-aminosalicylic acid) (1) useful in the treatment of ulcerative colitis.

Prednicarbate (topical antiinflammatory)(117-120)

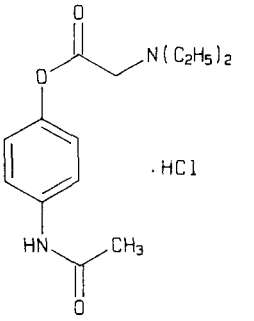
Country of Origin: **W. Germany**
Originator: **Hoechst AG**
First Introduction: **W. Germany**
Introduced by: **Cassella-Riedel**
Trade Name: **DERMATOP**



Prednicarbate is a potent topical antiinflammatory agent with minimal systemic effects. It is useful in the treatment of cutaneous inflammatory disorders such as eczema and psoriasis.

Propacetamol Hydrochloride (analgesic)(121,122)

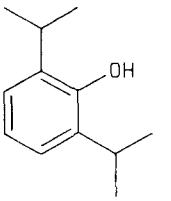
Country of Origin: **France**
Originator: **Hexachimie (UPSA)**
First Introduction: **France**
Introduced by: **Hexachimie (UPSA)**
Trade Name: **PRO-DAFALGAN**



Propacetamol is an injectable prodrug of paracetamol, useful in the symptomatic treatment of pain and fever.

Propofol (anesthetic)(123-126)

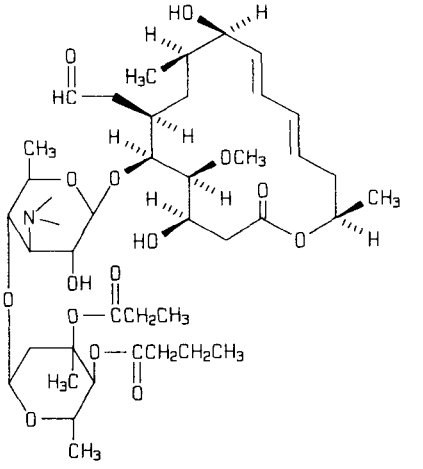
Country of Origin: **United Kingdom**
Originator: **ICI**
First Introduction: **United Kingdom**
Introduced by: **ICI**
Trade Name: **DIPRIVAN**



Propofol is an injectable, short-acting general anesthetic with a low incidence of side effects.

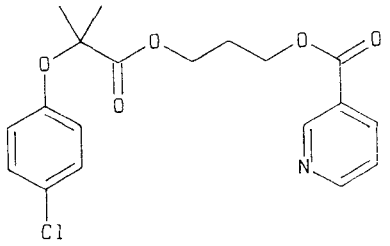
Rokitamycin (antibiotic)(127)

Country of Origin: **Japan**
Originator: **Toyo Jozo**
First Introduction: **Japan**
Introduced by: **Toyo Jozo**
Trade Name: **RICAMYCIN**



Rokitamycin is a semisynthetic macrolide antibiotic, closely related to miokamycin (2) both in structure and antimicrobial spectrum.

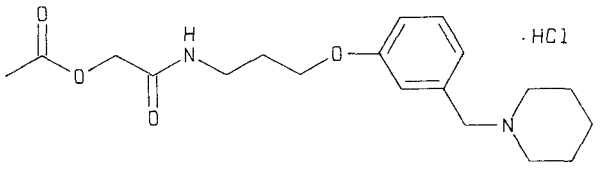
Ronafibrate (hypolipidemic)(128, 129)



Country of Origin: **Italy**
Originator: **Ibis**
First Introduction: **Italy**
Introduced by: **Ibis**
Trade Name: **CLOPRANE**

Ronafibrate, like earlier mentioned binifibrate, is a mutual prodrug of clofibrate and nicotinic acid, useful in the treatment of types II-IV hyperlipoproteinemia.

Roxatidine Acetate Hydrochloride (antiulcer)(130-132)



Country of Origin: **Japan**
First Introduction: **Japan**
Trade Name: **ALTAT**
Originator: **Teikoku Hormone**
Introduced by: **Teikoku; Takeda; Sumitomo**

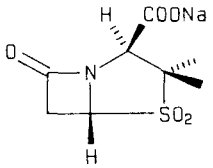
Roxatidine acetate hydrochloride is an H_2 antagonist, differing considerably in structure from other marketed agents (cimetidine, ranitidine and famotidine) in this category. It is useful in the treatment of gastric, duodenal and anastomotic ulcers, Zollinger-Ellison syndrome and peptic esophagitis.

Schizophyllan (immunostimulant)(133-135)

Country of Origin: **Japan**
First Introduction: **Japan**
Trade Name: **SONIFILAN**
Originator: **Taito**
Introduced by: **Kaken**

Schizophyllan is an immunostimulant polysaccharide related to lentinan, isolated from the culture filtrate of Schizophyllum commune. It is useful in combination with other antineoplastic treatments in the management of carcinomas of the lungs, stomach, uterus and breasts.

Sulbactam Sodium (β-lactamase inhibitor)(136, 137)

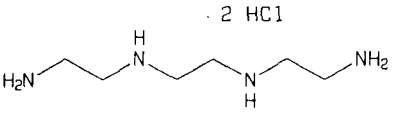


Country of Origin: **USA**
Originator: **Pfizer**
First Introduction: **Japan**
Introduced by: **Pfizer-Taito**
Trade Name: **SULPERAZONE**

Sulbactam sodium is a parenterally-active, β-lactamase inhibitor recently introduced as a 1:1 combination product with cefoperazone. Like clavulanic acid, the first agent of this type to be introduced, sulbactam enhances the effectiveness of β-lactam antibiotics against resistant strains.

Trientine Hydrochloride (chelator)(138-140)

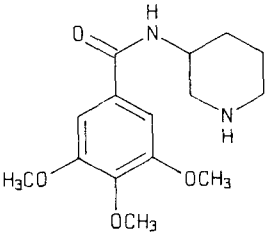
Country of Origin: **United Kingdom**
Originator: **Cambridge University**
First Introduction: **USA**
Introduced by: **Merck**
Trade Name: **CUPRID**



Trientine hydrochloride is a copper chelating agent recommended for the treatment of Wilson's Disease, especially in patients intolerant of penicillamine therapy. Unlike the latter agent, trientine is not indicated in cystinuria or rheumatoid arthritis.

Troxipide (antiulcer)(141-143)

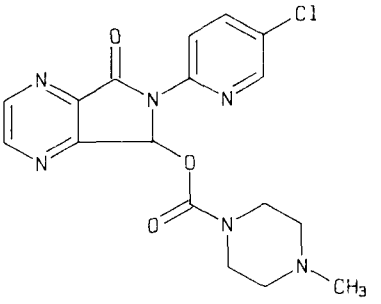
Country of Origin: **Japan**
Originator: **Kyorin**
First Introduction: **Japan**
Introduced by: **Kyorin**
Trade Name: **APLACE**



Troxipide is an antiulcer agent which is suggested to act via beneficial effects on gastric glycoprotein metabolism and blood flow.

Zopiclone (hypnotic)(144)

Country of Origin: **France**
Originator: **Rhone-Poulenc**
First Introduction: **Malaysia; Singapore**
Introduced by: **Rhone-Poulenc**
Trade Name: **IMOVANE**



Zopiclone is an effective hypnotic agent with a short duration of action. Although it interacts with the benzodiazepine receptor complex, it is reported to have minimal effects on memory, little synergy with alcohol, and low abuse potential.

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Chapter 32. Patents in Medicinal Chemistry

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Introduction - The majority of countries grant patents for inventions, giving the patentee a monopoly for a limited time in that country. The right given by a patent to exclude others from commercial exploitation of the invention may be extremely valuable. It is therefore desirable that medicinal chemists, whether working in industrial or academic research, should have some basic knowledge about patents. A brief review such as this can do no more than give a bare outline, and interested readers are referred to the listed books. These include books on patents for chemists and other non-patent specialists, dealing with all countries (1), or specifically with the patent law of the USA (2,3), Great Britain (4, 5), Germany (6), France (7) and the European Patent Convention (8), as well as books more suitable for patent specialists (9-18). However, when real-life problems arise, professional advice should always be obtained.

National and International Patent Systems - For over a century, international patenting has been regulated by the Paris Convention for the Protection of Industrial Property (1883). The basis of the Paris Convention is one of reciprocal rights, so that in each member country, foreign applicants will be treated on the same basis as home-country applicants. The most important provision of the Convention is that establishing Convention priority. If a patent application is made in one country, and corresponding applications are made in other countries within 12 months, then these later applications may claim priority from the first application. The later applications are treated as if they had been filed on the same day as the first application, so that any publication of the invention between the first filing date (the priority date) and the later filings will not affect the validity of the later applications.

Each country has its own patent law, and there are differences between the patent systems of different countries with respect to the term of patent protection, the type of protection available for chemical and pharmaceutical inventions, the strength of examination by the patent office, and many other points. Nevertheless there has been a considerable degree of standardization in recent years, particularly within Europe, based on the Strasbourg Convention (1963). This formed the basis for the European Patent Convention (EPC) of 1973, which led to the establishment of the European Patent Office (EPO) in 1978. It is now possible to file a single patent application at the EPO in Munich, designating up to 12 European countries, and to have patents in all these countries granted by a single examination procedure. The result is not a single multinational patent but a bundle of national patents, which must be maintained and enforced separately in each country, and there are differences in the type of protection available for some types of invention (see Table I). It is proposed to have a single multinational patent for the European Economic Community (EEC), but this has not yet become reality.

Table I. Member states of the European Patent Convention (EPC)

Status	Countries	reservations made
Original members (1977-8)	Belgium, France, W.Germany, Italy Luxemburg, Netherlands Sweden, Switzerland (with Liechtenstein) United Kingdom	None
Joined 1979	Austria	chemical compounds not patentable until October 1987
joined 1986	Spain	Chemical compounds not patentable until 1992
	Greece	pharmaceutical compounds not patentable until 1992
To join 1992	Portugal	None
Signatories of EPC who have not yet ratified	Ireland, Denmark Norway	

The Patent Co-operation Treaty (PCT) of 1978 simplifies the procedure for filing under the Paris Convention, and allows a single filing to be made, designating a number of contracting states and claiming priority from an earlier application. Most developed countries including the USA and Japan have ratified the PCT. Unlike the EPC, however, the PCT does not provide for a single central patent office, and at some stages a PCT application has to be translated into the languages of the individual designated countries and sent to their national patent offices for examination. It is possible to file a PCT application designating the EPO, and this route is often used by US applicants.

Table II indicates some of the differences between the patent systems of the USA and the European Patent Office. The national laws of the EPC countries and that of Japan resemble the EPO system more than the US one. The main difference is that in the USA, a patent is granted to the first person to make the invention, whereas in Europe, the first person to file an application is entitled to a patent. The European first-to-file system is followed by all countries of the world with the exception of the USA, Philippines and Canada; Canada is presently amending its law in this respect. The first-to-file system has the great advantage of certainty and simplicity, whereas in the USA conflicts

between two or more patent applications claiming the same invention have to be resolved by interference procedure, which is complicated, very expensive, and prolongs the uncertainty of the parties for several years. The US first-to-invent system provides that an inventor who works in the USA can establish an invention date by evidence such as laboratory notebooks, whereas an inventor outside the USA cannot do this and can only rely upon a US or foreign patent application date. Some proposals have recently been made to change the US patent law to a first-to-file system (19), but such a fundamental change is not likely in the near future.

Table II. Differences between the US and European (EPO) patent systems

	USA	EPO
term of patent	17 yr from grant	20 yr from application
patent awarded to:	first to invent	first to file
novelty	mixed, 1 year grace period	absolute, no grace period
early publication	no	18 months from priority date
opposition:	no, but re-examination possible	within 9 months of grant
amendment after grant:	by reissue	national laws apply.

Patentable inventions - Practically all countries specify that an invention, in order to be patentable, must be novel, involve an inventive step, and be useful or applicable in industry. The last of these criteria excludes, in Europe but not in the USA, methods of medical treatment of humans or animals.

The requirement that an invention be novel is defined differently in different patent systems. The EPC has adopted the principle of absolute novelty; that is, an invention is no longer new if it has been made available to the public by written or oral publication, use or any other way anywhere in the world before the priority date. Thus, novelty may be destroyed by a printed publication in an obscure language, by a thesis put on the shelves of a university library, or by an oral presentation at a conference. Some minor countries still have the old rule of local novelty, according to which an invention is new if it was not previously published in that country, even if it was known elsewhere.

Because of its first-to-invent system, the novelty requirements of US law are too complicated to deal with fully here (20). Briefly, an invention is not novel if it was published before the applicant's invention date, or more than 12 months before his US filing date. The novelty system may be described as mixed novelty. As is the case with

the absolute novelty system, a printed publication anywhere in the world can destroy novelty, but oral publication or use can do so only if it is within the USA. The practical consequence is that a US-based inventor may publish his invention before filing a patent application, and still obtain a valid US patent. He cannot, by definition, publish his invention before his own invention date, and so long as he files his US application within the "grace period" of 12 months from the publication, there is no bar to patentability in the USA. However, if he then wishes to file corresponding applications in other countries claiming priority from his US filing date, his own publication before his priority date will make it impossible to obtain valid patents outside the USA. Many US inventors have fallen into this trap, often with serious consequences. For example, Cohen & Boyer's basic gene-splicing invention, which is the basis of all recombinant DNA technology, was published (21) before a US patent application was filed (22), and Stanford was unable to file in other countries.

The requirement that an invention in order to be patentable must be non-obvious, or must involve an inventive step, is intended to prevent the patenting of trivial variations upon what is known to the ordinary man at the laboratory bench. Accordingly, legal definitions of obviousness involve the hypothetical "man skilled in the art" - in medicinal chemistry, a qualified medicinal chemist without any particular originality or inventive powers. An invention which is obvious to such a man having the prior art before him is not patentable. It must be remembered, however, that many good inventions may seem obvious with the benefit of hindsight. Particularly if the invention was a commercial success, it is relevant to ask why no-one had ever done it before.

Pharmaceutical Inventions - Although (or perhaps because) patent protection is more important in the pharmaceutical field than in any other, many countries restrict the protection available for pharmaceutical inventions (see Table III). This trend is being reversed in some countries at least; thus the new law soon to be enacted in Canada will introduce product protection for pharmaceuticals, and change the previous practice of granting automatic compulsory licences at a nominal 4 % royalty. Even Mexico will introduce per se protection for medicinal compounds no later than 1996.

Table III.

Examples of Countries Discriminating Against Pharmaceutical Inventions

No protection at all for pharmaceuticals; at least process protection for other chemicals	Brazil, Turkey, Ghana
Non-medicinal compounds patentable per se, only process protection for pharmaceuticals	Finland, Greece, Canada (to 1986), Mexico (1986-96)
Compulsory licence provisions for pharmaceuticals	Canada (to 1986) India
Shorter term for pharmaceutical patents	India
Medicinal inventions protectable by Certificates of Invention, not patents	Mexico (to 1986)

Inventorship and ownership - Invention is the mental act of conception of the inventive idea. The person making this mental step will be the sole inventor if from that point on it requires only routine work to put it into effect. Co-inventorship may arise when further inventive work is needed to reduce the invention to practice, or where for example one inventor has invented one group of compounds, another has invented a second group, and the two groups are closely enough related to be combined together into a single patent application. When a chemist makes a new compound and sends it for testing for a particular indication, if the compound is useful for this indication, the inventor will be the chemist alone and not together with the pharmacologist who carried out the tests. The situation would probably be different if the pharmacologist had the idea of testing the compound for an unrelated utility, and if this were successful he would be a co-inventor. Merely setting a problem for another to solve is not an inventive act, but making suggestions for a particular line of approach may well be so. Accordingly whether a research manager or supervisor should be a co-inventor is something to be decided on the facts of each case.

In most countries the actual inventorship is of relatively small importance as long as all inventors work for the same company, except when the inventor may be legally entitled to extra compensation, as in Germany. In the USA, however, the patent is applied for by the inventors, and if the inventorship is incorrect, the validity of the patent could be challenged. Although correct inventorship is still important in the USA, the former extremely strict rules on this subject have recently been relaxed. It is now easier to correct an innocent mistake in the naming of inventors, and it is now clear that joint inventors need not have physically worked together, nor have each contributed to every claim. A continuation-in-part application need no longer have the same inventorship as the parent case, so long as they have at least one inventor in common.

Under the common law, in both the UK and the USA, ownership rights in an invention basically belong to the inventor. However, if an employee is paid to invent, or if he is in a very senior position, then his inventions will belong to the employer. Otherwise, the inventions of an employee remain his property, even if they were made using the facilities of the employer. In the latter case, however, the employer in the USA has a shop right to use the invention in his business.

Because these common law principles could be overridden by contract, it became the rule for employees to have in their employment contract a clause by which they agreed in advance to assign all their inventions to the employer. In the UK an important case heard in 1977 held that such a clause was unenforceable in the case of a storekeeper who was not expected to make inventions as part of his job (23). The Patents Act 1977 essentially restored the common law rules as to ownership of inventions, and provided that these could not be dispensed with by a contract between the parties. A research chemist will still have to assign any invention he makes in the area of work he is employed to do, but an invention he makes in an unrelated area, even if it is one of interest to his employer, belongs to himself. In the USA, on the other hand, employment contracts requiring assignment of all inventions are generally enforceable.

The new British patent law of 1977 also introduced provisions for the payment of compensation to employee inventors. Germany has had such a law for some time, but whereas in Germany the employee inventor

basically has a right to receive payment for any invention which is made use of by the employer, in the UK compensation is payable only if the invention is of "outstanding benefit" to the employer. There have not yet been any decided cases on this part of the law, and it is not yet clear how valuable an invention must be in order for compensation to be payable. In the pharmaceutical field there is a good case for arguing that new market products are so rare that any invention which reaches the market must be of outstanding benefit. In the USA, the employee inventor has no legal right to anything more than his salary no matter how important an invention he makes.

Academic inventors are in a more complex situation, since not only are they employees of their university, but they are often in receipt of research funding from government or from industry.It is important in this situation to have clear written agreements stating who owns the rights to any inventions which arise out of the research. In the USA, where government funding is involved, the university generally may license the invention to industry. Any exclusive licence is subject to residual rights of the US government, which can also insist that additional licences be granted if the invention is not being properly exploited.

Universities in the USA are generally more patent-conscious than in the UK, where many universities do not wish to be involved in the trouble and expense of patenting. In Germany, academic chemists seem to be more free to dispose of their own inventions, and the universities seldom assert title.

Filing a Patent Application - Patentable inventions should normally be made the subject of a patent application. Exceptions to this general rule might be where the invention is an improvement to a process, which cannot be detected by analysis of the end product, and which is better kept as secret knowhow; or where all that is wanted is freedom to operate the invention, and not the right to exclude others. In the latter case a rapid publication of the results to prevent others from patenting might be the best course.

The inventor will normally prepare a written summary of his work, which will provide with a basis on which a specification and claims can be drafted. Usually there will be a need to have further information in order to decide what is the real invention and what should be the scope of the claims, and possibly more experimental work may be needed. There will always be a conflict between the requirements of filing as good a text as possible and of getting the earliest possible filing date. In the USA, because the requirements for a sufficient disclosure are quite strict and because the inventor can go back to his date of invention, there is sometimes a tendency to delay filing until every detail has been worked out. This, like self-publication, can cause problems in other countries. On the other hand, applicants based in countries other than the USA must be careful not to file too brief and superficial a text, even if the practice in their own country permits it. If they later file a Convention application in the USA, priority may be denied if the US sufficiency standards were not met.

After the first application has been filed, the twelve-month period provided by the Paris Convention begins to run. During this time, work will normally continue on the invention, and groups of new compounds may be made and tested, process conditions improved, etc. These new developments may be made the subject of further patent

applications which can be combined with the first at the foreign filing stage, priority being claimed from all of them. In the USA, such later applications may be filed as continuation-in-part (CIP) applications, which refer back to the first application and take its filing date for the subject-matter common to both.

Well before the end of the twelve-month period, a decision must be taken on whether or not to file the patent application in other countries (foreign filing). Since about two months should be allowed for preparation of the foreign filing text, mailing it abroad and preparation of translations, the decision should be taken 3-4 months before the end of the priority year if at all possible. There are four possible courses of action at this point:

1) Abandon the application completely

2) Continue with the application in the home country only

3) Abandon and refile

4) File in a number of additional countries.

The first two of these alternatives are clear, and will apply when continuing interest in the invention is zero or small. The third is a useful procedure to adopt when, as often happens, the normal decision point is too early to say whether there is a real prospect of commercializing the invention. The application is withdrawn, a new application (identical with the first or including new results) is filed in the same country, and the clock starts running again. The disadvantage is that the early priority date is lost, and any publication or filing by competitors within the priority year can be fatal. This alternative is not very popular in the USA, where practitioners do not like to abandon applications, and would generally prefer to file a CIP. However, it should be noted that it is not possible to claim priority from a CIP in respect of matter which was in the parent case, if more than twelve months have elapsed from the original filing date. The option of abandonment and refiling is ruled out if the invention has been published in the meantime, which is why it is desirable to avoid publishing one's own results not only before a priority application is filed, but also between the priority date and the time of foreign filing.

If the invention seems likely to be commercially important a decision will normally be made to file corresponding applications in a number of foreign countries. The choice of countries in which to foreign file will depend upon general factors such as the strength of protection available and the cost of patenting in each country. For a new pharmaceutical, most companies will attempt to cover all countries of any importance in which patent protection can be obtained for drug substances per se, possibly together with some countries in which patent protection is weaker, but which are particularly important markets. A list of 20 - 30 countries is quite common. To stop costs from getting out of control, patents and patent applications should as a general rule be abandoned as soon as they no longer cover compounds which are being actively developed.

If the application is to be filed in more than about four of the countries which have ratified the EPC, it will generally be less

expensive to file a single application at the European Patent Office than separate applications in each country. A single EPO filing has the disadvantage, however, that "all the eggs are in one basket" - if the European application is refused or successfully opposed, the applicant has nothing in any country, whereas if he had filed separate national applications, it is probable that some at least would have been granted.

Examination of Patent Applications - All patent applications are subjected to some form of examination before being granted. In some patent offices this may be no more than a purely formal examination which does not go into the merits of the invention in any way. In such countries, determination of the validity of a patent is left to the courts, and will arise only in the event of litigation between the patentee and an infringer.

The patent offices of most developed countries carry out some form of novelty examination to determine whether the invention for which protection is sought is in fact new. Some patent offices, for example those of the UK, Germany and Japan, carry out their own searches without additional material; others, e.g. in Canada and Denmark, may supplement their own search by requiring the applicant to notify the patent office of the result of searches on corresponding applications in certain other countries. Some patent offices do not carry out searches themselves, but accept the result of a novelty search done in another country (e.g. Ireland accepts a British search report) or require a search to be carried out by the EPO office at The Hague (e.g. France, Belgium, The Netherlands and Turkey).

The USA is unique in that, although the US Patent and Trademark Office (USPTO) performs its own searches, it also requires the inventors to bring to the attention of the Examiner all relevant prior art of which they are aware. Failure to do so constitutes fraud on the Patent Office, which if proven means that the patent so obtained will be incurably invalid. Attempts to enforce the patent could then be considered antitrust violations, and the attorney could be subject to disciplinary measures. With the USPTO, honesty is the best policy.

Only relatively few patent offices go into the question of whether the invention is obvious in the light of the prior art. These include the more important offices such as the USPTO, the EPO, and those of Japan, Germany, the UK and The Netherlands. The seriousness of such obviousness examination varies from country to country; in the UK objections on this ground are easily overcome by argument, whereas in the USA it may be necessary to submit showings of unexpected superior properties of the compounds of the invention as compared with the closest prior art.

In some countries, as we shall see, it is possible to postpone the examination process for a number of years. In the USA, this option does not exist. Once an application has been filed, it is assigned a serial number and allocated to a specific examining group. An examiner in this group will issue a first official action on the case after a period of anything from four to twelve months, depending on the workload in that group. The applicant need not be unduly upset if all of the claims are rejected in this first action; indeed this is the usual state of affairs at this stage. The applicant will reply, through his attorney or agent, within the set time limit of six months (preferably within three, since fees are charged for replies in the last three months), and will argue against the examiner's rejections. He may decide to narrow the scope of

his claims in order to distinguish the invention from the prior art, and may file evidence to establish an invention date earlier than the publication date of some crucial citation, or to show superior properties to overcome an obviousness rejection. If the examiner is persuaded, he will issue a Notice of Allowance, and the patent will be granted in due course once the Issue Fee has been paid.

If the examiner remains unconvinced of patentability, he will send out further office actions, culminating in a Final Rejection. This, in spite of its name, is not the end of the story, but it does mean that, for that particular application, only one further response will be considered by the examiner. If that does not convince him to allow the application, an appeal is necessary. However, the applicant also has the possibility of filing a new application, which may be identical with the first, and which can claim the priority of the original filing date. Examination of this new application then starts afresh, except that, if no new issues are raised, the first office action may be a final rejection. In any case, time will be gained in order to present further arguments or new evidence, and the whole process can be repeated as often as required.

The USPTO is trying to reduce backlogs and bring the average time from filing to grant down to 18 months. In many fields, this will be in the interest of the applicant, who often wants to take action as soon as possible against an infringer, and cannot do so until he has a granted patent. In the pharmaceutical field, however, the applicant usually has no interest in a rapid grant, since his product is most unlikely to have marketing approval from the FDA until 8 or 10 years from the patent filing date. Since the term of a US patent runs from the date of grant, there is every incentive to the applicant for a pharmaceutical patent to take advantage of the possibility of refiling, going on appeal, and using all legitimate ways of delaying grant. Pure delaying tactics, such as refiling an application which could be allowed in its existing form, are not legitimate and should not be attempted.

A quite different system, in which the examination itself may be deferred for up to seven years, is used in The Netherlands, Germany and Japan. After the application is filed, no examination is carried out until this is specifically requested, either by the applicant or by a third party. If no request is made by the end of the seven-year period, the application lapses irrevocably. This system is particularly advantageous for pharmaceutical applications, for which rapid grant is not important, and of which many will be abandoned during the course of seven years. The cost and effort of prosecuting these abandoned cases will be saved, for the patent office as well as for the applicant. However, as in these countries the patent term runs from filing and not from grant, the deferred examination system can give rise to situations in which a patent may have little or nothing left of its life by the time it is finally granted.

When the system of deferred examination was introduced, it was felt to be unacceptable that patent applications could remain in the pipeline for ten years or more and then suddenly emerge to be used against persons who had begun infringing activities in ignorance of the existence of relevant patent rights. An essential aspect of the system was therefore the early publication of pending applications. In all countries having deferred examination, publication of the application, as filed and before examination, takes place 18 months from the filing date or, if Convention priority is claimed, from the first priority

date. Early publication has also been adopted by the EPO and the British Patent Office, although neither have a deferred examination system.

These early publication documents, such as the German Offenlegungsschriften, Japanese Kokai or British or European Published Applications will often be the closest prior art against one's own applications and are useful sources of information about patenting activities of competitors. Although their claims are those filed by the applicant, and may bear little relation to what will eventually be granted, once early publication has occurred anyone may inspect the file of the application in the patent office and so monitor the progress of prosecution. This is in complete contrast to the USA, where pending patent applications are secret and there is no way of checking at the USPTO on the status, or even the existence, of an application before it is granted. Since US patent applications, can easily spend ten years or more in prosecution, it is not uncommon to receive an unpleasant surprise when a competitor's US application suddenly appears as a granted patent many years later.

Most countries having early publication give some rights to the applicant from the publication date. Although he cannot sue for infringement until he has a granted patent, he can recover back damages for infringing acts between the dates of publication and of grant.

Appeal and Opposition - Although a patent office examiner may refuse to grant a patent application, this refusal is not in itself final, since the applicant generally has a right of appeal to at least one higher authority - indeed often to several. In the US Patent Office, an applicant who receives a final rejection and cannot persuade the examiner to change his mind may file a Notice of Appeal. This must be followed within two months by an Appeal Brief setting out the claims on appeal, the reasons why they have been rejected and the arguments why the rejection should be reversed. The appeal brief goes first to the examiner, who may withdraw his objections after reading the appeal brief, but more usually will write an Examiner's Answer in reply. The applicant may reply to this with a Reply Brief; the case is then scheduled to be heard by a three-person panel of the Board of Patent Appeals and Interferences. The applicant has a right to an oral hearing if he wishes. The Board may uphold the examiner's rejection wholly or partly, or may overturn him, as it does in about 25 % of the cases it hears. If its decision is negative, the applicant may make a request for reconsideration, and/or may appeal further to the Court of Appeals for the Federal Circuit (CAFC) or to the U.S. District Court for the District of Columbia. The CAFC is a relatively new specialist court which hears appeals from the District Courts on cases of patent infringement, as well as appeals from the USPTO. The District Court is not a specialist patents court but can consider new evidence, whereas the CAFC must judge the case on the existing record. From the CAFC a petition for Certiorari can be made to the Supreme Court, but the Supreme Court will hear the case only if it considers that a particularly important point of law is involved. This was the case for example in Diamond v. Chakrabarty (24), in which the Court held that living microorganisms were themselves patentable. Similarly, from the DC District Court further appeal can be taken to the Court of Appeals for the District of Columbia and thence to the Supreme Court.

The procedure of appeal first within the patent office and from the patent office to the courts is also found in the UK and Japan. In the UK, an appeal from the Examiner is heard first within the Patent

Office by a Hearing Officer, and a further appeal can be taken to the Patents Court, a specialist division of the High Court which also has jurisdiction in patent infringement suits. The possibility exists of further appeal to the Court of Appeal and the House of Lords. Similarly in Japan, the first level of appeal is to an appeal board within the Patent Office, and from there appeals may be taken to the Tokyo High Court and thence to the Supreme Court. In Germany, there is strictly speaking no appeal within the Patent Office, since appeals from the decision of the examiner are heard by a panel of the Bundespatentgericht (Federal Patents Court), which although located in the same building, is not part of the Patent Office. From the Bundespatentgericht the unsuccessful applicant may obtain permission to appeal to the Bundesgerichtshof (Federal Supreme Court) if important points of law are involved.

The system in the European Patent Office differs from all of these in that there is an appeal from the Examining Division to a three-person panel of the Technical Board of Appeals, but no further possibility of appeal to any court. When there is a particularly important point to be decided (for example the decision on the patentability of second medical uses of known compounds), the appeal may be heard by the Enlarged Board of Appeals, but this is an alternative to the normal Board of Appeals, and not an additional stage in the appeal process.

In all of these patent offices, it is often very useful to have an informal interview with the examiner before reaching the stage of a formal appeal. Most examiners appreciate personal contact with patent attorneys and inventors, and even if an interview does not persuade the examiner to change his mind, it will often be of use in clarifying the issues and thereby making a subsequent appeal more likely to succeed.

Even when a patent application has been accepted or granted, the applicant cannot be certain that he has a valid, enforceable patent. In many countries, the validity of a patent can be challenged by other parties in the courts, either directly or by way of a defense against an infringement action. In addition, it is possible in a number of countries, and in the EPO, to challenge the grant of an accepted application or granted patent by a procedure before the patent office. When such action may be taken only within a limited time after acceptance or grant, it is generally referred to as an opposition. The classical form of opposition is met with in Japan. After the initial early publication (kokai), the application is published for a second time when it has been allowed by the examiner. This publication (kokoku) incorporates any amendments which have been made during prosecution. Within two months of the second publication, any party may file an opposition to the grant of a patent; if no one does so the application is granted and published for the third time as a patent. If an opposition is filed, the evidence brought by the opponent is considered, together with the applicant's counter-arguments, and the opposition decided. If the opponent is successful, grant of a patent will be denied, but the applicant may appeal this decision. If the opponent is unsuccessful, the patent is granted, and this cannot be appealed further.

Germany had until recently a very similar system, in which an accepted application was published as an Auslegeschrift, which was open to opposition before finally appearing as a Patentschrift. The procedure has now been somewhat simplified, and the Auslegeschrift has been omitted. On acceptance by the Patent Office, a patent is granted, but this may be opposed within a period of three months, and if the

opposition is successful, the newly granted patent is revoked. The same principle has been adopted by the EPO, where opposition may be filed within nine months of the grant of a European Patent.

Oppositions have always been an important feature of the German patent system. Approximately 16 % of all accepted German patents are opposed, a figure which is much higher than that of any other country. In the EPO, where a successful opposition could kill at one blow the patent rights in up to ten countries, it was thought that the opposition rate would be even higher than the German figure; this would have put a considerable strain on the whole European patent system. In practice, however, it has been found that about 10 % of all granted European patents are being opposed. Oppositions in the EPO are heard by an Oppositions Board, which may decide to revoke the patent, grant it unchanged, or grant it subject to amendment. The losing party has the right of appeal to the Technical Board of Appeal.

In the British Patent Office, there is in effect no time limit for opposition, and a patent may be revoked by the Patent Office at any time. However, very little use is made of this possibility. The USA never had the classical form of opposition, but has recently introduced a similar procedure called re-examination, which may be requested by anyone (including the patentee) at any time during the life of a US patent. Although similar in principle to opposition, re-examination proceedings are more limited in certain respects. Whereas opposition procedure in most patent offices allows the opponent to raise any ground of invalidity which could have been raised in prosecution, and also to argue that prior art considered by the examiner was not given sufficient weight, re-examination may be requested only on the grounds of lack of novelty or obviousness over a printed publication. The application for re-examination will be rejected out of hand if the USPTO does not consider that substantial new issues are raised. Furthermore, once re-examination has begun, the requester cannot take an active part in the proceedings.

Table IV summarizes the appeal and opposition system in a number of important patent offices. In Germany and Japan it is also possible to attack the validity of a granted patent even after the opposition period, by means of a nullity suit or invalidity trial before the patent office. The disadvantage of this from the viewpoint of the person attacking the patent is that while such a suit is pending, the patent remains valid and can be enforced against the attacker.

Maintaining a Patent in Force - Once a patent has been granted, it will be necessary in almost every country to pay renewal fees to keep the patent in force. For US patents applied for before December 12, 1980, no renewal fees are required, and such a patent will remain in force for its full term of 17 years unless it is abandoned by being "dedicated to the public". As of writing, this is also true of all Canadian patents, but it is expected that the new Canadian law shortly to be enacted will require renewal fees.

In most countries, renewal fees are due annually, but in the USA three fees are due over the lifetime of a patent, after 3 1/2, 7 1/2 and 11 1/2 years from grant. For granted European patents, renewal fees are collected by the national patent offices of the designated countries; the patent can be maintained in some countries and allowed to lapse in others. The EPO receives a share of the renewal fees from the national offices. The cost of maintaining a patent for its full lifetime varies

Table IV. Appeals and Oppositions

	USPTO	EPO	Germany	UK	Japan
1)	Board of Patent Appeals and Interferences	Tech.Board of Appeal or Enlarged Board of Appeal	-	Hearing Officer	Appeal Board
2)	CAFC or DC Dist Ct. ↓ Ct.of Appeal for DC ↓ Supreme Ct.	none	Bundespatent-gericht ↓ Bundesge-richtshof	Patents Court ↓ Court of Appeal ↓ House of Lords	Tokyo High Court ↓ Supreme Ct.
3)	--	within 9 mo. from grant	within 3 mo from grant	--	within 2 mo of kokoku
4)	re-examination	--	revocation action	revocation action	invalidity trial

1) Appeal within Patent Office

2) Appeal to Courts

3) Opposition

4) Other forms of review by Patent Office

greatly from one country to another, and the value received is by no means porportional to the cost. Some of the most expensive countries are those in Eastern Europe, where the protection available is very limited. Renewal fees generally are quite modest during the first few years and rise more or less steeply towards the end of the patent term. In this way, the operations of national patent offices are financed largely by those patentees who are making the most commercial use of their patents; those who are not making use of their patents are encouraged to let them lapse, thereby bringing inventions into the public domain earlier than would otherwise be the case.

In some countries it may be necessary not only to pay renewal fees but also to fulfill working requirements in order to maintain a patent in force. In these countries a patent lapses or can be invalidated if the invention is not worked in that country. In the case of pharmaceutical inventions, strict working requirements make patent protection worthless, since for economic and scientific reasons it is very seldom practicable to synthesize the active compound of a new drug in a number of different countries.

Extension of Patent Term - Before the present British patent law came into force in 1978, British patents had a term of 16 years from the application date. They could be extended for up to ten years if the

patentee could prove that he had not made a reasonable amount of money from his patent, taking into account the benefit of his invention to the public. A number of pharmaceutical patents were extended under this law, for example the patents of the National Research Development Corporation (NRDC) for cephalosporin C and its precursor 7-ACA (25, 26). After 1978, the term of new patents became 20 years, and existing patents less than 11 years old had their terms automatically extended by four years, but no further extensions were possible. In the additional four year period the patents were subject to licenses of right, meaning that anyone could obtain a license for a reasonable royalty, and the patent gave no effective monopoly.

The old British form of patent extension for "inadequate remuneration" still is possible in Australia, New Zealand, Ireland, Pakistan, and for old patents in South Africa, but it seems likely that even in these countries it will not exist for very much longer. More recently, attention has turned to the question of patent term extention for the special case of pharmaceutical substances. The long delay in obtaining regulatory approval from the FDA or similar national authorities for pharmaceuticals means that the effective patent life has been reduced to around half of the normal legal term.

Patent term extension was enacted in the USA by the Drug Competition and Patent Term Restoration Act of 1984 (27), commonly known as the Waxman/Hatch Act, as one half of a compromise measure which also made it easier for generic drug manufacturers to obtain an Abbreviated New Drug Application (ANDA) to market a product once the patent expired. Under the patent term restoration provisions of Waxman/Hatch, the patent for a drug for which the IND was filed after September 24, 1984, may be extended for a period corresponding to half the IND time plus all the NDA review time, up to a maximum of five years. The extended patent life may not extend beyond 14 years from the NDA approval date. Only one patent for each product can be extended; a patent cannot be extended more than once, even if it covers two new drugs; and a patent which has already lapsed cannot be revived. The scope of the patent in the extended period is restricted to cover only the marketed product, and sale of the compound for non-pharmaceutical uses is no longer infringement.

Proposals for legislation having similar effect are being made in Japan, and in the UK, a bill has been introduced in Parliament which would exempt pharmaceutical patents from the license-of-right provisions which apply to the last four years of all remaining pre-1978 patents.

Enforcement of Patent Rights - A patent does not give its owner the right to use the invention, but the right to prevent others from doing so. Having a patent gives no guarantee that carrying out the invention will not infringe patent rights of others; indeed it is quite common for the subject-matter of a patent to fall within the scope of an earlier patent. In this situation the owner of the later patent cannot use his invention without a license from the patentee of the earlier one, and conversely the earlier patentee would need a license in order to use the later invention.

Although the state grants patents, the state does not enforce them. In normal circumstances patent infringement is not a criminal offense, but an actionable wrong for which the person wronged must himself seek redress in the courts. If the infringement suit is successful, the patentee can obtain damages for past infringement, and,

what is often more important, an injunction to restrain future infringement. Breach of such an order is contempt of court which can be punished by heavy fines or imprisonment.

For an act to constitute infringement of a patent, it must amount to making, using or selling something falling within the claims of the patent, since it is the claims which define the scope of protection. In the pharmaceutical field, a patent may claim a composition of matter such as a new chemical compound or a mixture of two or more substances; a process, which may be a process for making or for using a substance; or an article, for example a tablet containing a drug. Often, the same patent will contain more than one type of claim, for example claims to a new substance and to pharmaceutical compositions containing it. If the claim is to a chemical substance, then the claim is infringed by anyone who makes, uses or sells the substance in the country of the patent. If the claim is to a process, the claim is infringed by carrying out the process and, in most developed countries, by selling the direct product of the process. The USA does not as yet have this "derived product protection", so that it is not infringement of a US patent to import into the USA a product made abroad by a process patented in the USA. However, such imports can often be prevented by the patentee applying to the International Trade Commission for an exclusion order under the Tariff Act.

There is considerable variation from one country to another as to how broadly the claims of a patent are interpreted. In the UK, claims have always been interpreted literally, so that there will generally not be infringement if the act in question does not fall under the strict literal wording of the claims. In the USA, the courts apply the Doctrine of Equivalence, which holds that equivalents which bring about the same result in the same way may infringe even if they do not strictly fall under the wording of the claim. The practice in the German courts is even more liberal in this respect. In both the USA and in Germany, a "pioneer" invention will be given a broader scope of equivalents than a patent for a relatively minor improvement. In the USA, the patentee will be prevented by so-called "file wrapper estoppel" from covering by equivalence anything which was in the scope of the claims he originally filed, but which was dropped when his claims were narrowed during prosecution in order to distinguish from the prior art. In Japan the court will never broaden the literal scope of a claim; indeed it may very well narrow the scope of protection if it feels that the claimed scope is not adequately supported in the specification, or is too close to the prior art.

Infringement Suits - In the USA, infringement actions are first heard by the Federal District Courts. Suit may be brought in the district in which the alleged infringer has his registered office or in any district in which he has a regular place of business and is infringing. The possibility of filing suit in more than one district gives the opportunity of "forum shopping", i.e. trying to have the case heard in a court thought to be favorably disposed to one's own side. This used to be very important when appeals were heard on patent cases by the Circuit Courts of Appeal, some of which were known to be very much less favourable to patentees than others. Now, however, appeals from all District Courts on patent cases are heard by the CAFC, and this has largely succeeded in unifying and strengthening patent jurisprudence in the USA.

The defendant in a patent infringement suit will normally base his

defense either on denying infringement, on contesting the validity of the patent, or on both of these. In the USA, as in England and other common law countries, the same court may deal with both issues, and consider the validity of the patent as well as the question of infringement. It is possible for the court to stay the infringement action while validity is challenged by the re-examination procedure in the USPTO, but this is unusual.

The cost of an infringement action in the USA is very high. A company intending to sue must consider not only the direct legal costs, which may easily run into the millions of dollars, but also the enormous amount of disruption to its normal business. The latter will be caused by the need to supply masses of documents to the other party in the "discovery" stage of the procedure, and by the need for executives to spend time in preparing and giving testimony in the form of depositions or in court. For this reason many infringement actions are settled out of court, after the preliminary stages have enabled the parties to estimate their chances of success. When an infringement suit is fought to a finish, the result can be catastrophic for the party held to have infringed a valid patent. In the recent case of Polaroid v. Kodak (28), the grant of an injunction to Polaroid meant that Kodak had to close down a factory employing over a thousand people and had to compensate all owners of Kodak instant cameras, for which they could no longer supply film - and this all before the question of damages for the infringement was even considered.

In the majority of Continental European countries, and in Japan, the court hearing an infringement action has no jurisdiction to declare the patent invalid. Consequently if the defendant wishes to challenge the validity of the patent, he cannot do so by way of defense in the infringement suit, but must start a separate process at the patent office (or, in the case of Germany, at the Bundespatentgericht). In Japan, the judge in the infringement case cannot declare the patent invalid even if completely novelty-destroying prior art is found; in this situation he can, and frequently does, interpret the claim so narrowly that the alleged infringement is no longer covered. The effect as far as the parties are concerned is the same.

The Patent Specification and Claims - A patent, strictly speaking, is a short document, which sets out the legal rights given by the patent grant. What we normally tend to refer to as a "patent" is in fact the patent specification, which describes the invention and defines the scope of protection which the patent gives. These two functions are provided by the body of the specification and the claims respectively.

A patent specification is a legal as well as a scientific document. It begins with a title after which there is usually a short account of the background of the invention and the prior art. This is not compulsory, although in many cases it is practically essential for an understanding of the invention. In other cases, particularly where the invention is a new chemical compound or compounds, it can do more harm than good. Often at the time of filing the patent application the closest prior art is not known, and it is not very helpful to insert a discussion of less relevant art. In the USA, admissions made in the specification as to what the prior art teaches may be binding on the applicant later, even if incorrect. Similarly, US patent specifications frequently include a long list of objects of the invention, which is not essential and generally conveys no useful information.

Following this introductory part comes the statement of invention, which will in most countries be identical with the main claim. In the USA it may be broader, since the US specification is generally not altered when claims are narrowed during prosecution. Following this will come a list of preferred subscopes of the invention, on which subgeneric claims may be based. If the invention relates to novel compounds, the next part of the specification will usually consist of general instructions for the preparation of the compounds from available starting materials.

The next section will be the utility statement, which describes the use of the compounds and states any advantageous properties. Although it may at some stage be necessary to supply to the patent office details of comparative testing against prior art compounds, it is preferable not to include such data in the specification as filed. However, for pharmaceutical uses, it is generally necessary to indicate in some detail the test methods used to establish the activity of the compounds, and to estimate the daily dosage range appropriate for administration to humans.

After the descriptive part of the specification will come the examples, giving detailed instructions for the preparation of at least one of the compounds. Unlike the experimental section of a scientific paper, there will often be only a few fully written-out examples, followed by a table listing many other compounds within the scope that can be prepared in the same way. Many of these may be so-called "paper examples" which were never actually made. Inclusion of paper examples in a specification is not a false representation that the compounds have been made, but an honest representation that they can be prepared as indicated.

The final part of the specification comprises the claims defining the scope of the monopoly given by the patent. They are numbered consecutively and each claim reads as if it were a separate sentence starting with the words "What I claim is...". In Europe, patent offices generally favour the two-part claim in which a pre-characterizing part sets out the features of the invention already known, and a characterizing clause adds the novel feature. An example of such a claim is "A process for the preparation of A by reaction of B and C under acid catalysis characterised in that the acid catalyst is acetic acid". Such a type of claim is not appropriate for claiming novel compounds. A claim may be independent in form, or may be dependent, in which case it refers back to one or more preceding claims, and incorporates all the limitations of any claim on which it depends.

In most countries patent specifications also contain an abstract, which is sometimes printed on the title page. This gives a summary of the invention, but it is not legally part of the specification, and cannot be used in interpreting the claims.

Chemical Inventions - A novel chemical compound will be a patentable invention if it is industrially applicable and unobvious. The first requirement means that it must be useful in itself, or at least as an intermediate in the production of a useful compound. Even if it is new and useful, it may still not be patentable because it is so close to the prior art that there is no inventive step involved in making it. Closeness to the prior art is a question not only of structural similarity, but also of the properties of the compounds involved. Thus although an ethyl ester is very close structurally to the corresponding

methyl ester, the ethyl compound could well be patentable if it was useful, for example as pharmaceutical, whereas the methyl homolog was merely described in an academic publication without any mention of such a use. On the other hand, if the methyl compound was previously known for the same use, the ethyl ester would not be patentable unless its properties could be shown to be surprisingly better than those of the prior art compound.

In considering obviousness, it is also necessary to consider the extent to which structure/property relationships were understood by the average worker in the field at the date of the patent application. Thus in an important case involving pharmaceuticals, heard in the UK in 1970, it was held that as of 1955, the date of the patent in question, it was not obvious to substitute $-CF_3$ for -Cl at the 2-position of a phenothiazine ring system, even though both the prior art compounds (e.g. chlorpromazine) and the novel compounds (e.g. trifluroperazine) were tranquillizers (29). After trifluroperazine itself was known, however, substitution of $-CF_3$ for -Cl in other compounds of similar type would have been considered obvious, and the results would be patentable only if there were surprising advantages.

In order to avoid any suggestion that the properties of new compounds may be expected or predictable, pharmaceutical patents will generally avoid any discussion of structure/property relationships, isosteres, etc. This is only a sensible application of the general rule that a patent should preferably not contain any theory as to why the invention works. If the theory is correct, it may make the invention appear obvious; if incorrect it can cast doubt on the reliability of the description of the invention.

Selection Inventions - When a group of compounds has already been described, for example by means of a general structural formula, it may still be possible to patent a narrower sub-group of compounds falling within the earlier group, so long as no individual compounds of the new sub-group, were specifically described. In this situation the new sub-group, described as a selection out of the original group, will generally be prima facie obvious, but may nevertheless be patentable if the compounds in it have some advantageous property not shown by the previously-known members of the group. If the disclosure of the group is in a patent which is still in force, then the patent for the selection invention will be dependent upon that earlier patent. Difficulties may arise when the original group is so small that a disclosure of the group is in effect a disclosure of all its members; thus in one British claim to a process using lithium was held not to be novel in view of an earlier description of the same process with "an alkali metal" (30).

Optical isomers of known racemic compounds may be considered an extreme case of a selection invention. Courts in both the USA and the UK have held optical isomers to be novel over the racemate and it was at one time easy to obtain a patent for a specific optical isomer by a showing of superior pharmaceutical properties. It is now generally realized that it is normal for one of the optical isomers to be more active than the racemate and the other to be less active, and patents for optically active forms have become more difficult to obtain. The British patent for amoxicillin (an optical isomer of a known racemate) was upheld only as regards claims to a composition for oral administration, since it was surprisingly found that amoxicillin had high oral activity whereas the racemate did not have this property (31).

In France and Germany the amoxicillin patent was held invalid.

Compounds of Unknown Structure - It may still be possible to patent a new compound even if its structure is not known. The problem is that of defining the compound in the claim, since this cannot be done by means of a structural formula. One approach is to define the compound in terms of its physical, chemical or biological properties in a so-called "fingerprint claim". This is often a suitable way of claiming a single novel compound, for example a new antibiotic obtained from a micro-organism, which has been purified, but whose structure has not yet been determined.

An alternative method which is more appropriate for a group of new synthetic compounds is that of defining the substances in terms of how they are made, i.e. as the products of reacting certain defined reagents under defined conditions. This type of product-by-process claim has the disadvantage that the compounds are not covered if made by an alternative route. Although patentees sometimes try to avoid this problem by claiming the compounds "obtainable by", rather than "obtained by" the given process, it is not clear that such claims would be interpreted by the courts as covering the compounds when made by a different process. It is dangerous to try to guess the structure of a new compound, if this is not certain. If the wrong structure is claimed, the mistake usually cannot be corrected later.

Natural Products - It is often alleged that naturally-occurring compounds are not patentable, but this is incorrect. A newly-isolated natural product may be patentable if it is claimed in such a way as to be distinguishable from the compound as found in nature, by specifying a certain degree of purity, or by giving physical properties in a fingerprint claim which are characteristic of the pure material. If the structure of a natural product is discovered for the first time, and the compound is synthesised, it should be possible to claim the synthetic material, however made. Similarly in the field of biotechnology, claims can be obtained to a known protein produced by recombinant DNA techniques. In all these cases, of course, the claims must be inventive as well as being novel. The mere purification by standard methods of a natural product which was readily available in an impure state is unlikely to lead to a valid patent.

Process Inventions - Chemical inventions include not only new chemical compounds, but also new processes for making or using compounds that may themselves be old. A new preparative process may be of broad applicability, for example the Cohen/Boyer gene-splicing invention, which can be used to make almost any protein, or may relate to the synthesis of one particular compound. In the latter case, the alternative of keeping the invention as a trade secret instead of patenting it must always be considered. The main question is whether or not the new process can be determined by examination of the end product, for example by the presence of characteristic trace impurities. If not, any patent which is obtained will be of very limited value because it will be practically impossible to enforce, and the information given away in the patent specification will make the process available to competitors. In the USA, where pending applications are kept secret, it is often a good plan to file an application in any case, and postpone the choice between patenting or trade secrecy until the patent is ready to be granted.

What one cannot do is have it both ways by trying to obtain patent

protection without giving away any useful information. The patent will be invalid if it does not give sufficient information to carry out the invention, and in the USA, the "best mode" must be disclosed.

Pro-drugs and Metabolities - The fact that a compound has a pharmaceutical effect indirectly through an active metabolite rather than directly should have no bearing on its patentability. If administration of the compound has a medicinal effect, the mechanism of the process is immaterial as far as the patent is concerned. However, if the active metabolite is itself patented, the question arises whether sale of its precursor is an infringement of that patent, since its use as a drug will necessarily cause the patented compound to be formed. In one English case, the House of Lords held that sale of the drug hetacillin, the acetone adduct of ampicillin, infringed the patent for ampicillin even though hetacillin did not fall under the structural formula in the claim (32). Hetacillin had no activity of its own, and was immediately hydrolysed *in vivo* to ampicillin. In these circumstances the court clearly considered hetacillin as an imitation designed to evade the ampicillin patent. If the precursor had its own activity and was not designed merely to give rise to the patented product, it is unlikely that infringement would be held. In the USA it is probable that the doctrine of equivalents would be applied to give the same result.

Pharmaceutical Compositions - Three types of patentable pharmaceutical compositions are those in which the invention lies in the combination of two or more active ingredients; drug delivery systems which can be used for many different drugs; and conventional compositions containing a new drug together with any pharmaceutically acceptable excipients.

The first of these presents special problems in the USA. Although in other fields of chemistry simple mixtures are readily patentable if novel and non-obvious, in the field of medicinal chemistry the USPTO often will not grant a patent unless a synergistic effect can be demonstrated between the active ingredients. A rigorous proof of synergism requires a comparison of dose-response curves of the single components and of various combinations of the two and if no animal model is available, such tests may have to be done in clinical trials on humans. It often happens that such proof cannot be demonstrated even if there are surprising advantages (e.g. less side effects) for the mixture. In this situation it must be emphasized to the USPTO that there is no statutory requirement for synergism, but only for non-obviousness, and synergism is no more than one way in which non-obviousness can be demonstrated.

Drug delivery systems such as sustained release forms, skin patches, microcapsules, etc. are becoming increasingly important as the cost of developing new chemical entities increases. They can often be claimed broadly, since many are suitable for use with a wide range of drug substances. Conventional compositions, on the other hand, are by definition not inventive when used with known pharmaceuticals. However a claim to "A compound of formula X in association with a pharmaceutically acceptable diluent or carrier" is in some countries an appropriate way in which to claim the invention that a known (and thus unpatentable) substance X has pharmaceutical utility.

In the EPO, the first pharmaceutical use of a known compound can be protected by a claim of the form "Compounds of formula X for use as an active therapeutic substance". The Technical Board of Appeal has held that such a claim covers the substance when sold for any medicinal use,

not only the one which was described in the patent specification (33). Similar claims had been allowed previously in the German Patent Office, but not in the British, where the law had always been that a claim for a substance could not be made novel by adding an indication of its use. However, in the field of pharmaceuticals only, the British Patent Office now follows the European practice in this respect.

Second Pharmaceutical Use - There has recently been considerable controversy in Europe on what protection can be given for a new unrelated medicinal use of a substance already known as a pharmaceutical. This problem does not arise in the USA, where a claim of the type "A method of treatment of disease Y comprising the administration, to a human in need of such treatment, of an effective dose of compound X" can be granted. In the countries of the EPC, however, methods of medical treatment of humans or animals are specifically excluded from patentability, and until very recently this was uniformly held to preclude the patenting of second medical uses of known drugs.

The situation began to change when the German Federal Supreme Court held patentable Bayer's invention that nifedipine, known as a cardiovascular agent, could be used in the treatment of cerebral disorders (34). The allowed claim had the form "Use of compound X for the treatment of disease Y". The Swiss Patent Office was then asked whether it would accept claims of this type, and replied that such a claim was not permissible under Swiss law, but a claim of the type "Use of compound X for the preparation of an agent for the treatment of disease Y" would be acceptable (35).

The same application on which Bayer was successful in Germany was also filed in the EPO, where it was eventually considered by the Enlarged Board of Appeals. In deciding this and other related cases, the Board held that although the German form of claim was not acceptable in the EPO, a claim of the Swiss type would be (36). This decision was perhaps more diplomatic than logical, since the Swiss claim has the undeniable defect that it lacks novelty, because the "agent" prepared for the treatment of disease Y will normally be identical with that already used for the treatment of disease Z by the same drug. However in the interests of European unity and in the desire to give protection for useful inventions, this difficulty has been ignored, and national patent offices in most of the EPC states now follow the practice of the EPO (37). It must be remembered that such patents can only be used to prevent a competitor from actively promoting the compound for the patented use. They cannot prevent doctors from prescribing for the new use a generic product that is already on the market for another indication.

Biotechnology - The patenting of inventions in the field of biotechnology would require a whole review in itself. The same rules apply to inventions in this area as to those in the field of classical medicinal chemistry, but these rules are harder to apply because of the complexity of the subject, the rapid development of the state of the art, the close competition between different companies working on the same projects, and the relatively short development time between patenting and clinical testing in man (38).

Conclusions - Ten years ago, anyone viewing the future of patent rights internationally would have been extremely pessimistic. A long list of developing countries were practically abolishing patents altogether,

aided and abetted by international organisations such as the United Nations Conference on Trade and Development (UNCTAD). The EEC Commission seemed to regard any attempt to enforce patent rights in Europe as contrary to the Treaty of Rome, and even in the USA, the Anti-Trust Division of the Department of Justice and the Federal Appeal Courts had a deeply anti-patent attitude. Today, things look very much brighter. Pushed by economic pressure from Washington, the more important developing countries are now strengthening their patent protection. The EEC Commission has brought out a Group Exemption for patent licence agreements which is much more acceptable than the earlier drafts (39), and with the new Court of Appeals for the Federal Circuit and the new approach of the Justice Department, the climate in the USA is also highly favourable to the patentee. The research-based pharmaceutical industry, as well as the enterprising academician, can with some confidence feel that research and investment will in future be protected from imitation.

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