ANNUAL REPORTS IN MEDICINAL CHEMISTRY Volume 20

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

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

STERLING–WINTHROP RESEARCH INSTITUTE
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PREFACE

Volume 20 of *Annual Reports in Medicinal Chemistry* contains 32 chapters in seven sections. The last of these sections, added for the first time to Volume 19, again provides a useful summary of worldwide market introductions and complements the traditional sections: CNS Agents, Pharmacodynamic Agents, Chemotherapeutic Agents, Metabolic Diseases and Endocrine Function, Topics in Biology, and Topics in Chemistry and Drug Design.

In addition to annual updates on the key areas of antianxiety agents, antidepressants, analgesics, antiallergy agents, antihypertensives, antineoplastics, and peptic ulcer therapy, this volume contains reviews of recent developments in the treatment of convulsive disorders, glaucoma, and sickle cell anemia. Also included are chapters covering fluoroquinolone antibacterials, dopaminergic agents, and LHRH analogs. A review of recent work on interleukin 1 appears as a companion piece to a chapter in Volume 19 on interleukin 2. Other endogenous substances such as growth hormone, protein kinase C, neutrophil elastase, platelet-activating factor, and renin are also covered in separate reviews. Two chapters on new technologies in NMR spectroscopy and imaging in living systems have been included in the Topics in Chemistry and Drug Design Section.

I am indeed grateful to the staff of section editors and the contributors to Volume 20 for their fine work. Additionally, I extend my sincere thanks to Martha Johnson for her significant contribution to the preparation of final copy.

Denis M. Bailey
Rensselaer, New York
May 1985

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Section I - CNS Agents

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Chapter 1. Anti-Anxiety Agents and Sedative-Hypnotics

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Introduction - Current evidence indicates that most anxiolytic and sedative-hypnotic drugs exert their pharmacological actions by binding to discrete neuronal recognition sites, consisting of benzodiazepine (BZ) "receptors", GABA receptors and chloride ion channels. Research efforts focussing on the molecular mechanisms of anxiolytic activity have aided medicinal chemists in the synthesis of more selective drugs with fewer side effects. Behavioral models continue to be essential to assess the anxiolytic, anticonvulsant, sedative and muscle relaxant properties of novel compounds despite considerable progress in understanding receptor-mediated events. The molecular aspects of BZ receptor interactions[1-6] and their clinical implications,[7,8] behavioral models,[9] and problems of tolerance and abuse[10] have been reviewed in recent publications.

Molecular pharmacology of benzodiazepine receptors - Binding of benzodiazepines (BZs) to the molecular complex increases the efficiency of GABA in opening chloride channels. Drugs acting at each component of the complex are known and have been used to label their respective recognition sites with either agonists or antagonists. The complex has been solubilized and partially purified.[11-16] Recently, it has been purified to homogeneity from bovine, rat and chick brain cortex and shown to retain binding sites for BZs, GABA agonists and antagonists, and for the convulsant t-butylcyclophosphorothionate (TBPS) which labels the chloride channel.,[11,17] Proteolysis of [^{3}H]flunitrazepam (Flu)-photolabeled membranes results in the isolation of the BZ recognition site on a small peptide fragment.[18] The molecular sizes of the receptors as they exist in their native membranes have been determined by high energy radiation inactivation studies to be 50,900, 54,800 and 137,000 for the BZ receptor, GABA receptor and chloride ionophore (^{35}S-TBPS), respectively; but functional coupling requires a highly integrated multimer of at least 500,000 daltons.[19]

$GABA_A$ receptors, probably of low affinity, mediate the actions of BZs on the chloride channel. In autoradiographic studies, [^{3}H]bicuculline (ligand for low affinity $GABA_A$ receptors) labels sites paralleling the distribution of [^{3}H]Flu binding sites in the brain.[20] GABA-induced electrophysiological responses, recorded from the superior cervical ganglion[21] and from mouse spinal cord neurons in primary cell culture[22,23], are modulated by BZ ligands in patterns correlating with their behavioral effects.[21-23] Chronic BZ treatment reduces postsynaptic sensitivity to GABA, as shown by electrophysiological responses following iontophoretic

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application of GABA on to serotonergic neurons; associated radioligand binding studies demonstrate a decreased ability of GABA to stimulate BZ binding, without significant effect on BZ binding itself.[24] Diazepam administered in vivo to mice increases ^{3}H-muscimol binding to brain membranes.[25] Preincubation of membranes with GABA at 37° stimulates [^{3}H]GABA binding by increasing the number of both high and low affinity sites, either by removal of an endogenous inhibitor or by conversion from another GABA receptor population, e.g., the "super-low" affinity type.[26] Chemical modification of GABA receptors by diazotization or thiocyanate selectively inactivates low and high affinity GABA receptors, respectively.[27] The chemically-modified membranes differentiate agonists from antagonists.[27] Agonists may also be distinguished from antagonists by their different effects on cerebellar cyclic GMP levels[28] and on GABA turnover rates.[29] Inactivation of GABA receptors by phenoxybenzamine reduces GABA-stimulation of ^{3}H-diazepam binding.[30]

The possibility of BZ receptor heterogeneity continues to be explored with great interest because it provides a mechanism for finding selective drugs. Evidence for BZ receptor heterogeneity has been reviewed.[31] It has now been demonstrated that even at 37°C, [^{3}H]Flu photolabels two proteins in hippocampus (P-51 and P-55; BZ-1 and BZ-2) but only one (P-51, BZ-1) in cerebellum[32]. CL 218,872 and some β-carbolines preferentially label cerebellar BZ-1 receptors.[32,33] CL 218,872 also exhibits shallow displacement curves and low Hill coefficients in binding to spinal cord membranes.[34] Muscimol enhances [^{3}H]Flu binding to BZ-2 receptors but not BZ-1 receptor sites in hippocampal membranes.[35] Photolabeling the BZ receptor with Flu reduces its affinity for BZ without preventing GABA-induced changes in BZ affinity, suggesting that photolabeling does not modify the effector system but only the Flu recognition site.[36] The ability of muscimol to enhance the binding affinities of ligands for the BZ receptor, rather than the affinities per se, correlated with their hypnotic activity.[37] Coupling of the molecular complex appears to differ in cerebellum and cortex: GABA agonists and depressants (pentobarbital, etomidate and etazolate) are much more potent inhibitors of ^{35}S-TBPS binding in cerebellum than in cortex; depressants enhance ^{3}H-diazepam binding in cortex more than in cerebellum.[38] The barbiturate and picrotoxinin recognition sites appear to be separate yet allosterically-linked, since all barbiturates, but not picrotoxinin, accelerate ^{35}S-TBPS dissociation.[39]

Ligands for BZ receptors can be characterized as agonists, antagonists or inverse agonists, on the basis of their behavior in three in vitro-binding assays.[40] It has also been proposed that selective pharmacological activity can be explained with a single receptor model: receptors induce either positive or negative efficacy by responding differently to receptor occupancy, depending on the nature of the ligand, generating a continuous spectrum of ligands with pharmacological activities ranging from full agonist to full inverse agonist.[41,42] Brain BZ receptors in the baboon have been labeled with [^{11}C]Ro 15-1788 in vivo and characterized using positron emission tomography (PET)-scanning techniques.[43]

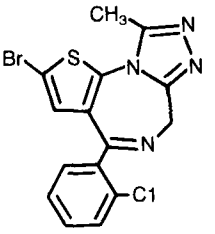
The BZ antagonists CGS 8216 (10a) and Ro 15-1788 (4a) block diazepam-induced decreases in plasma corticosterone levels in stressed rats, indicating involvement of BZ receptors.[44] Although low doses of diazepam reduce plasma corticosterone, high doses raise the hormone level[45]. ACTH reduces the potentiation by chlordiazepoxide of hexobarbital sleep-time.[46] Both stress and β-carbolines reduce binding at the low affinity GABA site;

diazepam blocks these effects, suggesting that stress might release an endogenous inverse agonist.[47] A neuropeptide with such pharmacological activity has been isolated from bovine brain and purified to homogeneity.[48,49] The BZ receptor might control "homeostatic levels of vigilance" via endogenous ligands with opposing activities.[50]

Flu antagonizes cholecystokinin-induced excitation of certain cortical and hippocampal neurons; Ro 15-1788 blocks the effect of Flu, suggesting the possible involvement of cholecystokinin in the anxiolytic action of BZ drugs.[51] Serotonin has also been implicated in the anti-anxiety action of BZ drugs in some[52,53] but not other[54,55] studies. TVX Q 7821 is effective in anti-anxiety models and binds to serotonergic (S-1) but not BZ receptors.[56] Norepinephrine and BZ's might utilize a common mechanism to enhance the effect of GABA on Purkinje cell neurons.[57]

Animal models of behavior - Blockade of pentylenetetrazole (PTZ) interoceptive discriminative stimulus (IDS) as a model for anxiolytic activity has been reviewed and found to be reliable.[58] Generalization to diazepam as an IDS has been demonstrated to be stereoselective for the S(+) isomer of 3-methylflunitrazepam.[59] Anxiolytic activity has been demonstrated in a social interaction model using a novel partner as an anxiogenic stimulus,[60] and in a weanling rat model in which exploratory activity is measured via ultrasound in an area inaccessible to the mother.[61] Animal models of anxiety have been reviewed,[62] and a novel model of anxiety in monkeys has been reported.[63] BZ, GABA and serotonin binding are the same in brain preparations from rats that respond or do not respond to diazepam in a conflict model.[64] Baboons provide good models to detect dependence potential of BZ drugs; precipitated withdrawal may be induced after 7 days of 0.25 mg/kg/day diazepam.[65] Ro 15-1788 exhibits weak anxiolytic activity in some behavioral models[66,67] and weak anxiogenic activity in other models.[68,69] Ro 15-1788, CGS 8216 and FG 7142 were inactive in an exploratory model of anxiety in mice; the species and paradigm may determine the pharmacological characteristics of drugs acting at BZ receptors.[70] Chlordiazepoxide increases food-anticipation; interestingly, the effect is greater in satiated than in food-deprived rats.[71] Diazepam increases food-intake; Ro 15-1788 blocks the effect.[72] In several models, CL 218,872 reduces motor activity at doses only slightly greater than those needed to demonstrate anxiolytic activity in rats and mice, although it did not impair rotarod behavior in mice even at high doses.[73] Valproate may be a partial agonist at the BZ receptor-GABA receptor complex.[74,75] The calcium antagonist nifedipine blocks flurazepam-induced sleep in rats.[76] BZ receptors with affinity in the micromolar range were proposed as regulators of voltage-sensitive calcium channels;[77] others were unable to find a micromolar-affinity BZ binding site.[78]

Benzodiazepine anxiomodulators - Relatively few articles concerning pharmacology, clinical and metabolic studies of [a]-annulated or plain 5-phenyl-1,4-benzodiazepines have been published in the past year, reflecting somewhat diminished interest in this class of compounds. Brotizolam (1) was more potent than diazepam in anticonflict and anticonvulsant tests in rodents.[79] The antianxiety activity of prazepam (2a) was found to be more effective and better tolerated than bromazepam in a controlled clinical study.[80] Recent publications include a review of the pharmacological properties and therapeutic efficacy of pinazepam (2b),[81] the pharmacokinetics of tetrazopam (2c)[82] and midazolam (3)[83] in man, and evidence for the interaction of cimetidine with various BZ drugs.[84-86] Interest in these classical BZ type compounds is concentrated in the areas of i) computer-assisted analysis of the receptor-ligand interactions,[87-89]



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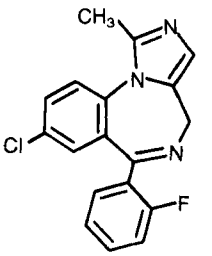

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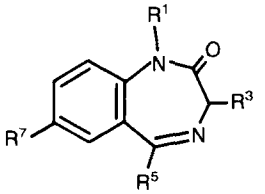
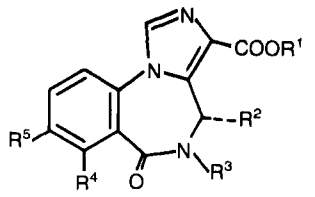


	R_1	R_3	R^5	R^7
2a		H	C_6H_5	Cl
2b	$HC{\equiv}CCH_2$	H	C_6H_5	Cl
2c	CH_3	H		Cl
2d	$Me_2N(CH_2)_2$	H	o-F-C_6H_4	Cl
2e	CH_3	OH	C_6H_5	Cl
2f	H	H	C_6H_5	NO_2

ii) neurochemical understanding of the mechanism of the pharmacological actions,[51] iii) the nature of peripheral receptors and their ligands,[90-92] and iv) studies on dependence liability[93-96] and ethanol interaction.[97]

[a]-Annulated 1,4-benzodiazepines lacking a 5-phenyl group, for example Ro 15-1788 (4a), remain of interest. Pharmacology and initial

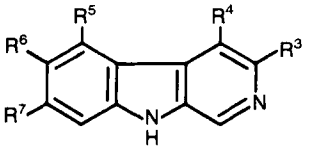


	R^1	R^2	R^3	R^4	R^5
4a	C_2H_5	H	CH_3	H	F
4b	t-C_4H_9	$-(CH_2)_3-$		Br	H
4c		$-(CH_2)_2-$		Cl	H

clinical results with Ro 16-6028 (4b) and Ro 17-1812 (4c) have been reported.[98,99] Both drugs are more potent in anticonflict tests and anticonvulsant tests (chemically induced) than diazepam but are much weaker in inducing ataxia and muscle relaxation.[98,99] Challenge with Ro 15-1788 did not precipitate withdrawal signs in squirrel monkeys treated for two weeks with either of these two drugs.[98] However, in humans, both Ro 16-6028 and Ro 17-1812 were potent sedatives and muscle relaxants, demonstrating the difficulties in extrapolating from animal data, at least with these partial agonists.[99] Ro 15-1788 has been successfully used in several hundred patients to reverse the effects of BZ drugs which had been used in premedication, induction and maintenance of anesthesia.[100] Ro 15-1788 has also been administered intravenously and orally to patients suffering from grand mal epilepsy and showed clear anticonvulsant properties without

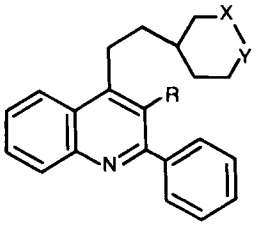
inducing drowsiness or muscle relaxation,[100] consistent with a partial agonist action in terms of its anticonvulsant activity.[101,102] Pharmacokinetic studies on Ro 15-1788 using GC and HPLC have been published; the elimination half-life of Ro 15-1788 from rat brain is 16 min.[103]

Non-benzodiazepine anxiomodulators - Earlier β-carboline derivatives were antagonists like β-CCPr (5a), partial inverse agonists like FG 7142 (5b), or full inverse agonists like DMCM (5c) and β-CCM (5d). More recent analogs of β-CCE (5e) include a full agonist (ZK 93423, 5f), a partial agonist (ZK 91296, 5g) and another antagonist (ZK 93426, 5h).[41,42,104] Thus, with appropriate substitution, the β-carboline series provides examples of the entire spectrum of BZ receptor ligands. A number of novel β-carboline derivatives including the 3-carbamate (5i), the 3-phosphate (5j) and the 3-sulfonamide (5k) were described in a recent German patent.[105]



	R^3	R^4	R^5	R^6	R^7
5a	$CO_2C_3H_7$	H	H	H	H
5b	$CONHCH_3$	H	H	H	H
5c	CO_2CH_3	C_2H_5	H	OCH_3	OCH_3
5d	CO_2CH_3	H	H	H	H
5e	$CO_2C_2H_5$	H	H	H	H
5f	$CO_2C_2H_5$	CH_3OCH_2	H	$PhCH_2O$	H
5g	$CO_2C_2H_5$	CH_3OCH_2	$PhCH_2O$	H	H
5h	$CO_2C_2H_5$	CH_3	Me_2CHO	H	H
5i	$NHCO_2CH_3$	H	H	H	H
5j	$PO(OC_2H_5)_2$	H	H	H	H
5k	$SON(CH_3)_2$	H	CH_3	H	H

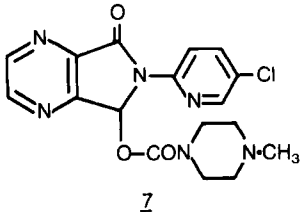
The quinoline derivatives, PK 8165 (6a) and PK 9084 (6b) do not bind to BZ receptors in vivo but are behaviorally active.[106,107] Other inves-



	X	Y	R
6a	CH_2	NH	H
6b	NH	CH_2	H
6c	CH_2	NH	Cl

tigators maintain that PK 8165 is a partial agonist at central type BZ receptors.[108] A recent patent describes the 3-chloro derivative (6c) of PK 8165.[109]

The pharmacology of zopiclone (7), an anxiolytic-hypnotic BZ receptor ligand, has been reviewed in several papers.[110] The pharmacological



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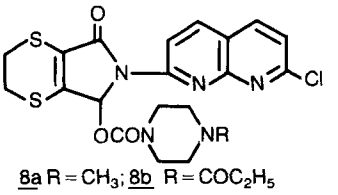
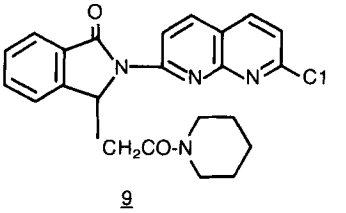


8a R = CH_3; 8b R = COC_2H_5



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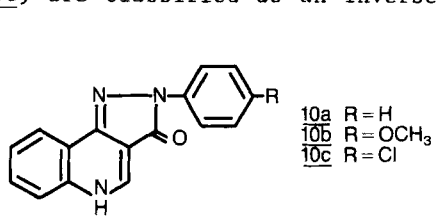
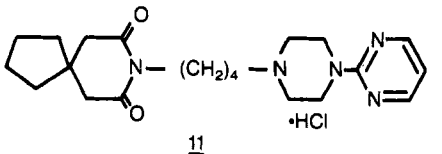
profile of suriclone (8a) is that of a typical agonist,[111] but its binding characteristics differ (independent of GABA concentration,[112] and unaffected by photolabelling the receptor with Flu[113]), suggesting that this compound might bind to a novel site linked allosterically to the BZ receptor.[114] A U. S. approved name (USAN), suproclone (8b), was recently assigned to the N-propionyl analog of suriclone.[115] Pyrrolone-like compounds (e.g. 9), with subnanomolar IC_{50} values for ^{3}H-diazepam receptor binding, were described in a recent European patent application.[116]

The pyrazoloquinolines CGS 8216 (10a), CGS 9895 (10b) and CGS 9896 (10c) are classified as an inverse agonist, a partial agonist and a mixed agonist/antagonist, respectively on the basis of in vitro binding characteristics.[40] CGS 8216 blocks the anticonflict but not the muscle-relaxant activity of phenobarbitol, whereas it antagonizes both the anticonflict and muscle relaxant activities of diazepam.[117] Antagonism of the diazepam discrimination cue by CGS 8216 is noncompetitive.[118] A pharmacokinetic study of CGS 8216 has appeared.[119] The potential for CGS 9896 to induce physical dependence following chronic dosing has been evaluated in baboons. Contrary to lorazepam-treated baboons (positive control), CGS 9896-treated baboons did not display Ro 15-1788-precipitated withdrawal, and only mild spontaneous withdrawal signs were apparent.[93] CGS 9896 did not generalize to diazepam in diazepam-cued drug discrimination studies, whereas in CGS 9896-cued paradigms, diazepam did generalize to CGS 9896, suggesting that the CGS 9896 discriminative stimulus is associated with an anxioselective effect.[120] The lack of sedation observed with CGS 9896 might result from a mixed agonist/antagonist profile, although other explanations are possible.[120]



10a R = H
10b R = OCH_3
10c R = Cl

An NDA approval for buspirone (Buspar™, 11) and its subsequent launching in the U. S. market appear imminent. Several reviews on various aspects of the anxiolytic action of buspirone have been published, but the mechanism has not yet been established.[121-123]



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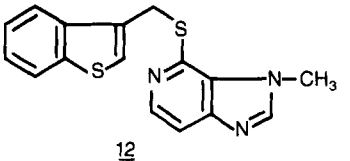

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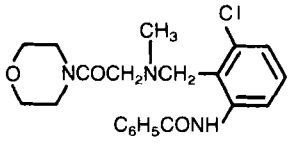
The ability of EMD 41717 (12) to antagonize the anticonflict but not the muscle relaxant and anticonvulsant activities of diazepam has been confirmed.[124] Antianxiety activity of fominoben (13) was observed in a mouse exploratory model for anxiolytics: this activity was blocked by Ro 15-1788.[125]



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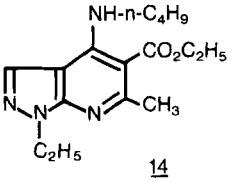

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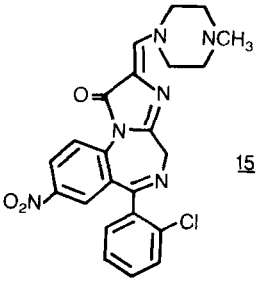
The neurochemistry and behavioral pharmacology of tracazolate (14) has been reviewed.[126] The picrotoxinin binding site may subserve tracazolate's anticonflict activity.[127] Analytical methodology, metabolism, disposition and pharmacokinetics of tracazolate have been reported.[128,129]

Sedative/hypnotics - Loprazolam (15, 1 mg) and flurazepam (2d, 15 mg) were equieffective in patients with disturbed sleep pattern. "Hangovers" were more frequent in the flurazepam group.[130]



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In another study, the "hangover" effects of temazepam (2e, 20 mg) were compared with those of nitrazepam (2f, 10 mg). It was concluded that 20 mg of temazepam is a suitable hypnotic for subjects whose daily work requires constant alertness.[131]

The relationship between the effects of muscimol on BZ receptor binding and the hypnotic activity of nine BZs has been investigated. BZs whose receptor bindings are strongly modulated by muscimol possess potent hypnotic activity, suggesting that the BZ-GABA receptor complex is involved in the hypnotic activity of BZ drugs.[37]

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Chapter 2. Anticonvulsant Agents

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Introduction - Numerous symposia, review papers and research advances in anticonvulsant therapy were published during 1984. Experimental advances are documented in reports of several symposia [1,2,3] and the electrophysiology of epilepsy has been reviewed in a definitive volume.[4] Of clinical, as well as preclinical, relevance are a symposium on valproate,[5] a workshop on metabolism of anticonvulsants,[6] a volume reviewing various aspects of epilepsy,[7] the annual Merritt-Putnam symposium,[8] and the XVth Epilepsy International Symposium.[9]

Currently marketed anticonvulsants are associated with a wide range of side effects, and polytherapy is often necessitated by the lack of complete control of seizures by selected anticonvulsants.[10] A need currently exists for improved anticonvulsant drugs. Mechanistic approaches are increasingly being facilitated by "the new wave of research in the epilepsies".[11] Major trends include new advances in the pharmacology of excitatory amino acid antagonists[12,13] and the continuing exploitation of mechanistic approaches based on γ-aminobutyric acid (GABA).[13]

Biological Models

Neuronal models - New biological models offer alternatives to traditional screens such as electroshock- and pentylenetetrazol (PTZ)-induced seizures, and portend significant progress in delineating the mechanism(s) of the epilepsies. In vitro neuroblastoma, spinal cord and cortical cell culture techniques have been developed for the investigation of anticonvulsant mechanisms.[14,15,16,17] Several review articles have documented in vitro hippocampal slice preparations.[18,19] Epileptic models are obtained by bathing the tissue in solutions containing convulsants such as penicillin[20] or ferrous salts,[21] or by recording from human epileptic cortex slices post-mortem.[22] The classical in vivo focal models of epilepsy are established by topical application of convulsant agents including alumina gel,[23] bicuculline,[24] $FeCl_3$[25] or cobalt[26] to the brains of living animals. Disruption of the blood-brain barrier surrounding these foci, by irradiation, permits parenterally-administered GABA (which cannot cross the blood-brain barrier) to suppress epileptic spike activity.[27]

Genetic models - Genetic animal models of epilepsy are increasingly used for anticonvulsant screening.[2,28] Spontaneous epilepsy models include mutant Wistar rats which show spontaneous petit mal-like seizures with recurrent spike-wave discharges.[29,30] These seizures are ameliorated by agents active against petit mal and by drugs acting on benzodiazepine receptors. Another correspondence to petit mal is that these seizures either are unaltered or aggravated by phenytoin and certain GABAmimetics. The most widely used model of reflex epilepsy for screening purposes is audiogenic seizures in immature DBA/2 mice.[28,31] Several neurochemical abnormalities are evident in brains of these mice but none is clearly causal.[32,33] False negatives are extremely rare in the audiogenic seizure model, but different classes of anticonvulsant drugs may not be well-

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discriminated.[28,31] Mongolian gerbils exhibit seizures in response to handling[34,35] and, more reliably, to an air puff.[35] Anticonvulsant potency against "minor" seizures in gerbils parallels that against PTZ-induced seizures; susceptibility of "major" seizures corresponds to that of electroshock-induced seizures.[35] The model is particularly sensitive to GABA-mimetic drugs. Seizures in photosensitive baboons, the only available primate epilepsy model,[36] are suppressed by experimental anticonvulsants[37,38] and drugs which bind to benzodiazepine receptors[39,40] but not by directly acting GABA-mimetics.[39] The pharmacological sensitivity of the model may differ from human epilepsy and no conclusive evidence points to an intrinsic neurotransmitter abnormality.[41]

Kindling - Kindled seizures have been comprehensively reviewed.[42,43] Differing pharmacological sensitivities have been described for the various seizure phases[43] and for the development of kindling as opposed to the expression of kindled seizures. For example, carbamazepine blocks kindled seizures but does not appreciably retard the development phase.[44,45] Spontaneous and stimulation-contingent seizures in previously kindled animals may also have a differing pharmacological sensitivity.[46]

Excitatory Amino Acids

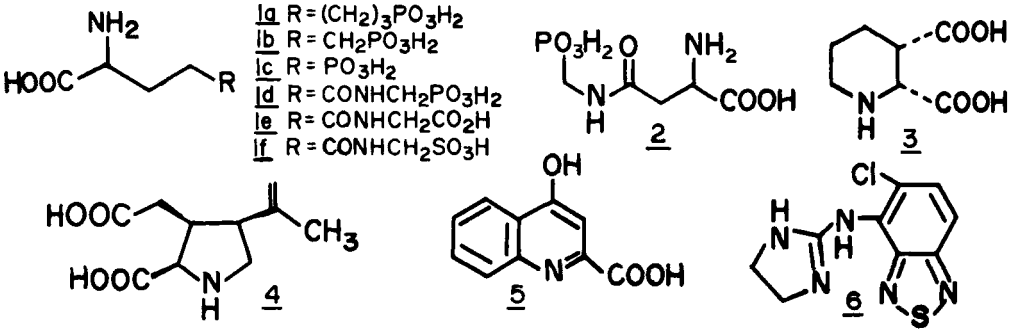
Among the new mechanistic approaches, the most novel is that of the excitatory amino acid antagonists.[12,13] Three classes of excitatory amino acid receptors, named after their most potent known agonists, are recognized: N-methyl-D-aspartate (NMDA), α-kainate and quisqualate-glutamate receptors.[47] Possible endogeneous ligands for these receptors include aspartate and glutamate.

Phosphonic acid derivatives - 7-Phosphono-2-aminoheptanoic acid (AP7, 1a) and 5-phosphono-2-aminopentanoic acid (AP5, 1b) are claimed to be potent, selective antagonists at NMDA receptors and are effective versus chemically-induced seizures (3-mercaptopropionic acid, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), thiosemicarbazide, picrotoxin and NMDA)[12,48,49] and in the audiogenic mouse and photosensitive baboon models.[13,48] In comparison with diazepam, AP7 is six times more potent in the audiogenic seizure model following i.c.v. administration but 100 times less potent when given systemically.[31] Although these drugs are only weakly effective against electroshock or PTZ-induced seizures when given systemically, intranigral administration of AP7 does block electroshock-induced tonic extensor seizures.[50] The more lipophilic analog (AP7) is more effective than AP5 in blocking audiogenic seizures[48] and in reducing afterdischarge in the kindled amygdala model of epilepsy[51] but, unlike 2-amino-4-phosphonobutyric acid (1c), is inactive in a kindled hippocampal seizure model, suggesting that kindling of different limbic areas may involve different neurotransmitter receptors.[52] Other NMDA-selective antagonists include γ-D-glutamylaminomethylphosphonic acid (1d) and β-D-aspartylaminomethylphosphonic acid (2).[53]

Other excitatory amino acid antagonists - Cis-piperidine-2,3-dicarboxylic acid (3), which blocks audiogenic seizures, is active at the NMDA, kainate and quisqualate receptors; the trans isomer, by contrast, has predominantly proconvulsant properties.[54] β-Kainic acid (4), the C-2 epimer of the excitant α-kainic acid, also protects against audiogenic seizures.[55] Kynurenic acid (5), a non-selective excitatory amino acid antagonist, blocks quinolinic acid-induced seizures and neuronal loss.[56] γ-D-glutamylaminomethylsulfonic acid (1f), the sulfonic analog of 1d, which appears to be selective for kainate and quisqualate receptors, protects against audiogenic seizures.[57] The carboxylic acid analog

γ-D-glutamyl glycine (1e) is NMDA-selective, but less potent than the corresponding phosphonic acid.[58,59]

Brain aspartate - The decrease in brain aspartate levels induced by valproic acid (VPA) and several anticonvulsant analogs correlates well with anticonvulsant potency;[60,61] surprisingly, elevations of GABA levels do not correlate as well. Moreover, VPA protects GABA-depleted and control animals equally well against seizures, indicating that the earlier hypothesis for the mechanism of action for VPA (elevation of GABA levels) may be incorrect.[62] Phenytoin inhibits potassium-evoked release of aspartate from rat cerebral cortex slices.[63] Tizanidine (DS 103-282,6) inhibits aspartate release from rat spinal cord slices[64] and is active against audiogenic and photic-induced seizures, but not against NMDA-induced seizures,[38] implicating aspartate in the mechanism of action of this drug.



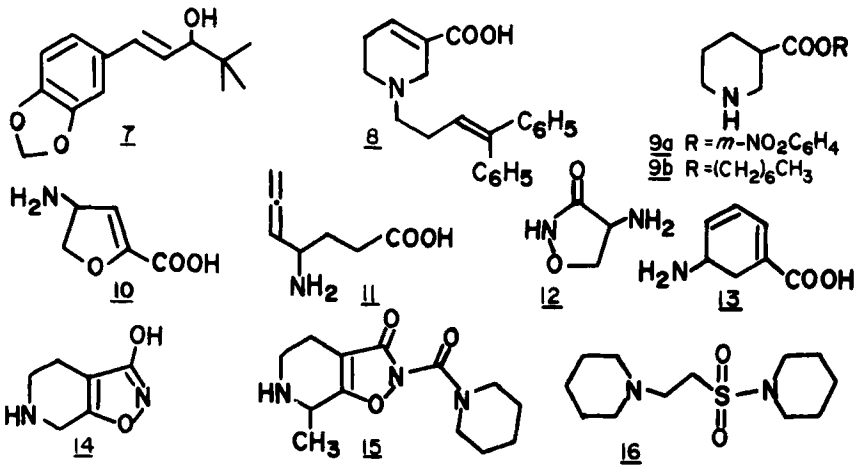
Inhibitory Amino Acids

Inhibitory amino acids, particularly GABA, continue to be a rewarding mechanistic target for the development of novel anticonvulsants. Notably, the enhancement of GABAergic neurotransmission can be accomplished by several means, and may enhance inhibition of seizures.[12]

GABA - Stiripentol (7), a new anticonvulsant, blocks synaptosomal GABA uptake and elevates mouse brain GABA levels.[65] The guvacine derivative (8), a highly potent GABA uptake inhibitor, exhibits good oral activity against chemically-induced seizures in rats, but not in mice.[66] Other GABA uptake inhibitors with anticonvulsant activity include the nipecotic esters 9a[67] and 9b.[68] Clinical studies in epileptics with the GABA-transaminase (GABA-T) inhibitor 4-amino-5-hexenoic acid (γ-vinyl-GABA) have been encouraging.[69] Novel GABA-T suicide substrates 10[70] and 11[71] have recently been prepared. The anticonvulsant activity of isoxazolidinone 12 has been attributed to GABA-T inhibition.[72] Microinjection of gabaculine (13) into the substantia innominata blocks amygdaloid kindled seizures.[73] Protection against PTZ-induced seizures has been observed with the chymotrypsin-resistant GABA prodrug N-pivaloyl-leucyl-GABA.[74] The GABA-A agonists THIP (14) and 3-aminopropanesulfonic acid suppress sound-induced seizures in DBA/2 mice but 14 is proconvulsant in photosensitive baboons.[39] An analog of THIP (15) is active against chemically-induced seizures.[75] The GABA derivative $CH_3(CH_2)_2CONH(CH_2)_3CONH(CH_2)_3CH_3$ exhibits an anticonvulsant profile similar to valproic acid but does not alter GABA levels.[37]

Glycine - Glycine potentiates the anticonvulsant effects of the GABA agonist muscimol[76] and the GABA-transaminase inhibitor γ-vinyl GABA.[77]

The glycine derivative 2-(pentylamino)-acetamide (CP 1552-S, milacemide) is particularly effective against bicuculline-induced seizures,[78] and partially active against cortically-kindled seizures.[79] Milacemide increases glycine and glycinamide levels in the brain.[80]



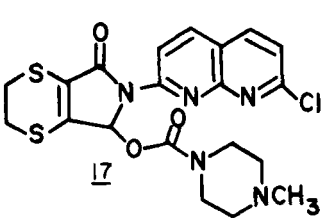
Taurine - The anticonvulsant profile of taurine has been extended to include penicillin-induced focal epilepsy.[81] The taurine analog (16) (one of 23 synthesized) blocks electroshock-induced seizures but has no effect on binding of ^{3}H-taurine to mouse brain membranes.[82] A possible deficit in brain taurine has been noted in brains of genetic seizure-susceptible rats.[83]

Other Anticonvulsant Approaches

Benzodiazepine (BZ) receptor modulation - Classical BZs are not widely used for long-term management of epilepsy because tolerance develops to their anticonvulsant efficacy. Nevertheless, BZ receptor modulation continues to be a prominent theme in the search for novel anticonvulsants. Suriclone (17), a non-BZ anxiolytic drug, protects baboons against photically-induced myoclonus.[84] Two β-carbolines ZK 91296 (18a) and ZK 93423 (18b), are active in this model,[40] and the former is also active in the rat genetic petit mal model, without producing concurrent sedation.[85] Two other β-carbolines DMCM (18c) and β-CCM (18d), enhance seizures in photosensitive baboons and audiogenic seizure-susceptible mice.[86] Surprisingly, the effects of DMCM can be reversed by AP7. The BZ receptor antagonist, RO 15-1788, (19) blocks the development of kindled seizures[87] and antagonizes genetic petit mal in rats,[88] again in the absence of apparent sedation. Fominoben (20), an antitussive agent, has been found to block chemically-induced seizures and to displace [^{3}H]-flunitrazepam binding from rat brain membranes.[89]

The "peripheral" BZ receptor ligand, RO 5-4864 (21), induces seizures in normal mice[90,91] and potentiates audiogenic seizures in susceptible mice.[92] The putative peripheral BZ antagonist, PK 11195 (22), selectively blocks the effects of low doses of RO 5-4864[92] and at higher doses protects mice against audiogenic seizures. The effects of higher RO 5-4864

doses, however, cannot be reversed by PK 11195[92] but can be blocked by clonazepam, which has very little affinity for peripheral BZ sites.[93] Thus, some of the proconvulsant effects of higher RO 5-4864 doses may result from mechanisms other than peripheral BZ agonist activity. DeLorenzo and collaborators have suggested that a micromolar BZ "anticonvulsant" receptor binding site is linked to Ca^{2+}-calmodulin systems.[94] However, this binding site has yet to be confirmed by others.[91]



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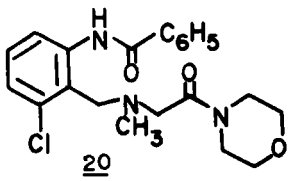

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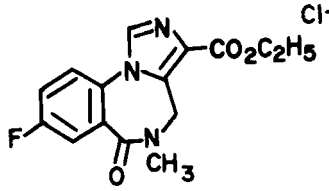

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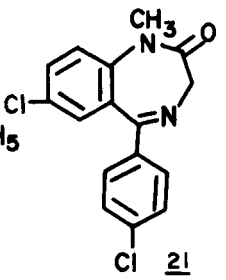

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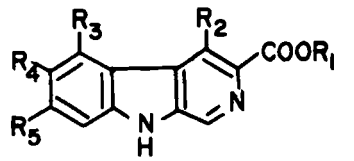
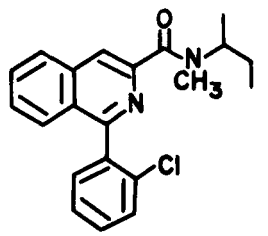


	R_1	R_2	R_3	R_4	R_5
18a	C_2H_5	CH_2OCH_3	$OCH_2C_6H_5$	H	H
18b	C_2H_5	CH_2OCH_3	H	$OCH_2C_6H_5$	H
18c	CH_3	C_2H_5	H	OCH_3	OCH_3
18d	CH_3	H	H	H	H



22

Adenosine - In the hippocampal slice preparation, adenosine postsynaptically inhibits neural discharges induced by penicillin or low calcium concentrations.[95] The development of kindled seizures is inhibited by papaverine, an adenosine uptake blocker, and accelerated by the adenosine antagonist, aminophylline.[96] The kindled seizures are intensified by adenosine antagonists and antagonized by adenosine agonists.[96,97] Carbamazepine inhibits the binding of [^{3}H]-N-6-cyclohexyladenosine to rat brain membranes[98] and its anticonvulsant activity is blocked by theophylline.[99] Other anticonvulsants do not show this profile.[98,99]

Opioids - The complex relationships between opioids and epileptogenic activity continue to generate controversy. A major review[100] distinguishes between two types of proconvulsant activity observed with opiates; one (associated with hippocampal activity and with i.c.v. opioid administration) is mediated by the opiate receptor, the other (associated with cerebral cortex and spinal cord) is not.[101,102] Anticonvulsant actions of opiates and opiate-related proconvulsant activity may occur at overlapping drug doses.[100] Cyclazocine, cyclorphan and pentazocine show moderate antiepileptic activity in certain models.[103,104] Intracerebroventricular administration of pentazocine, meperidine and the δ-opioid antagonist

ICI 154,129, (N,N-bisallyl-Try-Gly-Gly-Ψ-(CH_2S)-Phe-Leu-OH) raises dose thresholds for seizures induced by hexaflurodiethyl ether (flurothyl).[105,106] Postictal inhibition of seizure sensitivity in several animal models may be mediated by endogeneous opioids.[107,108] However, drugs active at opioid receptors have not yet generated clinical interest as potential anticonvulsants.

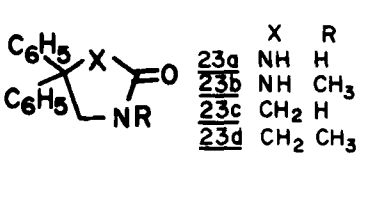
Non-opioid peptides - Kindled seizures in cats are ameliorated by TRH and the TRH analog, DN-1417, but the effects are transient, lack apparent dose-dependency and are secondary to catecholamine mediation.[109,110] Somatostatin (i.c.v.) slightly attenuates the intensity of PTZ-induced seizures.[111] Somatostatin content increases in temporal cortex of kindled rats[112] without a corresponding downregulation of temporal cortex somatostatin receptors.[112] In kindled rats, however, hippocampal somatostatin receptor binding is decreased. A possible association between somatostatin and seizures is supported by the ability of carbamazepine to increase cerebrospinal fluid somatostatin levels.[113] Structure-activity studies of cholecystokinin and certain fragments in blocking picrotoxin-induced seizures have been reported.[114]

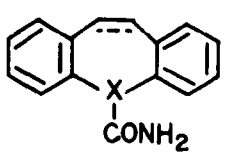
Ion channels - Phenytoin and carbamazepine inhibit batrachotoxin-activated $^{22}Na^+$ influx into cultured neuroblastoma cells and into rat brain synaptosomes, indicating partial blockade of Na^+ channels at clinically relevant concentrations.[115] In vitro electrophysiological studies show that phenytoin preferentially interacts with inactivated (as opposed to open) Na^+ channels, stabilizing these channels in an inactive state. This mechanism can explain why phenytoin inhibits repetitive firing without affecting normal neuronal activity.[16] Glial (Na^+/K^+)-ATPase is deficient in feline and human epileptogenic brain tissue[116] and in brains of mice prone to audiogenic seizures[117].

New, ion-selective electrodes have been used to demonstrate rapid decreases of extraneuronal Ca^{2+} concentrations during single epileptic spikes, possibly reflecting large inward intraneuronal Ca^{2+} currents.[118] Calcium accumulation in neuronal soma and mitochondria may account for hippocampal cytotoxicity induced by experimental status epilepticus.[119] A genetic analysis shows that Ca^{2+}-ATPase activity is deficient in mice susceptible to audiogenic seizures.[120].

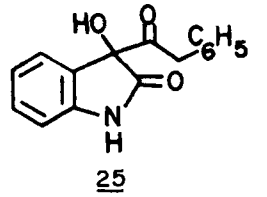
Miscellaneous - Comparison of the anticonvulsant activity of phenytoin (23a) with analogs 23b-23d shows potency to increase in the order 23d<23b<23c<23a suggesting that hydrogen bonding is important for anticonvulsant activity.[121] The lower potency of 23c may also reflect the greater than optimal hydrophobicity of this compound.[122] Comparison of the recently determined X-ray structures of cyheptamide (AY-8682, 24a) and 3-hydroxy-3-phenacyloxindole (25) with phenytoin and carbamazepine (24b) shows good overlap between the amide moieties and one of the hydrophobic groups.[123] Nafimidone (26) increases plasma levels of phenytoin and carbamazepine in epileptic patients by inhibiting microsomal oxidation of these drugs.[124] The pharmacological profile of etomidate (27), a potential drug for the treatment of status epilepticus, has recently been reviewed.[125] Fluzinamide (AHR-8559, 28), similar in profile to VPA, also inhibits kindled amygdaloid seizures.[126] The barrier to rotation about the N-aryl bond in methaqualone (29) is sufficiently high to permit resolution into (+)-29 and (-)-29. Both enantiomers are active, but not equipotent, against electroshock-induced seizures.[127] Of 50 substituted ω-alkoxystilbene analogs, 30 exhibits activity superior to phenytoin or carbamazepine.[128] The corresponding 4-hydroxy piperidine analog is

inactive. CM 40907 (31) has activity comparable to that of carbamazepine in the kindling model of epilepsy.[129] The γ-lactone 32 is equipotent to ethosuximide in preventing PTZ-induced seizures, while regioisomer 33 is proconvulsant. It has been proposed that 33 exerts its effect via chloride channel blockade in the BZ receptor complex.[130]





24a X=CH (no C=C)
24b X=NH (with C=C)



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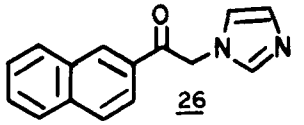

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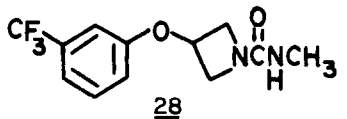

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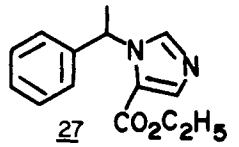

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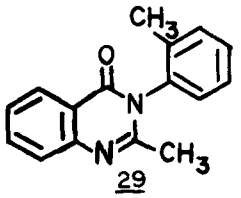

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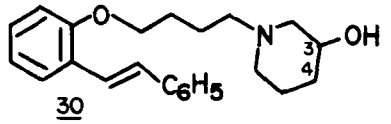

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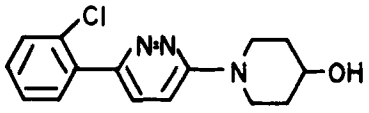

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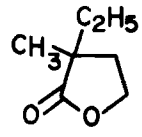

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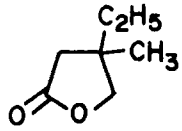

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Chapter 3. Analgesics, Opioids and Opioid Receptors.

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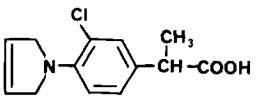
Introduction - An extensive yet incomplete literature concerning the role of receptor mediated events and inflammatory processes in nociception continues to accumulate. The use of strong analgesics in severe pain has been reviewed[1] and the proceedings of a symposium on the management of pain in primary care practice have been published.[2] Reviews have been provided on opioid drugs and their receptors,[3] receptor classification[4] and the biological and clinical significance of endogenous opioids.[5] The concept that endogenous opioids behave as classical neurotransmitters in pain mechanisms has been suggested to be an oversimplification.[6] Reviews of the receptor pharmacology,[7] psychobiology[8] and electrophysiology[9] of opioids and their actions on mammalian spinal neurones[10] and pancreatic islet function[11] have been published. A new overview of structure-activity relationships amongst synthetic opioids[12] and a comprehensive survey of peptides and nociception[13] have been provided. The proceedings of the 1984 International Narcotic Research Conference have been published[14] together with those of a symposium on opioids held in 1983 to mark the 80th birthday of Hans W. Kosterlitz FRS.[15]

CLINICAL HIGHLIGHTS

Non-steroidal antiinflammatory drugs (NSAIDs) - Although concerns about the safety of this class of drug have been expressed,[16] the search for new agents with reduced side effects continues. Comprehensive surveys of the therapeutic applications,[17,18] drug interactions[19] and mode of action[20] of NSAIDs and the role of arachidonic acid oxidation products in pain and inflammation[21] have appeared. The proceedings of a symposium on the use of ibuprofen, now available OTC, have been published.[22] The clinical profiles of suprofen,[23] piroxicam,[24] isoxicam[25] and piroprofen (SU-21524, 1)[26] have been reviewed. Additional studies on the analgesic efficacy of suprofen,[27] piroxicam,[28] diflunisal[29] and etodolac[30] have been reported. Nabumetone appears to be an effective and well tolerated treatment for rheumatoid arthritis,[31] and is transformed into two naphthylacetic acid metabolites which may be responsible for the in vivo activity.[32] Volunteer studies have suggested central antinociceptive effects for zomepirac in man[33] and animal data support the concept of central and peripheral analgesic components for other acidic NSAIDs.[34] The pharmacology of imidazole salicylate (ITF-182), has been reviewed[35] and a preliminary pharmacokinetic study reported.[36] The preclinical pharmacology of loxoprofen (CS-600, 2)[37] and animal studies with a novel N-phenylacetamide FX205-754 (3) have been described.[38]



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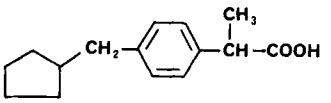

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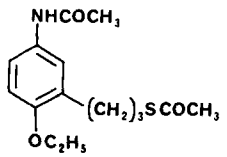

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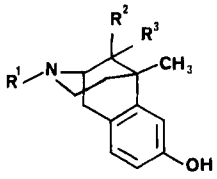
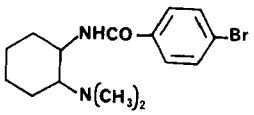
Opioid analgesics - Reviews of the use of opioid analgesics in cancer treatment[39] and anesthesia[40] have been provided. Long-lasting pain relief, free from serious side effects, has been demonstrated by epidural administration in a variety of chronic conditions.[41] Epidural morphine has been given for 13 months without the development of tolerance.[42] Effective post-operative analg sia was provided for about 12 hours by epidural buprenorphine.[43] When administered sc or po, the major metabolites of this drug are norbuprenorphine and conjugates of this and the parent molecule.[44] Dezocine (10mg, im) was as effective as morphine (10mg, im) in post-operative pain[45] and butorphanol (2mg, im) in the treatment of chronic cancer pain.[46] An appraisal of the use of sufentanil for high-dose opioid anesthesia in cardiac surgery has been published.[47] A review of the pharmacokinetics, clinical efficacy and adverse effects of naltrexone has appeared[48] and rapid sc absorption has been demonstrated in man.[49] The potential use of opioid antagonists in the treatment of disorders such as shock and stroke, which may involve endogenous opioids, has been reviewed.[50] Newer analgesics include MR-1268 (4), shown to be as effective as pentazocine in severe postoperative pain.[51] Zenazocine (5)[52] and bromadoline (6)[53] are both reported to be in phase II clinical trials.



4 $R^1 = -CH_2$ (furan-2-yl, 5-CH_3), $R^2 = H$, $R^3 = CH_3$

5 $R^1 = R^2 = CH_3$, $R^3 = (CH_2)_3COCH_2CH(CH_3)_2$



6

OPIOID RECEPTOR STUDIES

Classification and pharmacology - The existence of three types of opioid receptor, μ, δ and κ, is now firmly established.[54] The σ-receptor is considered to be non-opioid as actions mediated here are not reversed by naloxone, and other CNS ligands appear to have high affinity for this site.[55] Caution has been urged in using opioid ligands of varying selectivity, affinity and intrinsic activity in studies which ascribe the effects of such ligands to the occupation of one receptor site *in vivo*.[56]

μ-Receptors - The effect of Na^+ and GTP, or its non-hydrolyzable analogue GppNHp, on opioid receptor binding has been the focus of much recent attention.[57-60] The affinities of opioid agonists and, to a lesser extent, partial agonists for the μ-receptor are markedly reduced in the presence of these agents. The μ-receptor affinities of seventeen opioid drugs in a binding assay incorporating Na^+ and GppNHp correlated well ($r = 0.959$) with the affinities obtained in a rat vas deferens (rvd) assay.[57] The rvd is reported to contain μ-receptors with very high intrinsic activity requirements such that opioids normally considered to be partial agonists for the μ-receptor (including morphine) behave as antagonists in this tissue.[61]

Conflicting evidence for the existence of μ_1- and μ_2-subsites has emerged from studies in the rat. Quantitative ligand binding and mathematical modeling have provided independent support for a high-affinity site in brain, consistent with the proposed μ_1-receptor.[62] Pretreatment of rats with naloxonazine, claimed to be an irreversible μ_1-antagonist, markedly attenuated morphine-induced analgesia without significant effect on withdrawal signs.[63] Approximately 40% of the total specific binding of [^{3}H]-naloxonazine to brain homogenate is not freely reversible.[64] However, when administered icv, naloxonazine produced a long-lasting antagonism

of the inhibition of spontaneous bladder contractions induced by the selective δ-agonist [D-Pen^{2,5}]enkephalin (DPDPE) given by the same route, suggesting the irreversible component may be δ-mediated.[65] It has been proposed that μ_2-subsites are involved in the antagonism of the morphine-increased production of dopamine metabolites in striatal slices by the κ-agonists MR2034, ethylketocyclazocine (EKC), U-50488H and tifluadom.[66] Earlier reports that naloxonazine and MR 2034 were able to antagonize the effects of morphine on pain threshold (μ_1) and respiration (μ_2), respectively, have not been confirmed by others.[67]

δ-Receptors - The hamster vas deferens is reported to contain opiate receptors exclusively of the δ-type.[68] The inhibitory effect of δ-agonists on electrically stimulated contractions of this tissue could be attenuated by naloxone and equally effectively by a new δ-receptor antagonist, ICI 174,864 (N,N-diallyl[Aib^{2,3},Leu^5]enkephalin). The latter compound appears to be highly δ-selective in the mouse vas deferens assay with Ke values of ~30nM against [Leu]enkephalin and >5000nM against normorphine (μ) and tifluadom (κ).[69] In binding studies DPDPE and [D-Pen^2,L-Pen^5]-enkephalin (DPLPE) were found to be the most δ-selective agonists and ICI 174,864 was the most δ-selective antagonist.[70] In each case the binding of these analogues at the δ-site comprises 99% of the total binding.

The role of the δ-receptor in nociception remains unclear. Several studies using a range of μ- or δ-selective peptides, show that analgesic potencies correlate well with affinities for the μ-receptor but not for the δ-receptor.[71-73] In contrast, another study, in which highly selective agonists (DPDPE, DPLPE, [D-Ala^2,Glyol^5]enkephalin (GLYOL), U-50488H) were given to rats (icv), provides evidence that analgesia (hot plate) is mediated centrally by μ- and δ-receptors.[74] Similar results were obtained in mice although it was also observed that following intrathecal administration the rank order potencies of the agonists were changed.[75] The latter results were interpreted to indicate that at the spinal level inhibition of analgesia is mainly mediated through δ-receptors. Evidence continues to accumulate that δ-receptors play a role in opioid-induced respiratory depression.[72,76] A study using [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) and H-Tyr-D-Ala-Gly-NHCH(CH_3)$CH_2CH(CH_3)_2$ as selective δ- and μ-agonists respectively (ip, rats) indicated that the reduction of respiratory frequency was δ-receptor mediated, while the reduction in tidal volume was μ-receptor mediated.[77]

Evidence for type I (low affinity) and type II (high affinity) δ-receptor subtypes has been obtained using the irreversible ligands FIT and BIT.[78] The low affinity site is postulated to be a receptor complex consisting of physically associated μ- and δ-binding sites at which μ-agonists inhibit [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin ([^{3}H]-DADLE) binding non-competitively. It was speculated that the low affinity δ-site and the μ_1-receptor are the same.

κ-Receptors - It has been reported that 84% of opioid binding sites in guinea pig cerebellum are of the κ-type ([^{3}H]-bremazocine binding, μ and δ suppressed).[79] A single component of binding activity with characteristics of a κ-site has been demonstrated in solubilized binding sites from this tissue.[80] [^{3}H]-Dynorphin A has been shown to bind to apparent κ-receptors in deep layers of the guinea pig cerebral cortex, confirming earlier studies with less selective tritiated benzomorphans.[81] [^{3}H]-EKC binding (μ and δ suppressed) to mouse brain membranes has been reported to reveal a small population of high affinity κ-receptors.[82] The observed low sensitivity of compounds which have κ-affinity to the effect of added sodium

in binding assays, reflected in the "double sodium ratio", has provided a correlation with the pharmacological profile of these agents.[83]

Studies with U-50488H, claimed to be a selective κ-agonist,[84] continue to provide an insight into the pharmacology which may be associated with the κ-receptor. The analgesia observed in rodent thermal stimulus models was antagonised by pre-treatment with p-chlorophenylalanine and reserpine and by several reputed serotonin antagonists, inferring a secondary involvement of serotonergic mechanisms.[85] U-50488H failed to inhibit intestinal transit in the rat[74,86] and did not increase non-propulsive contractions of the dog small intestine,[87] supporting a lack of κ-involvement in opioid-induced constipation. A dose-dependent diuresis was observed for U-50488H in the rat.[88] In the dog this effect was abolished by an infusion of antidiuretic hormone.[89] κ-Induced diuresis has been proposed as a simple in vivo test for both κ-agonists[90] and antagonists.[91]

ε- and λ-receptors - The ε-receptor of the rvd has been likened to the "benzmorphan-site" of rat brain membranes.[92] Thus for β-endorphin and fragments thereof a reasonable correlation (r=0.81) was seen between binding affinities and ED_{50} values in an isolated-tissue assay. Other studies have demonstrated stereospecific binding of four (-)-4,5-epoxymorphinans to the putative λ-site in rat brain homogenates.[93] Preliminary opioid receptor assays in intact rats also support the existence of the λ-site, the pharmacological significance of which remains to be established.[94]

Receptor isolation - Considerable progress has been reported in the isolation and characterization of opioid receptors. Affinity chromatography on a sepharose 4B gel modified with β-naltrexyl-6-ethylenediamine (7) has been used to enrich binding sites from brain 300- to 450-fold.[95] When combined with sucrose density gradient centrifugation, digitonin treatment of guinea pig whole brain homogenates afforded two well separated components, one of which appeared to comprise both μ- and δ-sites with a molecular weight estimated at 750,000-875,000 from apparent sedimentation coefficients.[80] The other showed the characteristics of a κ-site with an apparent molecular weight of 400,000, suggesting a different molecular species.[80] A molecular weight of 300,000 has been reported for κ-binding sites solubilized with digitonin from frog brain, said to be rich in κ-receptors.[96] Studies with intact membranes from rat brain have afforded significantly lower molecular weight estimates for μ- and κ-sites.[97,98] The use of polyacrylamide gel electrophoresis indicated a molecular weight of 58,000 for the μ(GLYOL)-site when labeled with the irreversible ligand [^{3}H]-Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl ([^{3}H]-DALECK).[97] Radiation inactivation studies suggested a minimum molecular weight associated with biological activity of about 110,000 for sites defined by [^{3}H]-GLYOL and [^{3}H]-DADLE respectively, the former compatible with a dimer of the [^{3}H]-DALECK site.[98]

ENDOGENOUS OPIOIDS

Physiology and pharmacology - Reviews on general physiology and pharmacology[99] of endogenous opioids and associated implications for clinical practice[100] have been published. More specific reviews on endogenous opioids discuss their involvement in nociception,[101] endogenous pain control,[102] placebo analgesia,[103] endocrine actions in mammals[104] and humans,[105] blood pressure regulation in man,[106] control of food, salt and water intake,[107] the menstrual cycle in primates,[108] the immune system[109] and the gut.[110] The problem of characterizing which opioids are responsible for modulating various forms of endogenous analgesia and where in the body they act is complex. Studies on electroacupuncture analgesia in rabbits and rats have

indicated that in the spinal cord dynorphin A, [Met]enkephalyl-Arg-Phe-OH (MERF) and [Met]enkephalin may be involved whereas in the periaqueductal gray area MERF, [Met]enkephalin, and to a lesser extent β-endorphin, but not dynorphin A, may be implicated.[111,112]

Biosynthesis, distribution and release - The existence of three high molecular weight peptide precursors of the endogenous opioids is now firmly established.[113] In brief, proopiomelanocortin (POMC) gives rise to the β-endorphins; prodynorphin to dynorphin A(1-17 and 1-8), dynorphin B(1-13) and α- and β-neoendorphin; proenkephalin A to [Met]- and [Leu]enkephalin, MERF and [Met]enkephalyl-Arg-Gly-Leu-OH (MERGL).

The POMC system has been the most extensively studied and a comprehensive review has appeared on the enzymic biotransformation of POMC and its derivatives.[114] A combined review and study of β-endorphins in the human pituitary has cited evidence that the processing of POMC in man and animals may be significantly different.[115] In rats and pigs, POMC-derived peptides in the anterior and neuro-intermediate lobes occur in different forms; β-lipotropin (β-LPH) and β-endorphin(1-31) predominate in the anterior pituitary whereas in the intermediate lobe the major products are β-endorphin(1-27) and the non-opioid α-N-acetylated versions of β-endorphin (1-31,1-27,1-26). In humans the pituitary lacks a functional neuro-intermediate lobe and appears not to contain N-acetylated β-endorphins,[116,117] although β-endorphin(1-27) does occur and has been shown to be an antagonist of β-endorphin.[118]

In common with peptides derived from POMC, the highest concentration of prodynorphin derived opioids in rat brain are found in the pituitary gland.[119] Unlike POMC the distribution of prodynorphin and its derivatives seems to be more widespread.[119-122]

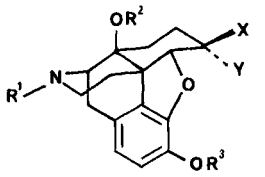
Proenkephalin A and associated opioid peptides are also widely distributed and there is significant regional overlap of immunoreactive(ir)-dynorphins and ir-enkephalins.[123] In rat and human brain highest levels of MERF and MERGL occur in the striatum and hypothalamus but neither could be detected in the pituitary.[124] Co-localization of ir-proenkephalin A and ir-prodynorphin has been demonstrated in certain rat brain medullary neurones.[125] The [Leu]enkephalin sequence occurs in proenkephalin A and prodynorphin but it is generally considered that [Leu]enkephalin is biosynthesized from the former. However, there is now evidence that in the rat substantia nigra [Leu]enkephalin is derived from prodynorphin.[126] The role of membrane-bound enzymes in the processing of dynorphins and MERF has been examined[127] and an enzyme which converts dynorphin B(1-29) to (1-13) has been described.[128]

Metabolism - Three enzyme systems are thought to be responsible for the degradation of enkephalins; an aminopeptidase, a dipeptidylaminopeptidase, and a metalloendopeptidase (EC 3.4.24.11, also called enkephalinase). These cleave the Tyr^1-Gly^2, Gly^2-Gly^3, and Gly^3-Phe^4 amide bonds of the enkephalins, respectively. Whether any of these enzymes are responsible for deactivation of enkephalins at the neuronal or synaptic level is not clear, and it has been suggested that it may not be necessary to invoke such a mechanism.[129] EC 3.4.24.11 has been shown to have a thermolysin-like specificity[130] and occurs widely in the brain and the periphery, with particularly high levels occurring in the kidney.[131] In addition it has the ability to hydrolyse various neuropeptides, in some cases more efficiently than the enkephalins.[130,131]

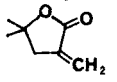
A potent inhibitor of EC 3.4.24.11, N-[1-(R,S)-carboxy-2-phenylethyl]-L-phenylalanyl-p-aminobenzoate, was shown to elicit a weak but long-lasting analgesia in rats (tail flick, ip) but this was not thought to be an enkephalinase mediated effect.[132] This compound does not inhibit angiotensin converting enzyme (ACE).[131] Bestatinyl-L-arginine is reported to be 100-fold more potent than bestatin as an inhibitor of a membrane-bound enkephalin-degrading aminopeptidase.[133] A new inhibitor of enkephalin-degrading enzymes, [(R)-3-(N-hydroxy)-carboxamido-2-benzylpropanoyl]-L-alanine (kelatorphan) has been described.[134] It inhibits all three enzymes and when coadministered icv with [Met]enkephalin, it increased the analgesic potency of the latter 50,000-fold.[134] Analgesia induced by intracerebral injection of MERF is potentiated by bestatin but not by thiorphan, an inhibitor of EC 3.4.24.11.[135] Others have shown that the analgesic effects of MERF are potentiated by the ACE inhibitor, captopril.[111,112]

NOVEL COMPOUNDS, OPIOID RECEPTOR PROBES AND ANALGESICS

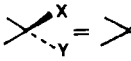
Opioid receptor probes - The characteristics of β-funaltrexamine (β-FNA) binding to mouse brain membranes have been shown to be consistent with its pharmacological profile as an irreversible μ-antagonist/reversible κ-agonist.[136] Although the binding affinities of various δ-agonists to rat brain membranes pre-treated with β-FNA were not significantly different from controls, the ability of GTP to attenuate [^{3}H]-[Leu]enkephalin binding was blocked.[137] The long-lasting μ-antagonist effects of β-FNA have been confirmed in the guinea pig ileum (GPI) in vitro, however, no change in [^{3}H]-GLYOL (μ) binding was observed between untreated and treated ileum homogenates, suggesting that coupling between binding and effector response may have been disrupted.[138] Structure-activity considerations support the hypothesis that the irreversible effects of β-FNA involve covalent binding which depends upon secondary recognition at the receptor.[139] The α-methylene-γ-butyrolactone 8 has been claimed to be an irreversible μ-antagonist,[140] and the ligands 12 and 13, based on the endoethenooripavine framework, have been synthesized.[141] [^{18}F]-3-Acetyl-6-deoxy-6β-fluoronaltrexone (9) has been used to visualize opioid receptors in the baboon brain in vivo, where it is retained in the basal ganglia and thalamus.[142]



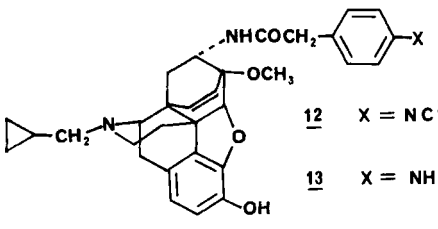
7 $R^1 = CH_2$-cyclopropyl, $R^2 = R^3 = Y = H$, $X = NH(CH_2)_2NH_2$

8 $R^1 = CH_2$-cyclopropyl, $R^2 = R^3 = H$, X, Y = 

9 $R^1 = CH_2$-cyclopropyl, $R^2 = Y = H$, $R^3 = COCH_3$, $X = {}^{18}F$

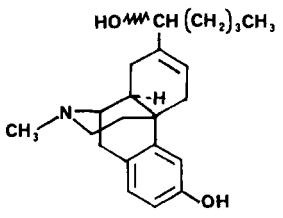
10 $R^1 = R^2 = CH_3$, $R^3 = H$, X, Y = 

11 $R = CH_2$-cyclopropyl, $R^2 = R^3 = Y = H$, $X = NHCOPh$



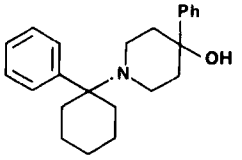
12 X = NCS

13 X = NHCOCH=CHCOOCH$_3$

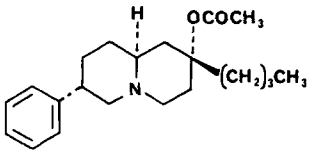


14

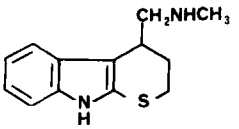
Novel compounds - The oxymorphone ether 10 was the most potent of a series of 14-alkoxymorphinans in a hot plate assay (400 x morphine, sc).[143] Acylation of β-naltrexamine afforded the benzamide 11, a potent κ-agonist in vitro[139] and other studies have demonstrated that the 6-position can accommodate a variety of substituents without loss of opioid activity.[144] In the evaluation of a series of 7-alkyl-6,7-di-dehydromorphinans, the epimeric alcohols 14 proved equipotent as analgesics in a writhing assay (100 x morphine, sc).[145] The phencyclidine analogue 15 has been described as an analgesic possessing high affinity and μ-selectivity in binding assays.[146] Studies with butaclamol analogues have demonstrated potent opioid activity in analgesic tests and binding assays for the (+)-isomer 16.[147] The absolute configuration (2R,7S,9aS), determined by X-ray analysis, is identical to that of active prodine analogues. The indole 17 was equipotent with morphine in a writhing assay and effective in a number of analgesic tests but was said to be devoid of opioid activity.[148]



15



16



17

Modifications of Tyr^5 in dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2),[149] or dermorphin(1-5)[150] yields analogues with diverse selectivities for μ- and δ-receptors. A tetrapeptide, [D-Arg^2,Sar^4]dermorphin-(1-4), is reported to be 20-50 times more potent than morphine as an analgesic in mice (sc).[151] [$\Delta^E$$Phe^4$,$Leu^5$]Enkephalin was found to be 150- and 260-fold less potent respectively in μ- and δ-receptor binding assays than the corresponding $\Delta^Z$$Phe^4$ analogue.[152] It was postulated that binding of enkephalins to the opioid receptor is favored by trans-orientation of the phenyl and carbonyl groups of the Phe residue.[152] Analogues of [Leu]-enkephalin in which each amide bond was replaced in turn by a thioamide linkage have been described by two groups.[153,154] Both report high δ-receptor selectivity and increased potency for the analogue in which the Gly^2-Gly^3 amide bond was modified. An enkephalin dimer, bis-[N,N-diallyl-[D-Ala^2,Leu^5]enkephalyl]-cystine, is reported to be a selective δ-antagonist.[155] [D-Pro^{10}]Dynorphin A(1-11) appears to be a highly selective κ-agonist, similar in selectivity to U-50488H but 220-fold more potent in a binding assay.[156] An analogue of β-endorphin(1-31) containing only D-amino acid residues in positions 13-31 is 2-fold more potent than β-endorphin in a GPI assay and has a tenth of the potency as an analgesic.[157]

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Chapter 4. Antidepressants

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Introduction - Several monographs have been published which present the current state of knowledge of the biology of mood disorders[1-4] and on circadian rhythm disturbances in affective disorders.[5] Biological markers in mental disorders[6-8] and the role of antidepressants (ADs) in the treatment of depression[9] have been reviewed. The use of hormones of the thyroid axis,[10] peptides,[11] monoamine precursors,[12] and monoamine oxidase inhibitors (MAOIs)[13] in the treatment of depression have been reviewed, as has atypical depression, the subclass in which MAOIs have been stated to be superior to tricyclic antidepressants (TCAs) and ECT.[14] Forty-nine new drugs are reported to be currently undergoing clinical trials as ADs.[15]

Antidepressant "receptors",[16,17] in particular ^{3}H-imipramine (IMI) binding sites,[18,19] continue to be of interest. A nonpeptidic endogenous ligand for the ^{3}H-IMI recognition site has been extracted from rat brain and partially purified.[20] The number of ^{3}H-IMI binding sites was significantly reduced in the hippocampus and occipital cortex from a series of depressed patients;[20] however, two reports of seasonal variations in human platelet ^{3}H-IMI binding concluded that, when annual rhythms are taken into account, there is no difference in ^{3}H-IMI binding between depressed patients and normal controls.[22,23]

Mechanism of action - Numerous review articles on the mechanism of ADs have appeared.[24-28] The delayed clinical onset of activity led to the currently held hypothesis that one or more "adaptive" changes occur at central receptors as a result of long-term treatment.[29] In animal studies, ß-receptor subsensitivity is observed with effective AD therapies neurochemically,[30] electrophysiologically,[31] and behaviorally[32,33] and may depend on "passage of time" rather than chronic exposure to drug, since one dose of desipramine (DMI) showed effects after 17 days.[34] Although most ADs desensitized the adenylate cyclase system after subacute treatment, several TCAs failed to do so.[35] In addition, the potency for inducing ß-subsensitivity is inversely correlated with therapeutic potency.[36] ß-Agonists (e.g., clenbuterol) also produce ß-subsensitivity[37] and are active in laboratory AD tests[38] and in man.[39] Coadministration of α_2-antagonists accelerates ß-receptor down-regulation.[32,40] ACTH can influence ß-receptor adaptation, potentiating both the reduction with IMI and the enhancement in receptors after denervation.[41]

Although tricyclic antidepressants (TCAs)[42-44] and ECT caused α_2-subsensitivity in animals, this was not common to all ADs;[42,43] furthermore, some laboratories reported either no changes[45,46] or increased sensitivity.[46] Similar inconsistencies have been found clinically: by monitoring clonidine-induced effects (e.g. sedation), α_2-subsensitivity was observed with DMI[47] and amitriptyline (AMI)[48] but not with mianserin.[48] Intact noradrenergic neurons

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appear necessary for α_2 down-regulation to occur since the effect of AMI was antagonized by locus coeruleus (LC) lesions or 6-OHDA treatment.[44] α_1-Supersensitivity has been reported[49] and it has been suggested that the final common effect of ADs is facilitation of neurotransmission regulated by α_1-adrenoceptors.[50]

5-Hydroxytryptamine (5-HT) mechanisms in depression have been reviewed.[51] Most ADs down-regulate 5-HT_2 receptors neurochemically,[52] behaviorally (5-HTP head twitch)[53] and in man (tryptophan-induced prolactin release).[54] Coadministration of α_2-antagonists with ADs accelerates 5-HT_2 subsensitivity.[52] In contrast, enhanced 5-HT_2 receptor activity has been observed with ECT and some ADs.[55] Enhanced behavioral responsiveness to 5-MeODMT occurred after subacute ADs;[56] both increased and decreased behavioral responsiveness to 5-HT agonists is observed after AD withdrawal.[57]

The possible role of dopamine (DA) in depression has been reviewed.[58] There is some evidence that chronic AD treatment produces presynaptic DA-subsensitivity,[59] but this has not been consistently observed[60] nor confirmed neurochemically by measures of apomorphine-induced alterations in DA metabolism.[61] Although bupropion is a weak inhibitor of DA reuptake, it is unlikely that this is its mechanism of action.[62]

Cholinergic-catecholaminergic interactions may play a role in the etiology and treatment of affective disorders.[63] The anticholinergic effects of ADs appear to be correlated with their ability to facilitate amphetamine-induced locomotor stimulation,[64] but the effects of ADs on muscarinic receptor density after subacute treatment appear related to anticholinergic side-effect potential rather than therapeutic efficacy.[65]

The report of elevated ^{3}H-$GABA_B$ binding (B_{max} increase) in rat frontal cortex after chronic ADs has renewed interest in a GABA hypothesis;[66] $GABA_B$-receptors are located on NE nerve terminals.[67] GABA agonists are effective clinically[68] and ADs and progabide have been compared.[69] ECT may act by enhancing GABA transmission.[70]

Numerous other systems have been studied for the role they may play in the mechanism of action of ADs. Many ADs are H_1 antagonists; however, this may be related to their sedative activities.[71] Methylation processes may influence mood/affective disorders: S-adenosyl-methionine exhibited clinical AD activity and depression is a complication of folate deficiency.[72] Since ADs produce REM sleep deprivation (RSD) and the latter has AD effects in man, RSD has been proposed as a mechanism for ADs.[73] Changes in calcium metabolism occur in depressive states, thus another hypothesis emerged.[74] Opiate receptor interactions may be important in the mechanism of TCAs since they inhibit ^{3}H-naltrexone binding[75] and their actions in the behavioral despair model are antagonized by naloxone;[76] however, non-TCAs do not exhibit these effects. Lithium inhibits norepinephrine (NE) stimulated adenylate cyclase in both animals and man.[77] An enhancement of GTP (G-unit) activation of adenylate cyclase was observed after long-term AD treatment.[78]

Screening methods - In the more than 25 years since the chance discovery of the ADs, no single model has been discovered which incorporates most of the features of human depression.[79,80] One type of animal model is based on separation, which precipitates depression in man. Although TCAs significantly diminish the despair portion of the mother-infant separation syndrome in Rhesus monkeys, increased vocalization (protest behavior) was

noted after acute or subacute IMI.[81] Male-female pair bond separation in hamsters causes weight gain and decreased social interaction and exploratory behavior;[82] although clorgyline was ineffective,[82] subacute IMI significantly reversed the exploratory deficits.[83]

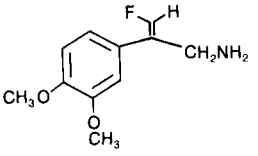
Recent reviews support learned helplessness (LH) as a model of certain features (stress and coping) of depression.[84,85] In some paradigms, subacute or chronic administration of ADs is required to reverse LH deficits;[86] in others, single doses of TCAs or nomifensine are effective.[87] Decreased cortical ^{3}H-IMI binding was observed in LH rats.[88] Several studies attempted to determine the mechanism of action of ADs in the Porsolt behavioral despair model. Decreased tonic immobility in rats following subacute DMI was antagonized by some DA blockers (e.g., clozapine) but not others (e.g., haloperidol).[89] Noradrenergic mechanisms are implicated since both phenoxybenzamine[90] and LC lesions[91] antagonized DMI's effects and LC stimulation reduced tonic immobility in rats.[91]

Most ADs inhibit mouse-killing behavior by rats; ß-receptor mechanisms may be involved since propranolol enhanced the effects of DMI when both drugs were administered for 21 consecutive days but was inactive acutely.[92] In olfactory bulbectomized killers the effects of TCAs were enhanced following chronic systemic or intraamygdaloid administration.[93] Noradrenergic mechanisms appear relevant since both acute and chronic activity of DMI were antagonized by phenoxybenzamine.[92] The reversal of reserpine activity by ADs has long been used as a screen. However, the reversal of reserpine hypoactivity tolerates upon chronic AD administration.[94] Chronic low dose reserpine causes hyperactivity in rats which can be blocked by co-administration of mianserin.[95] Postsynaptic α_2-receptors may be involved in the antagonism of reserpine-induced hypothermia by TCAs.[96]

Interest in α_2-antagonists as ADs continues. Several new clonidine reversal procedures (e.g., suppression of operant responding in rats,[97] AD reversal of locomotor depression in gerbils[98] and suppression of audiogenic seizure activity[99]) have been proposed as *in vivo* models for α_2-antagonism or AD potential. Beagles that exhibited abnormal behavioral patterns (e.g., lack of responsiveness) exhibited improvement after acute anxiolytic or subacute AD treatment.[100] A reassessment of the yohimbine-induced lethality model in mice concluded that it detected all clinically effective ADs.[101] A recent study confirmed that many ADs exhibit common characteristics upon power spectral EEG analysis in rats.[102]

Clinical studies - Cyanopramine,[103] amineptine,[104] dosulepin,[105] amitriptyline-N-oxide,[106] fluotracen,[107] lofepramine,[108] amoxapine,[109] fluoxetine,[110] fluvoxamine,[111] clovoxamine,[112] indalpine[113] and binedaline[114] were effective in double-blind studies versus TCAs. Amitriptyline-N-oxide, fluotracen, amoxapine and indalpine were reported in these studies to have a faster speed of onset than the reference agents. The difficulties in the evaluation of speed of onset were discussed.[115] The mianserin analog aptazapine (CGS 7525 A),[116] alaproclate[117] and tomoxetine (LY139603)[118] were active in open clinical trials. The α_2-antagonist idazoxan (RX 781094) reduced Hamilton rating scores by approximately 50% in a small four week open trial in which most patients were severely depressed.[119] Zimelidine was withdrawn in six European countries due to reports of Guillian-Barre syndrome.[120]

The role of MAO-B in depression remains controversial. In a clinical trial using two doses of the irreversible MAO-B selective inhibitor l-deprenyl, only the higher, non-selective dose was superior to placebo.[121] The lack of "cheese effect" seen clinically with l-deprenyl may not be due to MAO-B selectivity (i.e. lack of peripheral MAO-A inhibitory activity) but rather to the (unknown) mechanism by which l-deprenyl blocks tyramine-induced release of ^{3}H-NE from rat cortical brain slices.[122] Clinical trials of several more selective MAO-B inhibitors, including MDL-72145 (1),[123] almoxatone (MD 240928, 2)[124] and MD 220661 (3),[125] may help clarify the role of MAO-B in depression.



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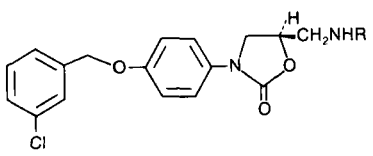
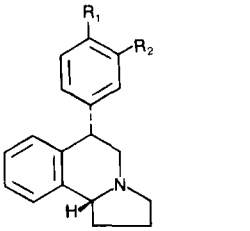


2 R = CH_3
3 R = H

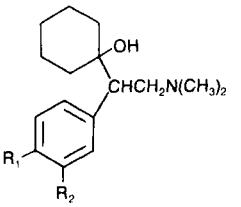
Several compounds clinically useful in other therapeutic areas were found active in AD trials. The antipsychotic agents sulpiride[126] and fluperlapine[127] were effective in open clinical trials. The β_2 agonists salbutamol (iv) and clenbuterol (po) were as effective as clomipramine and had a more rapid onset, particularly in bipolar and certain unipolar patients.[38]

Potential new antidepressants - Much research is still directed toward the discovery of reuptake inhibitors which are active in rodent AD models and have little or no side effects (i.e. anticholinergic, cardiovascular, stimulant, MAOI, etc.). Down-regulation of the rat brain ß-adrenergic and/or 5-HT_2 systems following chronic administration is often reported.

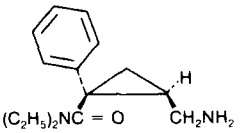
A series of pyrroloisoquinoline derivatives (4-6) is active against tetrabenazine ptosis and hypomotility and includes the most potent inhibitors of DA, NE and 5-HT uptake ever reported.[128] McN-5558 (4) and McN-5652-Z (5) are selective blockers of NE and 5-HT uptake, respectively. Virtually all the anti-tetrabenazine and reuptake activity of the prototype compound (6) resides in the (+)-enantiomer (6S,10bR).[128] Wy-45,030 (7) and Wy-45,881 (8) are inhibitors of NE, 5-HT and DA reuptake. Both acute and chronic administration of these compounds to rats resulted in diminished isoproterenol-stimulated cAMP formation in the pineal gland; these compounds should have reduced antimuscarinic, antihistaminic and antiadrenergic side effects.[129] F 2207 (9) is an inhibitor of 5-HT and NE uptake in vivo and in vitro and is 5 to 10 times as active as IMI in the tetrabenazine ptosis, yohimbine toxicity and learned helplessness rodent models. Chronic administration of F 2207 produced a down-regulation of 5-HT_2 but not ß-receptor binding sites.[130]



4 R_1 = H R_2 = CF_3
5 R_1 = SCH_3 R_2 = H
6 R_1 = R_2 = H



7 R_1 = CH_3O R_2 = H
8 R_1 = R_2 = Cl



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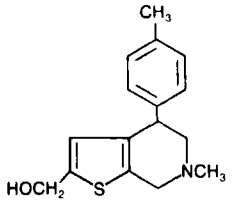
Tetrahydrothienopyridine 10 shows significant inhibition of 5-HT and NE reuptake and is active against tetrabenazine ptosis with less propensity for anticholinergic (^{3}H-QNB binding) and cardiovascular (isolated guinea pig atrium) side effects than AMI.[131] Hexahydroindanopyridine 11 prevents tetrabenazine ptosis and blocks DA and NE reuptake but has stimulant properties.[132] Amidines 12 and 13 are as potent as the TCAs in tetrabenazine ptosis and NE reuptake inhibition, and they are devoid of anticholinergic, antihistamine and MAOI activity.[133]



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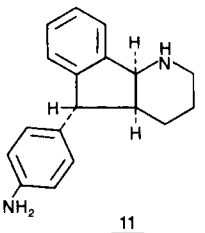

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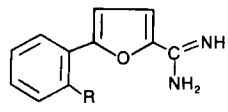
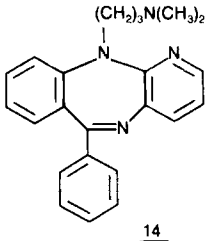


12 R = NO_2
13 R = NH_2

AHR-9377 (14), a potent and selective inhibitor of NE uptake, is active in the tetrabenazine ptosis and yohimbine-toxicity models of depression and induces ß-receptor down-regulation in rat cerebral cortex on chronic administration. In dogs, AHR-9377 is less cardiotoxic than either AMI or IMI.[134] MD 260185 (15), a selective NE reuptake inhibitor, is active in reserpine antagonism and behavioral despair, has less anticholinergic activity than reference TCAs and shows potent analgesic activity in rodent models, equal to or greater than TCAs.[135] CGP 4718 A (4-[5-chlorobenzofuran-2-yl]-1-methylpiperidine) is both a 5-HT uptake inhibitor and a competitive reversible inhibitor of MAO-A at comparable doses.[136]



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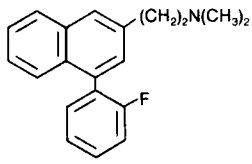

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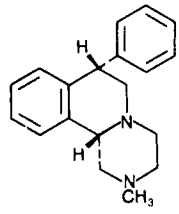
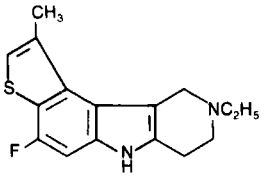


16

Several potential new ADs are effective in animal models and/or affect NE, 5-HT or DA systems, but are devoid of significant uptake inhibitory activity. PR 881-884A (16) is an atypical AD developed through modelling techniques, with TCAs and (+)-mianserin as models; it causes ß-receptor subsensitivity and has an AD profile in EEG studies in conscious beagles.[137] Tiflucarbine (TVX P 4495, 17) antagonizes tetrabenazine and reserpine ptosis and is active in a behavioral despair model. However, it does not potentiate yohimbine toxicity. Chronic administration of tiflucarbine diminished NE-stimulated cAMP formation in rat cerebral cortex.[138,139] Tiflucarbine has no NE reuptake or MAO inhibitory properties.[139] (±)-CGP 15 210 G (18) has little effect on 5-HT or NE reuptake but antagonizes the depletion of 5-HT caused by 4-methyl-α-ethyl-m-tyramine and p-chloroamphetamine with a potency equal to zimelidine.[140] Medifoxamine (N,N-dimethyl-2,2-diphenoxyethylamine) is active in the reserpine hypothermia, yohimibine toxicity and learned helplessness models, but lacks NE or 5-HT reuptake inhibition, MAO inhibitory, anticholinergic or cardiodepressive effects.[141] RS-2232 (19) is approximately equipotent to IMI in the reserpine ptosis and hypothermia models and is without anticholinergic or cardiodepressant effects.[142]



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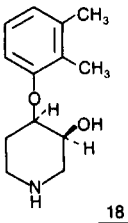

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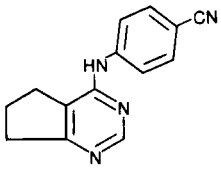
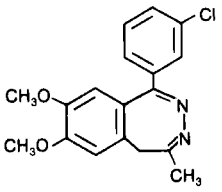


19

The 2,3-benzodiazepine GYKI 51189 (20) has both anxiolytic and AD activity (reserpine hypothermia, tetrabenazine ptosis and behavioral despair) in animal models.[143] Another anxiolytic, BMY 13805 (21) causes down-regulation of 5-HT_2 receptors on chronic administration.[144] Zometapine (22) is clinically effective and is active in a noise-induced stress model of depression in animals. It has neither amine uptake nor MAO inhibitory activity.[145]



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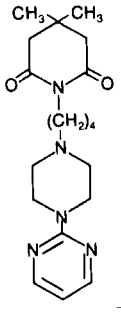

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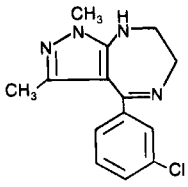
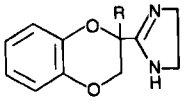


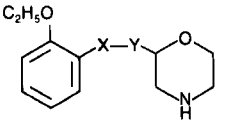
22

The potential for α_2-antagonists as ADs is being investigated clinically with idazoxan (see "Clinical Studies") and imiloxan (RS-21361). Imiloxan produced a more rapid onset of ß-adrenoceptor desensitization (rat cerebral cortex) than DMI.[146] More potent and selective (α_2/α_1) analogs of idazoxan, particularly RX 811033 (23)[147] and RX 821002 (24)[148] have been described. The α_2-antagonist FCE 20124 (25) is equivalent to DMI as an inhibitor of NE reuptake and is 20 times more potent than IMI against reserpine-induced hypothermia.[149]

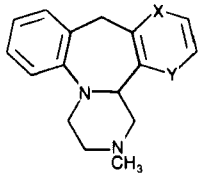
Clinically effective ADs continue to serve as templates for further chemistry. Two thiophene analogs of mianserin, CGS 11049A (26) and CGS 15413A (27) exhibit similar profiles to mianserin.[150] The reverse ether (28) of viloxazine is more potent than viloxazine in preventing reserpine ptosis and hypothermia but has approximately the same acute toxicity.[151]



23 R = C_2H_5
24 R = CH_3O (S-isomer)



25 X = O Y = CHC_6H_5
28 X = CH_2 Y = O



26 X = bond Y = S
27 X = S Y = bond

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Chapter 5. Dopamine Receptors and Dopaminergic Agents

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Introduction - The last two years have seen rapid developments in research related to dopamine (DA) agonists, antagonists and their receptors. Reviews of the pharmacology,[1-10] chemistry[11-14] and clinical experience[15,16] in this field have appeared. The study of DA receptor subtypes and the development of agents acting specifically at these receptors have been especially fruitful areas of research. It is the aim of this review to place recent developments in the field of DA receptors and dopaminergic agents in the context of agents acting at specific receptor types.

DA receptors - Classification of DA receptors has been simplified considerably. Earlier receptor binding studies had indicated the existance of four distinct receptor subtypes, identified as D-1, D-2, D-3 and D-4. Evidence now indicates that both the D-1 and D-2 receptors exist in high affinity and low affinity states. The high affinity state of each may be converted to the low affinity state in the presence of guanosine triphosphate. The low affinity D-2 binding site corresponds to the D-2 receptor while the D-4 receptor is now believed to be the high affinity D-2 site.[2,3,5,10,17] The high affinity D-2 receptor has been identified as the DA autoreceptor in the CNS and the DA receptor of the pituitary mammotroph.[3,7] Similarly, the low affinity D-1 binding site is the D-1 receptor and the D-3 receptor is the high affinity D-1 site.[2] Although the function of the high affinity D-1 site is not known, it is unusual in that depletion of striatal DA stores by several means reduces binding to this state of the receptor.[2]

The D-1 and D-2 DA receptors are biochemically and pharmacologically distinct. The D-1 receptor is linked to the stimulation of adenylate cyclase (AC) as its second messenger.[4] In the rat corpus striatum and anterior pituitary, the D-2 receptor has been shown to be negatively linked to AC.[18] In other areas of the brain, the secondary messenger for the D-2 receptor has not been clearly established. The D-2 receptor may be linked to the polyphosphoinositide cycle. Activation of the high affinity D-2 receptor on anterior pituitary mammotrophs has been shown to inhibit the phosphatidyl inositol (PI) cycle.[19] Drugs that block the PI cycle or interfere with arachidonic acid release from phospholipids have been reported to inhibit prolactin release.[20] Thus, hydrolysis of polyphosphoinositides may be the fundamental transducing mechanism in the pituitary mammotroph. In the rat hippocampus, dopaminergic agents have been reported to alter polyphosphoinositide metabolism.[21] Although the action of many neurotransmitters depends on the hydrolysis of membrane polyphosphoinositides,[22] little information is available on the action of DA on membrane phospholipid turnover in other areas of the brain and the role of this transducing mechanism for the activation of D-2 receptors in the CNS is not clear.

The D-1 receptors that stimulate AC and the D-2 receptors that inhibit AC in the rat corpus striatum are abolished by lesioning with kainic acid.[18] The remaining non-AC linked D-2 receptors appear to be

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located on the axon terminals of glutamate afferents from the frontal cortex. D-2 agonists appear to have a higher affinity for the D-2 receptors on dendrites or cell bodies of striatal neurons while D-2 antagonists have a higher affinity for D-2 receptors on glutamate terminals.[18]

DA autoreceptors are located on the axon terminals and on the cell body and dendrites of the DA neuron and have a high affinity for both DA and neuroleptics. Activation of DA autoreceptors inhibits the synthesis and release of DA. The DA autoreceptors on the terminals of nigrostriatal neurons regulate terminal excitability.[23] When the rate of impulses reaching the terminals of nigrostriatal neurons was increased by medial forebrain bundle stimulation, a pronounced decrease in terminal excitability resulted. This decrease could be blocked by haloperidol infusion into the terminal field. Terminal excitability is also reduced by infusion of apomorphine or amphetamine.[24] It is most likely that the decreased terminal excitability is due to an autoreceptor-mediated hyperpolarization and an alteration in ionic conductance of the membrane.[24]

Many electrophysiological studies have demonstrated that functionally active dopamine autoreceptors exist in the somatodendritic region of the A9 and A10 areas of the CNS. The somatodendritic autoreceptor has been shown to be responsive to all dopamine agonists studied to date.[25] The ability of dopamine agonists to suppress the firing of dopamine neurons is thought to be due primarily to activation of these impulse-regulating somatodendritic dopamine autoreceptors.[26] Recent evidence suggests that the firing rates of A10 neurons is inversely related to the density of somatodendritic autoreceptors.[27] The autoreceptors appear to have higher affinity for dopamine agonists than do the postsynaptic D-2 receptors. A large number of agonists have been shown to stimulate autoreceptors at doses below those which elicit postsynaptic effects.[25,28]

The clearest medicinal application for autoreceptor stimulants is use in schizophrenia since neuroleptic treatment is based upon the hypothesis that a reduction in dopaminergic activity is useful in the alleviation of some psychotic symptoms. Several preliminary reports using postsynaptic agonists at doses believed to stimulate primarily autoreceptors are suggestive of an immediate beneficial effect in schizophrenics.[29-31]

Methods for assaying dopaminergic activity - A wide variety of tests have been developed to determine the activity and degree of selectivity of compounds at the DA receptor subtypes. The stimulation of AC activity is a widely used assay for D-1 agonist activity.[32-34] D-1 antagonists inhibit DA-stimulated AC. Depending on the tissue preparation used in the assay, D-2 agonists either decrease or have no effect on AC. Binding studies employing ^{3}H-cis-flupentixol[35,36] or ^{3}H-cis-piflutixol[37] as ligands are also used to assess D-1 activity.

The effect of a compound on prolactin release is the most general test for D-2 activity. Both postsynaptic and presynaptic (autoreceptor) D-2 stimulants inhibit prolactin release, while D-2 antagonists stimulate the release of prolactin.[38,39] D-1 agonists and antagonists have no effect on prolactin release.[40,41] Stereotypy and locomotor activity measurements have been utilized as indices of dopaminergic activity.[42,43] Postsynaptic D-2 agonists increase both stereotypy and locomotor activity while D-2 antagonists cause decreases. Autoreceptor agonists also cause a decrease in locomotor activity.[44] The induction of turning behavior in rats with unilateral 6-hydroxydopamine lesions of the nigro-neostriatal

dopaminergic pathway (6-OHDA rat) is a commonly used measure of central dopaminergic activity.[45] Both D-1 and postsynaptic D-2 agonists can cause turning. Another specific assay for central postsynaptic D-2 activity is the stimulation of corticosterone release.[46] The measurement of acetylcholine and DA release from striatal tissue has been used as a test to differentiate D-2 postsynaptic agonists from autoreceptor agonists, respectively.[47] Compounds with autoreceptor stimulant properties decrease L-DOPA accumulation in the brains of rats pretreated with γ-butyrolactone.[48]

New methodology - During the last few years two powerful new techniques, in vivo voltammetry[49] and in vivo brain dialysis,[50] gained acceptance in the study of neurochemistry. The advantage of both of these techniques is that they can be used in conscious, unrestrained animals to determine the regional selectivity of drugs. Unlike the conventional single point, steady-state measurements, time dependent changes in the concentration of neurotransmitters and their metabolites in extracellular fluid can be measured.

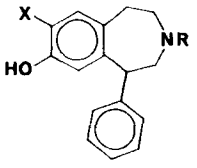
The voltammetric technique measures the concentration of easily oxidizable species such as ascorbate, catecholamines, serotonin, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA). Since all catechols oxidize at the same potential, it is difficult to be completely certain which catechol is being measured unless the electrode is placed in a brain area containing primarily only one of the catechols (i.e. the striatum for dopamine). Catechols and ascorbate have nearly identical oxidation potentials but each can be measured independently under certain conditions.[51-53] This is important because ascorbate is present at concentrations at least 1000-fold higher than dopamine. The in vivo voltammetry technique is more powerful when coupled with in vivo brain dialysis. A dialysis probe is stereotaxically implanted in the brain and saline or artificial CSF pumped through it. The dialysate output is analyzed for neurotransmitters, metabolites, etc. by HPLC or radioenzymatic assay. This technique is preferable to push-pull dialysis because it is a closed system.

Application of these methods has produced several interesting findings. D-2 agonists and amphetamine release ascorbic acid in conscious rats and inhibit its release in anesthetized rats.[51] Amphetamine was found to increase dopamine and decrease DOPAC and HVA output, while reserpine was found to induce the opposite effects.[54] γ-butyrolactone (GBL) rapidly reduced dopamine, DOPAC and HVA output.[54] Autoreceptor stimulating doses of apomorphine reduced dopamine release by 50% while doses sufficient to cause postsynaptic stimulation reduced it 100%.[55] These and many other studies have documented these techniques as new and valuable tools for neurochemical research.

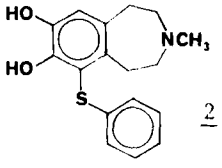
D-1 agonists/antagonists - Earlier some doubt existed whether the central D-1 binding site could actually be called a receptor since no central effects were known to be mediated through it.[56] Now, however, with the advent of agonists and antagonists selective for the D-1 receptor, the D-1 receptor has been shown to be involved in some behavioral responses. SCH-23390(1a), the first selective D-1 antagonist to be described, is effective in blocking stereotypy induced by apomorphine, amphetamine or methylphenidate, and in blocking locomotor activity induced by amphetamine -- activities formerly believed to be mediated exclusively through the D-2 receptor.[57-60] Mouse locomotor activity must involve interaction between D-1 and D-2 receptors since a combination of SKF-38393(1b) (a selective

D-1 agonist) and LY141865 (a selective D-2 agonist) was able to restore locomotor activity in the reserpinized mouse but either alone, even at higher doses, was ineffective.[61] Turning behavior in the 6-hydroxydopamine-lesioned rat (6-OHDA rat) has also been proposed to involve both D-1 and D-2 receptors.[62] SKF-38393 induced turning in 6-OHDA rats and SCH-23390 blocked turning induced by D-1 agonists but not by D-2 agonists.[57,59,61,63] Grooming behavior was induced by SKF-38393 and could be blocked by SCH-23390 but not by a selective D-2 antagonist.[60] In a possible model of tardive dyskinesia, SKF-38393 was seen to induce oral dyskinesias in naive rats.[64] SCH-23390 was able to reverse the self-mutilative behavior observed in ventromedial tegmental lesioned monkeys treated with apomorphine, inferring the involvement of D-1 receptors in Lesch-Nyhan syndrome.[65]

SCH-23390 demonstrates a >500-fold greater affinity for the D-1 receptor than the D-2 receptor and is very potent at inhibiting dopamine stimulated adenylate cyclase.[57,59,66,67] Its specificity for the D-1 receptor has led to the development of ^{3}H-SCH-23390 as a ligand for binding studies.[68,69] In animal models, SCH-23390 displays many pharmacological effects which are indicative of neuroleptic activity, e.g. suppression of CAR, block of amphetamine-induced lethality in grouped mice (block of aggressive behavior), block of amphetamine and apomorphine-induced stereotypy and induction of catalepsy, implying a possible involvement of D-1 receptors in the therapeutic effects of neuroleptic drugs.[57,59,70] It should be cautioned, however, that in some systems SCH-23390 may exert D-2 effects at concentrations close to those used to examine D-1 effects.[71]



1a R=Me, X=Cl
1b R=H , X=OH
1c R=Me, X=OH
1d R=H , X=Cl
1e R=Me, X=Br



2

A study of analogs of SCH-23390 (1a) has illuminated the structural requirements for D-1 receptor activity. SKF-38393 (1b) is a D-1 agonist. N-methyl substitution, as in SKF-75670 (1c), yields a partial agonist, while the 7-chloro derivative SKF-83509 (1d) is a pure antagonist of moderate potency. SCH-23390 incorporates both of these changes and is a much more potent antagonist.[72] The 7-bromo derivative, SKF-83566 (1e) is also a selective D-1 antagonist.[73] The D-1 antagonist activity of SCH-23390 and SKF-83566 is stereo-specific with the R enantiomers being the more active.[73] SKF-83742, (2), has also been reported to be a D-1 dopamine antagonist.[74]

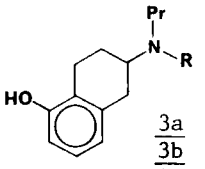
D-2 antagonists and neuroleptic drugs - A characteristic common to all drugs that are effective neuroleptics in man is the ability to block D-2 receptors. In addition, many neuroleptics are also D-1 antagonists. However, there is not a clear correlation between a neuroleptic's selectivity for D-1 and D-2 receptors and its tendency to produce extrapyramidal side effects (EPS) or tardive dyskinesias (TD). Haloperidol, cis-flupentixol, thioridazine and clozapine are all neuroleptics with mixed D-1/D-2 antagonist activity.[75] However, while haloperidol and cis-flupentixol have high side effect profiles, thioridazine and clozapine are much less likely to induce EPS.[76] Both sulpiride and metoclopramide are selective D-2 blockers.[75] Sulpiride is relatively free of side effects but metoclopramide has a strong tendency to cause

EPS.[76] The EPS caused by metoclopramide may be due to the drug acting selectively on the striatum (vide infra).[77]

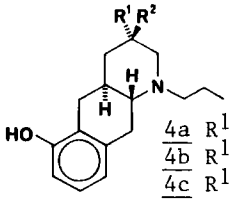
An important consideration for the development of neuroleptics with low side effect profiles may be the selectivity of drugs for specific brain regions. Neuroleptic drugs with a low incidence of side effects (thioridazine, clozapine and sulpiride) were reported to antagonize apomorphine-induced locomotion but not apomorphine-induced gnawing.[76] Locomotor activity is mediated via the limbic forebrain, and gnawing is mediated via the striatum.[76] In support of the regional selectivity hypothesis, Huff and Adams reported that clozapine released DA in the nucleus accumbens but not in the neostriatum, whereas chlorpromazine released DA in both areas.[78] Turnover studies have also indicated that the atypical neuroleptics appear to have a preferential action on the mesolimbic-cortical DA systems.[79]

Another source of the lack of dyskinetic side effects of the atypical neuroleptics may be associated with their lack of blockade of the DA autoreceptor. Clozapine, even at high doses, did not significantly reverse the inhibitory effect of apomorphine on DA neuronal firing.[80] Furthermore, neuroleptic drugs with low EPS liabilities did not potentiate the increase in brain DOPAC levels in response to amfonelic acid as did drugs possessing a high side effect profile.[81] The ability to potentiate the action of amfonelic acid may be related to enhanced neuronal firing through autoreceptor blockade. Persistent blockade of DA autoreceptors leads to the condition of depolarization inactivation. Neuroleptics which induce EPS cause depolarization inactivation of both A9 and A10 neurons, whereas antipsychotic drugs which lack this property inactivate only A10 neurons.[82,83] In contrast to normal neurons, the depolarization blocked neuron is activated by GABA application rather than glutamate.[82] The gradual development of depolarization inactivation could explain the delayed onset of therapeutic effects and the gradual development of supersensitivity.

D-2 agonists - Several new compounds have been synthesized which exhibit potent and, in some cases, selective D-2 agonist activity. The dipropylaminotetralin 3a is a known D-2 agonist,[84,85] although reports of its D-1 activity are conflicting.[85,86] Replacing one of the propyl groups with an arylethyl group leads to more potent and selective D-2 agonists. The phenylethyl derivative, N-0434 (3b), is two orders of magnitude more



3a R=Pr
3b R=$CH_2CH_2C_6H_5$
3c R=CH_2CH_2(2-thienyl)



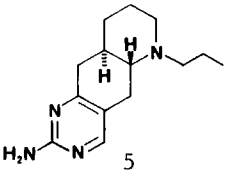
4a R^1=CH_2SCH_3, R^2=H
4b R^1=H, R^2=$NHSO_2NEt_2$
4c R^1=R^2=H

potent in inhibiting prolactin secretion in vitro.[85,87] The isosteric thienylethyl derivative, N-0437 (3c), is ten times more potent still. D-1 agonist activity was not observed with either N-0434 or N-0437.[85,87]

Sandoz workers reported the synthesis and prolactin secretion inhibiting activity of CV-205-503 (4a) and CV-205-502 (4b), benzo[g]-quinolines in which the sidechain at C-3 corresponds to the C-8 substituent of the ergolines.[88] Indeed, the methylthiomethyl group of 4a is conceptually derived from pergolide while the sidechain of 4b is the

same as that found in CQ32-084. While neither compound is as potent an inhibitor of prolactin secretion as its corresponding ergoline analog, both show decreased affinity for α_1, α_2, 5-HT_1, and 5-HT_2 receptors. Comparison of the biological activity of 4a and 4b with the parent compound 4c has not been reported. The 4aS,10aR enantiomer of 4b was found to possess all of the dopaminergic activity of the racemate.

The synthesis and potent D-2 agonist activity of LY137157 [(±)-5] and LY163792[(±)-6] have been reported.[89] LY137157 and LY163792 are equipotent with pergolide in inhibiting prolactin secretion, with LY163502 ((-)-5) being of equal or greater potency to pergolide in inducing turning in the 6-OHDA rat, and in inhibiting DA and acetylcholine release from superfused rat caudate slices.[90,91] Both compounds display stereoselectivity with the levorotatory enantiomers of each (absolute con-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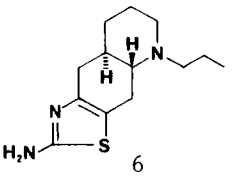
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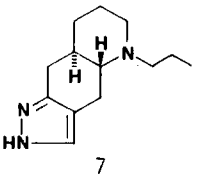
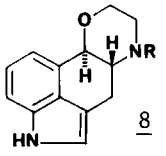

7

figuration depicted above) possessing all the dopaminergic activity of the racemate. A similar stereospecificity has been reported for quinpirole (LY171555,(-)-7).[92] The heteroaromatic rings of 5, 6, and 7 must be considered bioisosteric with the phenol of 4c.[89]

A study of partial structures of EOE (8, R=Et) led workers to develop PHNO (9).[93] (+)-PHNO, the dopaminergic enantiomer, is remarkably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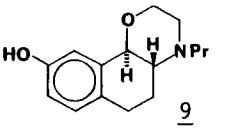
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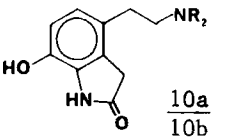
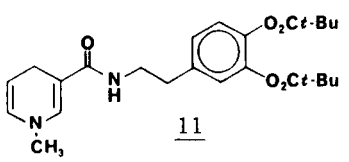

10a R=H
10b R=Pr

potent at both pre- and postsynaptic D-2 receptors, causing turning in the 6-OHDA rat, inducing emesis in dogs and inhibiting DOPA accumulation in the GBL treated rat. Carp retina adenylate cyclase was not stimulated by (+)-PHNO and its affinity for α_1, α_2, 5-HT_1, and 5-HT_2 receptors was negligible, indicating it is a highly selective D-2 agonist.[94,95]

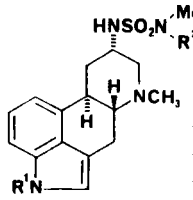
The indolones SKF-88827 (10a) and SKF-89124 (10b) were reported to have D-2 agonist activity in assays of peripheral drug action but no assays for central activity were reported.[96]

The dihydropyridine 11 was synthesized as a potential prodrug which was expected to allow DA to be delivered selectively into the CNS.[97] The study did not, however, demonstrate that 11 liberated DA in the brain since direct assays of brain DA were inconclusive and the assay of dopaminergic activity utilized, prolactin inhibition, is not a central effect.

Work on previously known DA agonists continues to be reported. A recent publication reviewed the effectiveness of bromocriptine in hyperprolactinemia, acromegaly and Parkinson's disease.[98] Pergolide has



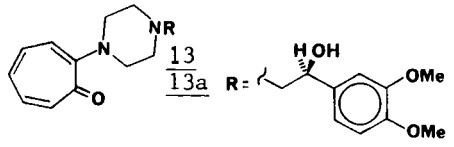
11



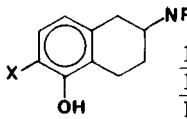
12a $R^1=R^2=Me$
12b $R^1=H$, $R^2=Me$
12c $R^1=R^2=H$

proven effective in the treatment of Parkinson's disease either alone or in combination with L-DOPA.[99-103] Pergolide is also effective in treating hyperprolactinemia.[104] Pergolide, LY141865, and mesulergine (CU32-085, 12a) controlled tremors in monkeys with ventromedial tegmental lesions, with mesulergine producing fewer abnormal involuntary movements.[105] Lisuride, another ergoline DA agonist, is well and rapidly absorbed by patients with Parkinson's disease. Wide variations in blood levels of the drug are attributed to differences in liver enzyme levels since the compound is eliminated almost exclusively by the liver.[106] Mesulergine (12a) has been demonstrated in vitro to be a DA antagonist. The desmethyl derivatives 12b and 12c, the latter of which has been demonstrated to be a metabolite of 12a in the rat, are DA agonists.[107,108] This explains the biphasic dopaminergic response observed with 12a in vivo.[109,110] Clinical studies in Parkinson's disease with 12a have shown it to be effective in controlling symptoms either alone or in combination with L-DOPA.[111,112] Mesulergine is also effective in lowering serum prolactin levels in normals, puerperea, and prolactinoma patients.[113]

Groups from both Merck[114] and Roussel-Uclaf[115] have published their work on the oxaergolines 8. The most active analog, 8 (R=Pr), has potency approximately equivalent to that of pergolide. The SAR of a series of troponylpiperazines 13 has been published.[116] AY-27110 (13a) has been found to be active in a variety of assays of dopaminergic activity.[117] LY141865 ((±)-7) was the first known selective D-2 agonist. Recent studies indicate that the previously reported[118] H-2 agonist activity of LY141865 is not significant at physiologically relevant doses.[119] The hypothesis that the activity of aminotetralin 14a is due to metabolic activation was supported by the finding that 14b and 14c are DA



13
13a R=



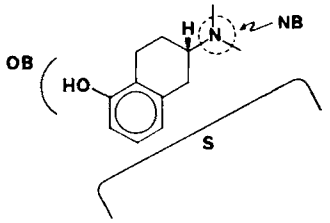
14a X=CH_3
14b X=CH_2OH
14c X=CHO

agonists.[120] A QSAR study of hydroxylated aminotetralins has been published.[121] The most important predictor of dopaminergic activity is the presence of a 5-hydroxy substituent. According to these QSAR calculations, at least one propyl group on the nitrogen is required for optimal activity.

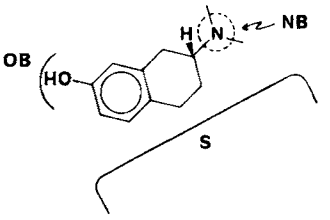
Models of the D-1 and D-2 receptors - Several reviews outlining the structural requirements for agonist activity at DA receptors have been published.[9,11-14] Current models of both the D-1 and the D-2 receptors are based on a model proposed by McDermed, et al in their studies of the dopaminergic activity of some aminotetralins.[122] The principle features of this model (see Figure) include binding sites for the basic nitrogen and the "meta hydroxy" of the aminotetralins (denoted NB and OB,

respectively) and a zone of steric intolerance (denoted S). The Figure displays the interaction of S-5-hydroxy-2-aminotetralin and R-7-hydroxy-2-aminotetralin (the enantiomers which possess DA agonist activity) with the proposed receptor model.[122]

FIGURE



S-5-hydroxyaminotetralin bound to model of DA receptor



R-7-hydroxyaminotetralin bound to model of DA receptor

Summary and conclusion: Significant advances have been made in the study of DA receptors and the agents which act at them. The receptor classification scheme now appears to be much simpler than was previously believed. Progress has been made in defining the biochemical steps leading from receptor activation to elicitation of a physiological response. The development of new techniques to detect in vivo, site-specific dopaminergic activity and the development of new, highly selective, dopaminergic agents is expected to lead to a greater understanding of central dopaminergic systems and to the effective treatment of DA-related CNS disorders.

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Chapter 6. Cotransmitters in the CNS

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Introduction - The traditional concept of neuronal transmission involving the release of a single neurotransmitter from a nerve terminal has served as the underlying principle of CNS drug research in the past. More recent investigations, however, have revealed numerous examples of the coexistence of two or more neuroactive substances in a single neuron. Evidence now suggests that, in particular, the coexistence of classic neurotransmitters with neuropeptides in the same neuron may be a very common occurrence, and as a result, a refined concept of neurotransmission may involve an intricate balance of the regulatory effects of two or more agents synthesized, stored and released from the same nerve ending. Recent reviews on the chemical anatomy of the brain[1], peptidergic neurons[2,3,4], and the interrelationship of neurotransmitters[5] have described the localization of peptides and classic transmitters in the peripheral and central nervous systems. Other reviews have concentrated on the coexistence of multiple transmitters in single neurons and the possible functional significance of this coexistence.[6,7,8]

This review will emphasize examples of the coexistence of neurotransmitters in the CNS, though many examples can be found in the periphery as well. It should be made clear that the term "cotransmitter" as used here is intended to be generic and does not imply functional distinctions from alternative terms such as "neuromodulator" or "neuroactive substance." We have chosen to highlight certain transmitter pairings because of their potential relevance to disease states or drug actions (e.g. dopamine/CCK, acetylcholine/somatostatin) or because of the substantial number of studies bearing on possible functional interrelationships between the cotransmitters (e.g. serotonin/substance P/TRH, dopamine/CCK). We have also included mention of GABA/benzodiazepines; even though there is no evidence to date for coexistence of GABA with the putative endogenous benzodiazepine ligand, this system illustrates the type of functional interactions which might apply to other cotransmitter pairs as well. The information contained in Table 1 more broadly catalogs cotransmitter occurrence and will serve to direct the reader to pertinent references in areas which are not dealt with here in detail. It should be stated at the outset that there are currently few answers to several key questions in this rapidly growing area including – (1) How do the neurons with coexisting transmitters differ structurally and functionally from neurons which contain each transmitter separately? (2) Are the levels or release of each cotransmitter independently regulated in response to varying physiologic demands? (3) Are interactions between cotransmitters pre- or postsynaptic (or both) in a given case? (4) Is the effect of one cotransmitter relative to the other inhibitory, excitatory, "modulatory", trophic, or perhaps no effect at all? This review will hopefully help identify areas for future research on such topics which may suggest new directions for novel CNS drug design and improved therapies.

Experimental techniques – Since the elucidation of the coexistence of neurotransmitters relies heavily on visual identification using the techniques of immunohistochemistry, a few methodological comments would seem appropriate. Various aspects of this rapidly developing field have been reviewed[1,4,6] with recent reports of refined techniques of radiolabelled monoclonal antibodies,[9] ultrastructural localization[10] and double immunogold-staining.[11] The antibodies used for establishing transmitter coexistence are usually raised against the transmitter itself (particularly in the case of peptides) or against enzymes involved in neurotransmitter metabolism (e.g. choline acetyl transferase for acetylcholine or phenyl-N-methyl transferase for epinephrine). Most commonly, adjacent tissue thin sections are alternately stained for each antigen. Although a powerful tool for the detection of coexisting agents, immunohistochemical techniques suffer from limitations in specificity and sensitivity.

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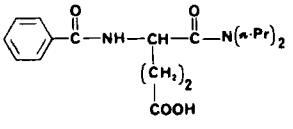
The cross-reactivity of antibodies limits the specificity of the technique, particularly in the case of peptides, where antigens structurally related to but not identical with the immunogen may exist. A limitation in sensitivity results from the high intraneuronal concentrations required for immunohistochemical visualization. Failure to detect immunoreactivity of certain neurons, therefore, does not preclude the existence of targeted neurosubstances in low concentrations. A common methodologic varation is to combine immunochemical and enzymatic analyses with surgical or chemical (e.g. 6-OH-dopamine) lesioning or depletion (e.g. p-chlorophenylalanine) to demonstrate concurrent reduction in two or more transmitters (or their receptors). Again, results must be interpreted with caution; differences in intraneuronal compartmentalization could result in differential sensitivity to chemical depletion, and transsynaptic effects resulting from altered input after lesioning may also occur.

Dopamine – One of the most extensively studied cotransmitter relationships is that of dopamine (DA) with peptides related to cholecystokinin (CCK), a 33 amino acid peptide originally isolated from the GI tract where it induces pancreatic enzyme secretion. CCK is present in high concentrations in many brain regions, primarily as a sulfated octapeptide (CCK8). Using indirect immunofluorescence histochemistry together with retrograde tracing of fluorescent dyes, CCK8 was shown to coexist with DA in the cell bodies and nerve terminals of a subpopulation of mesencephalic neurons.[12,13] Cells displaying this relationship are concentrated in the ventral tegmental area (VTA) and parts of the substantia nigra and project to limbic structures (the medial nucleus accumbens (NAc) and the central amygdaloid nucleus). There is no evidence for mesencephalic DA/CCK projections to the striatum.[13,14,15] This finding has aroused particular interest in light of the postulated involvement of DA in the pathophysiology of schizophrenia and recent evidence suggesting that the therapeutic actions of antipsychotic drugs may involve a reduction in DA function of mesolimbic and mesocortical, but not nigrostriatal, pathways.[16] Thus, CCK may have a role in both the etiology and treatment of schizophrenia which could be related to its coexistence with DA. These concepts have fueled intensive recent study of the functional relationship between DA and CCK, including biochemical, behavioral, electrophysiological and clinical investigations. Some of these have been summarized in a recent article.[17] Metabolic evidence for DA/CCK coexistence in mesolimbic structures has been shown in a study reporting a 36% decrease in the level of CCK8 after i.p. treatment of rats with reserpine in regions where coexistence has been determined histochemically (posterior NAc) but not in regions where DA and CCK exist only in separate neurons (anterior NAc, striatum).[18] Studies combining lesions with radioimmunoassay measurements, however, confirm the fact that not all the CCK and DA present in regions of known DA/CCK coexistence (e.g. the medial NAc) is present in DA/CCK neurons. A substantial amount may be present in separate neurons as well.[15] Such a complex situation, which is probably not unique to DA/CCK and is likely to apply to other cotransmitter pairs as well, obviously complicates interpretation of functional studies in relation to the coexistence phenomenon *per se*, particularly when substances are administered intracerebroventricularly (i.c.v.) but even when a specific nucleus (or sub-nuclear region) is targeted. Work summarized here will include studies of DA/CCK interactions in the mesolimbic system (e.g. the NAc) but will not deal with the large number of studies focussed on other regions, principally the striatum, since only the former pertains to the issue of true coexistence of transmitters in single neurons.

Not surprisingly, functional results have been conflicting. Two general hypotheses have emerged, one holding that CCK8 and DA are functional antagonists in the mesolimbic system and another that CCK8 facilitates DA function. Clearly, these have very different implications for the therapeutic consequences of pharmacologically-modulating DA/CCK interactions. In support of the functional antagonist theory, CCK8 injected into the dorsal medial (but not lateral) NAc increased neuronal firing, while DA reduced spontaneous and CCK8-elevated firing.[19] Furthermore, CCK8 applied to the NAc suppressed K^+-induced DA release *in vivo*.[20] Behaviorally, CCK8 injected into the VTA (cell bodies) or NAc (terminals) was reported to block the behavioral effects of a systemically administered DA releaser, amphetamine.[21] In a number of studies CCK8 was administered either systemically[22-24] or directly in to the NAc[24,25] and was found to exhibit neuroleptic-like effects (although in some cases both the sulfated and non-sulfated forms were active even though the sulfate group is generally believed to be required for most actions of CCK8). One of these studies suggests that CCK may be acting to produce DA

autoreceptor supersensitivity (similar to that produced by chronic neuroleptic treatment?), possibly resulting in decreased DA release from limbic terminals.[22] In contrast to the above studies, others have found that i.c.v. CCK8 decreased DA turnover only in areas where it does not coexist with DA and found no behavioral effects.[26] Another recent study injected the amphibian CCK analog ceruletide into the NAc and found no effect on resting or K^+-stimulated DA release, on spontaneous locomotion or on hyperlocomotion from DA injected in to the NAc.[27] A similar lack of effect on DA turnover and behavior has been reported elsewhere.[28] While the validity of the functional antagonist theory for coexisting DA and CCK remains to be established, other studies have suggested that enhanced CCK activity may be involved in the therapeutic actions of neuroleptic drugs. Thus, chronic, but not acute, treatment with haloperidol increased the number of CCK binding sites in mesolimbic but not striatal areas,[29] and chronic haloperidol, chlorpromazine or clozapine all increased CCK levels in both brain regions.[30] Furthermore, decreased levels of CCK in the cerebrospinal fluid[31] and postmortem brain regions[32,33] of schizophrenic patients have been reported. A number of clinical studies have directly addressed the possibility of antipsychotic action following CCK (or ceruletide) administration with mixed results. Both single[34,35] and multiple dose[36] treatment regimens have been claimed to produce significant and long-lasting (2 weeks) improvements in schizophrenic patients. Another reported improvements at 24 hours but worsening thereafter.[37] A recent placebo-controlled, double-blind crossover trial in neuroleptic-resistant schizophrenics found no effect of multiple doses of ceruletide or of continuous infusion of CCK.[38]

In contrast to the functional antagonist theory of DA/CCK interaction in the mesolimbic system, CCK8 has been reported to activate mesolimbic neurons[39] and to enhance the suppressive effect of DA on fimbriae-evoked firing in the NAc.[40] Also, while having no effect alone, CCK8 injected into the NAc (but not into the striatum) was found to potentiate DA-induced hyperlocomotion and apomorphine stereotypy.[41] This effect was seen with low but not high doses of CCK8, leading the authors to speculate that studies where higher CCK8 doses were used may have failed to obtain this result due to depolarization blockade. *In vitro*, CCK8 lowered the affinity but increased the number of D-2 (but not D-1) binding sites in NAc membranes, suggesting a postsynaptic interaction.[42] Also *in vitro*, CCK8 has been reported to increase ^{3}H-DA release from NAc slices in response to methamphetamine (MP) but not elevated K^+; this correlated well with the ability of coinjected CCK8 to enhance the hyperlocomotion resulting from injection of MP into the NAc.[43] Overall, this group of studies suggests that a CCK antagonist might have potential therapeutic application in schizophrenia since it would block the DA-enhancing effects of CCK. Two such antagonists have been discovered by virtue of their ability to block CCK stimulation of pancreatic secretion: proglumide (**1**), a glutamic acid derivative, and benzotript (**2**), a derivative of tryptophan.[44] Proglumide is used in Europe to treat GI disorders and has been shown to block some of the electrophysiological,[45] and behavioral[19,41,47] actions of CCK8 in mesolimbic areas. In a double-blind clinical trial, however, proglumide up to 3.2 g/day did not improve schizophrenic patients.[47] New peptide antagonists of CCK (**3**) have been reported which are 20-200 times more potent than proglumide based on inhibition of gastrin-induced acid secretion *in vivo*.[48] It will be of interest to determine the CNS, and perhaps clinical, effects of these potent CCK antagonists.



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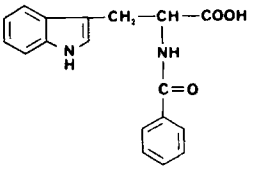

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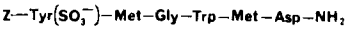

3

Another source of regulation for mesolimbic DA neurons may lie in the recent description of DA/neurotensin (NT) coexistence in the mesencephelon, hypothalamus and medulla.[49,50] A brief but broad overview of this peptide has appeared recently.[51] As with DA/CCK, mesolimbic DA/NT coexistence may also be somehow related to the pathology or drug therapy of schizophrenia. Indeed, a group of schizophrenics was shown to have decreased CSF concentrations of NT which were normalized after neuroleptic treatment and clinical improvement.[52] In rats, the consequences of i.c.v. injection of NT were similar but not identical to those of neuroleptic treatment: both decreased behavioral responses to amphetamine and increased DA turnover, but, unlike haloperidol, NT had no effect on apomorphine-induced behavior or on DA receptor binding or DA-linked adenylate cyclase activity.[53,54] As with DA/CCK, discerning the cellular mechanisms underlying mesolimbic DA/NT interactions is complicated by the existence of multiple and possibly opposing sites of action. Thus, NT injected into the VTA (location of DA cell bodies) increased DA activity while injection into the NAc (DA terminals) decreased DA activity.[55] There is evidence that the latter effect is mediated by a postsynaptic mechanism and may not be specific for DA since a DA-independent increase in locomotor activity induced by injection of an enkephalin analog into the NAc was also blocked by NT.[56] NT may thus be a general antagonist of behavioral hyperactivity in the NAc, regardless of the type of neurochemical initiation. One interesting possibility is that NT may have different effects on "normal" *versus* elevated (by intraNAc amphetamine) DA function since it is largely stimulatory in the former case while in the latter it showed 1000 fold greater inhibitory potency.[57] With a complete lack of pharmacological tools (stable NT agonists/antagonists), the therapeutic implications of DA/NT coexistence and its relation, if any, to mesolimbic DA/CCK coexistence remain to be elucidated.

Norepinephrine – Norepinephrine (NE) has long been known to coexist with somatostatin in the peripheral nervous system (PNS) and with enkephalin in the PNS, CNS (locus coeruleus) and the adrenal medulla.[6] Coexistence has also been demonstrated in the mammalian PNS and CNS with avian- and bovine-pancreatic polypeptide-like material.[58] This material has more recently been isolated as a 36 amino acid peptide and named neuropeptide Y (NPY).[59,60] In the CNS, NE/NPY (and possibly epinephrine/NPY) coexistence has been detected in the locus coeruleus and the medulla, where it may be involved in the regulation of blood pressure and other autonomic functions.[61] Intracerebroventricular injection of NPY has been reported to have marked hypotensive action which mimics that of adrenergic agonists.[62] Evidence of a functional interaction between NE and NPY also comes from studies in the periphery where NPY has been shown to enhance greatly the adrenergically-mediated contractile response of blood vessels to electrical stimulation (probably by a postsynaptic mechanism)[63] and to inhibit the electrically-induced contraction of rat vas deferens, possibly due to presynaptic inhibition of NE release.[59] An interesting suggestion regarding the molecular basis of NE/NPY interactions has been made whereby the C-terminal Try-NH_2 of NPY may adopt a catechol-like configuration resulting in adrenergic receptor affinity.[58]

Serotonin – An area of substantial research has been the investigation of the coexistence of serotonin (5-HT) with substance P (SP) and thyrotropin-releasing hormone (TRH) both in the peripheral[64,65] and central nervous systems.[9,66-75] The majority of investigations have focussed on the coexistence of 5-HT and SP in the medulla oblongata (rat and cat), primarily in the raphe nuclei and its projections into the medulla pons and spinal cord. Interestingly, entire serotonergic neuron populations within this region do not share this coexistence with SP.[1] For example, the lower medullary 5-HT neurons in rats contain SP but the dorsal raphe does not. Similarily, double-staining experiments have shown the raphe nuclei and caudal nucleus in cats to be rich in coexisting SP and 5-HT neurons while within the ventrolateral rostral cell group of the medulla, neurons contain SP only.[72] The heterogenous distribution of similar 5-HT/SP neurons is further complicated, as seen in the raphe nuclei (rat), by the coexistence of a third agent, TRH, in a subpopulation of SP/5-HT containing neurons.[1]

The functional significance of the coexistence of 5-HT, SP and TRH in the CNS, as with all cotransmitters, remains unclear and speculative.[1,9,76] The possible functional role of this example of coexistence has been reviewed and is best summarized as the overall enhancement of 5-HT transmission by the two neuropeptides.[1] The 5-HT effect is amplified by the interaction

of SP at the presynaptic autoreceptor thus preventing the suppression of 5-HT release by 5-HT itself. The 5-HT transmission is further enhanced by co-released TRH, presumably by direct interaction of TRH on the postsynaptic receptor. This concept of enhanced 5-HT transmission has been further supported by studies of the stretch reflex in rat,[77] the release of 5-HT from rat brain slices[78]and the effects of coadministration of these three cotransmitters on the sexual behavior of male rats.[79] More recent studies have also examined the functional roles of SP, TRH and 5-HT.[80-82] The report of SP-induced analgesia coupled with 5-HT depletion[80] and the effects of p-chlorophenylalanine (a 5-HT depleting drug) on SP levels in rat brain[81] lends further credence to the direct physiologic relationship between 5-HT and SP systems.

Acetylcholine - Numerous examples of the effects of neuropeptides on peripheral cholinergic systems have been reported including the potential coexistence and functional interactions of somatostatin,[83,84] CCK,[84] substance P[85,86] and NT[87] with acetylcholine (ACh) in the guinea pig myenteric plexus of the small intestine. Other studies have examined cotransmission and cholinergic mediation of secretion in the cat submandibular gland.[6,88-91] Lundberg and Hokfelt have reviewed the parasympathetic and sympathetic innervation of this gland with special emphasis on the functional significance of coexistence of ACh, vasoactive intestinal polypeptide (VIP) and neuropeptide Y (NPY).[6] In parasympathetic neurons, ACh coexists with VIP and the secretory effects of ACh are enhanced by the co-release and vasodilatory effects of VIP. In contrast, NPY is co-released in the parasympathetic neurons of the salivary gland, resulting in vasoconstriction and an overall reduction of salivary secretion. Electrophysiology studies[6] have further shown the subtle dynamics of low or high frequency nerve stimulation on the storage and release of these cotransmitters. More recent work suggests that VIP may not be the only mediator of the observed vasodilation in the cat submandibular gland and that a totally new mediator or mechanism of vasodilation may exist.[92]

Until quite recently, few reports had appeared on the coexistence of ACh with other transmitters in the CNS. Studies of the localization of VIP,[93] SP,[86] somatostatin[93] and CCK8[93] in the brain and their effects on ACh release are suggestive, but do not conclusively demonstrate, coexistence in the same neuron. A recent report on the coexistence of ACh with somatostatin, however, represents the first description of this pairing in the mammalian CNS (cortex) with direct functional implications in the regulatory mechanism of the normal brain and diseases such as senile dementia of the Alzheimer's type (SDAT).[94] Sequential immunohistochemical staining of rat cerebellum cell-cultures demonstrated the coexistence of acetylcholinesterase activity (indicative of ACh) and somatostatin. This intriguing discovery, together with previously reported decreases in cholinergic activity[95,96] and somatostatin levels[97,98] in the cerebral cortex and hippocampus of SDAT patients, has direct implications for the further elucidation of the pathophysiology of SDAT.[94]

GABA - The inhibitory neurotransmitter γ-aminobutyric acid (GABA) continues to be the focus of many investigations due to its implicated role in anxiety and other disorders such as schizophrenia, Huntington's chorea and Parkinson's disease. As with other classic neurotransmitters, GABA has been shown to coexist with other transmitters and neuropeptides in single neurons. The application of autoradiography (^{3}H-GABA and ^{3}H-muscimol) and immunohistochemical detection of glutamic acid decarboxylase (primary enzyme for the production of GABA) and GABA-transaminase (major metabolic enzyme) has demonstrated the coexistence of GABA with 5-HT in the raphe nuclei,[10,99-101] somatostatin in thalamic neurons[102,103] and hippocampus,[104] and motilin and taurine in the Purkinje cells of the cerebellar cortex.[76,105] Functional studies of the latter set of cotransmitters have been reviewed, focussing on the possible functional significance of this pairing to nerve transmission in the cerebellar cortex.[76,105]

Studies have also focussed on the regulation of GABAergic transmissions by the family of benzodiazepine anxiolytics.[106] The benzodiazepines are by far the most investigated examples of a drug which may act by modulating the effects of an endogenous cotransmitter (whose receptor is marked by benzodiazepines) as a means of enhancing the classic neurotransmitter's (GABA) action.[107,108] Evidence for the coexistence of an endogenous ligand for the benzodiazepine receptor with GABA continues to grow with recent reports and reviews on its

Table 1. Cotransmitters in the Peripheral and Central Nervous Systems.

Cotransmitters*		Location	Refs
Dopamine	- CCK	Vent. tegmental area	12,13
	- Neurotensin	Vent. tegmental area	49,50
	- Enkephalin	Carotid body	134
Norepinephrine	- Somatostatin	Sympathetic ganglia	133
	- Neurotensin	Adrenal medulla	135
	- Enkephalin	Superior cervical ganglia	136
	- NPY	Medulla oblongata, locus coeruleus	61
Epinephrine	- Neurotensin	Medulla oblongata	50
	- NPY	Medulla oblongata	61
Serotonin	- SP	Medulla oblongata	9,66-75
	- SP and TRH	Medulla oblongata	9,66-75
	- Enkephalin	Medulla oblongata, pons	137
Acetylcholine	- Somatostatin	Cerebral cortex, myenteric plexus	83,84,94
	- CCK	Myenteric plexus	84
	- VIP	Cerebral cortex, myenteric plexus	88-91
	- SP	Pons, myenteric plexus	85,86
	- Neurotensin	Myenteric plexus	87
GABA	- Somatostatin	Thalamus, hippocampus	102-104
	- Motilin	Cerebellum	105
	- Motilin and taurine	Cerebellum	105
	- Serotonin	Medulla oblongata	99-101
Somatostatin	- CCK	Submucosa plexus	138
	- APP	Cerebral cortex, hippocampus	128,139
	- Enkephalin	Median eminence	140
Substance P	- CCK	Dorsal root ganglia, caudal gray area	129,130
	- leu-Enkephalin	Avian ciliary ganglia	132
	- met-Enkephalin	Myenteric ganglia	131
Enkephalin	- APP	Spinal cord	115,141
	- Neurophysin	Posterior pituitary	142
	- Oxytocin	Neurophyses	143
Met^5-enkephalin	- MEAP	Pituitary	127
ACTH	- β-Endorphin and β-LPH	Pituitary, hypothalamus	120-125
	- β-Endorphin	Myenteric plexus	144
β-Endorphin	- β-LPH	Hypothalamus	120
	- MSH	Pituitary	126
Dynorphin	- Vasopressin	Hypothalamus	145
	- α-Neoendorphin	Hypothalamus	146
VIP	- Peptide histidine isoleucine	Autonomic ganglia	147,148

*Abbreviations used for all entries are those described in the text.

identity,[107,110,111] the heterogeneity of the benzodiazepine receptor,[112-114] and the selective regulation of the receptors by various benzodiazepines as a means of preferentially inhibiting or potentiating GABAergic synaptic function.[109,112] Investigations to date have thus demonstrated that benzodiazepines and GABA modulate neuronal transmission at postsynaptic sites with overall potentiation of the GABAergic inhibitory effect. Functional studies suggest that the putative endogenous cotransmitter may function as a mediator of GABA activity by down-regulating the postsynaptic high-affinity receptor for GABA. Benzodiazepines may in turn function as specific antagonists of the endogenous cotransmitter, thus effectively enhancing overall GABAergic transmission.[107]

Coexistence of peptides – The known examples of coexistence are by no means limited to the pairing of classic transmitters with peptides in single neurons. Investigations of the regional localization of peptides have demonstrated a wide distribution of numerous peptides in the CNS with substantial overlap in regions such as the pituitary and the hypothalamus.[4,115-119] Recent reports have clearly demonstrated that this overlap represents coexistence in single neurons (a complete summary is listed in Table 1). Early investigations focussed on the coexistence of peptides which potentially were derived from a common biosynthetic precursor, pro-opiomelanocortin (POMC). The peptides adrenocorticotropin (ACTH), β-endorphin and β-lipotropin (β-LPH) were found to coexist in single neurons both in the pituitary[120] and the hypothalamus,[121-125] thus substantiating their common generation from POMC. An extensive report of the biosynthesis, anatomy and distribution of the POMC system by O'Donohue and Dorsa described the coexistence of another POMC-derived peptide, melanocyte stimulating hormone (MSH), with β-endorphin in the intermediate lobe of the pituitary.[126] Of particular note, the authors address the functional role of this example of coexistence and present a working model which encompasses the synthesis of MSH and β-endorphin in the same neuron and the effects of the co-release of these compounds and their precursors on the postsynaptic endorphin and MSH receptors. Another example of the coexistence of peptides derived from a common precursor has been described for enkephalins.[127] The opioid heptapeptide Met^5-enkephalin-Arg^6-Phe^7 (MEAP) has been shown to coexist with Met^5-enkephalin in the pituitary and has been postulated to have several possible roles either as a biosynthetic precursor to Met^5-enkephalin, as a cotransmitter with Met^5-enkephalin, or as a single transmitter in neurons where Met^5-enkephalin is not produced. The further elucidation of the independent role of MEAP and the significance of its coexistence with Met^5-enkephalin awaits further studies of the biosynthesis of this peptide and Met^5-enkephalin from the common precursor preproenkephalin A and the investigation of the postsynaptic effects (physiological significance) of MEAP.[127] More recently, peptides of different biosynthetic origins have been found to coexist in single neurons. Of particular note is the coexistence of somatostatin with avian pancreatic polypeptide (APP),[128] SP with CCK,[129,130] and SP with enkephalins.[131,132] The functional role of most peptide coexistence, as with all cotransmitters, is unknown. The ubiquitous distribution of peptides both in the peripheral and central nervous systems and their potential implication in neurological diseases may provide opportunities in future drug research for the selective regulation of peptide-modulated neurons.[117]

Conclusion – The significance of the existence of cotransmitters in the CNS awaits further detailed functional studies of the biochemical and physiological effects of cotransmission. Future directions of drug discovery can no longer focus on the modulation of a single transmitter but must address the integrated effects of multiple transmitters in each synapse.

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Section II - Pharmacodynamic Agents
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Chapter 7. Antihypertensive Agents

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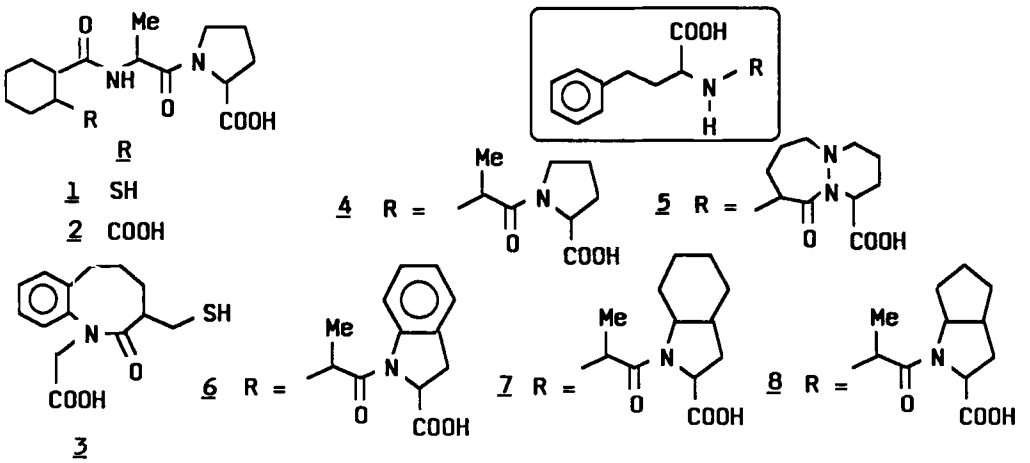
Introduction - The role of dietary factors in human essential hypertension is still an issue. A survey of over 10,000 subjects revealed that hypertensives had a significantly reduced intake of Ca^{++}, K^{+} and vitamins A and C compared to normotensives.[1] Lower Ca^{++} consumption was the most consistent factor in hypertensives. Interestingly this study found an inverse relationship between dietary Na^{+} and high blood pressure. A positive correlation between free cytosolic Ca^{++} in platelets and blood pressure has been described with hypertensives showing a 63% greater platelet calcium concentration compared to normotensives.[2] Antihypertensive therapy with calcium entry blockers, beta blockers, or diuretics lowered platelet Ca^{++}.[3] Altered handling of Ca^{++} by vascular smooth muscle in hypertension has been reviewed.[4]

Inhibition of the Renin-Angiotensin System - The present status of renin inhibition is reviewed in Chapter 26. Angiotensin converting enzyme (ACE) inhibition is a safe and effective mechanism of antihypertensive therapy.[5] Conformationally-constrained dipeptides 1 and 3 appear to be more potent than captopril *in vitro*, whereas 2 is about 100 times less potent.[6,7] A number of carboxyalkyldipeptides modelled after enalapril (4) have been described. Bicyclic compound 5 as a diacid is equivalent in potency to the diacid of enalapril *in vitro*, and as a prodrug is well-absorbed in rats.[8] Compound 6 has been taken to man and oral doses of 1, 2, or 5 mg



inhibited plasma ACE for up to 72 hrs.[9] The perhydroindoline 7 showed *in vitro* potency similar to 4 and inhibited an angiotensin I (AI) pressor response in dogs for over 24 hrs after 1 mg/kg.[10] Hoe 498 (8) has intrinsic activity similar to 4 but is 4-10 times more effective in rats and dogs after oral administration.[11] Compound 8 was 100 times more active in SPSHR than was 4 and it normalized blood pressure in

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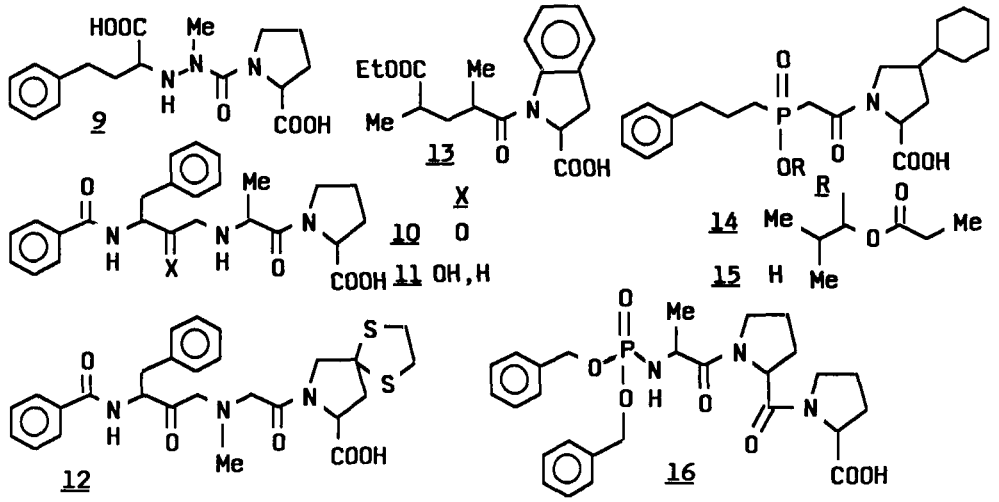
renal hypertensive dogs after 5 daily doses of 1 mg/kg.[12] Compound 8 was found to cause persistent inhibition of tissue ACE relative to 4 and this may contribute to greater antihypertensive efficacy.[13] Compound 8 given to human volunteers at 10 mg caused serum ACE inhibition which persisted for 14 days.[14] Using radioactive 8, it was found that 56% of an oral dose was absorbed and the main metabolite was the active diacid.[15] Compound 8 was effective in hypertensive patients at doses of 1, 2, or 4 mg bid.[16] Azapeptide 9 is about 20 times less potent than the diacid of 4 demonstrating that the basic amine nature of the nitrogen of 4 is not essential for tight binding to ACE.[17] Compounds 10, 11, and 12 are tripeptide analogs which are among the most potent ACE inhibitors described that do not possess a strong Zn ligand.[18,19] Aminoalcohol 11 is thought to be a transition state analog.[20] Compound 13 is intrinsically more potent than captopril but much less active *in vivo*.[21,22]



Compound 14 (SQ 28,555) is the prototype of a new class of ACE inhibitors in which the Zn ligand is a phosphinic acid. Compound 14 is a prodrug which is deesterified *in vivo* to yield 15 which is twice as potent as captopril *in vitro* and longer lasting *in vivo*.[23,24] Compound 14 produced AI inhibition in man similar to that seen with captopril but of a longer duration.[25] Compound 16 is a phosphinoyl peptide which in spite of weak potency as an ACE inhibitor caused marked long-lasting antihypertensive effects in SHR.[26]

Attempts to interrupt the renin angiotensin system by antagonism of the effects of angiotensin II (AII, 17) have been made. Compound 18 possessed about 10% of the antagonistic potency of Sar-Ile^8-AII in the rat uterus.[27] Compound 19 antagonized the effects of AII both *in vitro* and *in vivo*.[28]

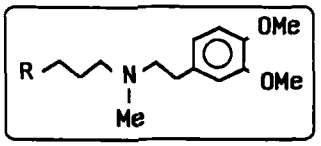
17 Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

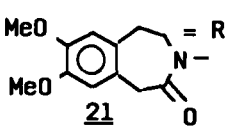
18 Cys-Arg-Val-Tyr-Ile-His-Pro-Cys

19 Ser-Arg-Val-Tyr(Me)-Ile-His-Pro-Phe

Calcium Entry Blockers - Several pertinent reviews have appeared emphasizing the molecular properties of calcium entry blockers (CEB)[29], correlations between receptor binding and pharmacology[30], mechanisms of Ca^{++} flux in smooth and cardiac muscle[31] and the relevance of Ca^{++} channels in hypertension.[32]

Compound 20, a carboxylic acid analog of verapamil, is only 1/10 as potent in inhibiting slow action potentials in guinea pig papillary muscle.[33] The benzazepinone 21 (UL-FS-49), a new fused-ring analog of verapamil related to AQA-39 (22), has been shown to be 20 times more potent than 22 in producing a bradycardic effect in guinea pig atria and 15 fold more selective for this tissue over rabbit aorta.[34] AQA-39,





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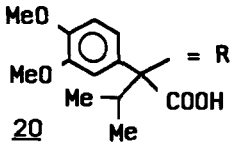

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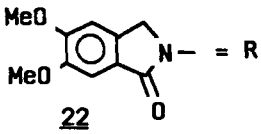

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long characterized as a specific bradycardic agent, has been shown to have a positive inotropic effect on puppy ventricular trabeculae which may be attributable to an antimuscarinic effect of the drug.[35]

A new analog of prenylamine (23) with pharmacology very similar to prenylamine and verapamil has been reported.[36] A detailed paper on the pharmacology of HA 1004 (24) has appeared. This compound is approximately 100 fold less potent than verapamil *in vitro* and is relatively non-specific, blocking contractions of rabbit aorta induced by K^+, histamine, phenylephrine, angiotensin II, 5-HT and PGF_{2a}.[37]

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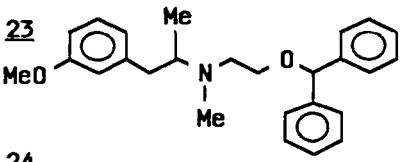

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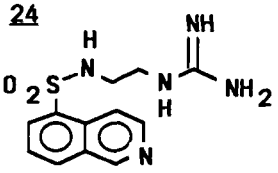

The perfluorophenyldihydropyridine 25 (WY-44,705) has been shown to possess 4-fold less atrial depressant activity than nifedipine and is hypotensive orally in SHR with an increased duration of action.[38] A series of new cyclopropyl and 2-ketopropyl esters of dihydropyridines (DHP) including 26 and 27 have been reported to have activity similar to felodipine in SHR and a model of regional blood flow in dogs.[39] Felodipine (28) has been shown to be 1000-fold more potent than nifedipine in preventing Ca^{++} stimulated contraction of denervated rat mesenteric resistance vessels and more selective against the phasic part of the contractile response.[40] This compound lowers blood pressure in man with a long duration that may make once a day dosing feasible.[41] Dosing in man is accompanied by reflex tachycardia that can be prevented by simultaneous administration of a ß-blocker.[42] Studies in anesthetized cats and dogs have appeared that suggest absence of myocardial depression and vasodilator activity of a long duration for PN 200-110 (29).[43]

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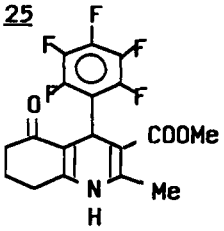
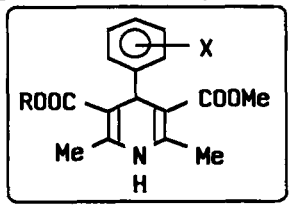


Because of their potential as pharmacological tools, calcium agonists BAY K-8,644 (30) and CGP-28,392 (31) have received close scrutiny during 1984. Specific binding sites for 30 have been documented in rat skeletal muscle[44], rat brain[45] and dog heart.[46] Single channel patch clamp studies in rat and guinea pig ventricular cells suggests 30 enhances membrane Ca^{++} transport by stabilizing the open channel state.[47] Lactone 31 enhances $^{45}Ca^{++}$ uptake by cultured neuronal cells[48] and platelets.[49] In platelets, the effect is reversed by nitrendipine, is absent when the *para*-difluoromethoxy analog of 31 is used, and is concentration dependent. Ca^{++} uptake by rabbit aorta correlates with tension development in this tissue and both effects are abolished by administration of an antagonist (PY108-068).[50] When compared on a rat heart cell preparation, 31 is 25 fold less potent than 30 at stimulating $^{45}Ca^{++}$



26 X= 3-NO_2

R=

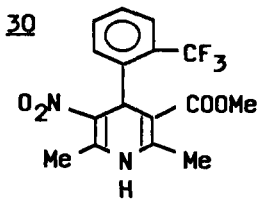
27 X= 2,3-Cl_2

R=

28 X= 2,3-Cl_2 R= Et

29 X= 2,3- R= *i*-Pr

30



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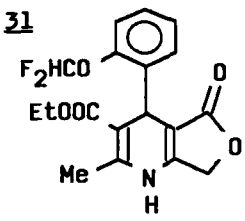
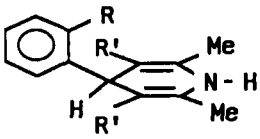


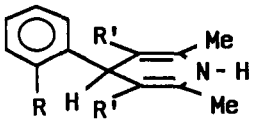
influx and the two compounds do not show additive effects.[51] In rat tail artery, both compounds are partial agonists at low concentrations (31 is 100-fold less potent than 30) and 30 is an antagonist at high concentrations.[52]

The dihydropyridines nifedipine, nitrendipine, niludipine and nisoldipine have been compared in conscious dogs for effects on baroreceptor sensitivity[53] and hemodynamics.[54] Only niludipine and nisoldipine decreased the baroreceptor response significantly. Nitrendipine was the most potent in lowering blood pressure while niludipine was least effective but also caused the lowest level of tachycardia and was the most effective coronary vasodilator. The mechanism by which these agents lower blood pressure is of major interest. Studies comparing DHP binding efficiency and PDE inhibition could show no correlation between these two activities, suggesting that elevation of cAMP is not an important part of the DHP effect.[55] Turkey gizzard myosin light chain kinase (MLCK) is inhibited by felodipine which apparently interferes with the binding of Ca^{++}-calmodulin to MLCK.[56] However, the concentrations of drug that are effective are 1-10-thousand fold higher than required to inhibit Ca^{++} flux. Variable temperature NMR experiments have shown that 2'-substituted DHPs (nifedipine) can exist in equilibrium in solution with the most favored conformation being synperiplaner.[57] Very clear binding studies in cardiac and arterial tissue have shown the receptor sites in these tissues to be the same even though the sensitivity for DHPs is very different (10,000-fold in the case of nicardipine).[58] Two different groups have reported isolation of Ca^{++} channel proteins from rabbit skeletal T-tubules.[59,60] There are three proteins, one of 130,000-142,000 daltons, and two smaller molecules (32,000-55,000 daltons) that are not covalently attached.

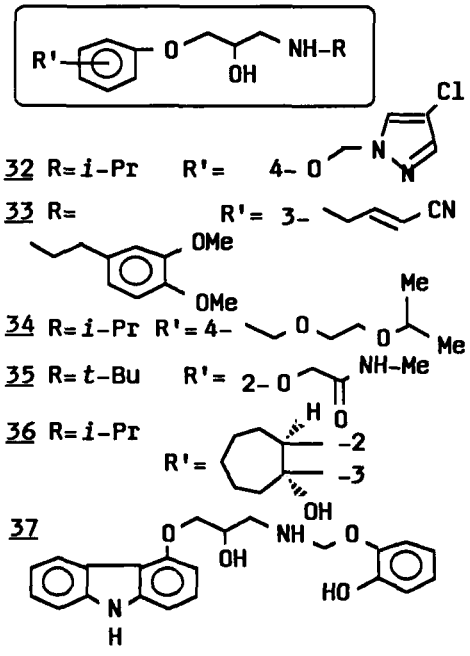
antiperiplaner

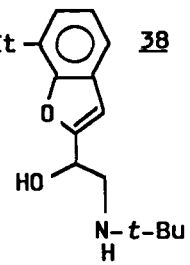


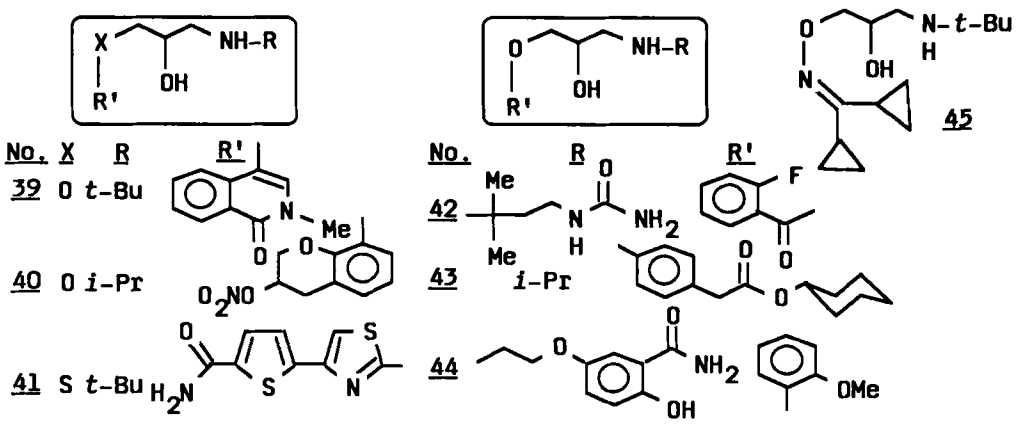
synperiplaner



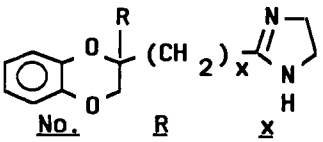
ß-Adrenoceptor Blocking Agents - Impressive $ß_1/ß_2$ selectivity ratios of >100/1, 4000/1, and 147/1 have been reported for 32, 33 (HOE 224) and 34 (bisoprolol) respectively.[61-63] Cetamolol (35) is $ß_1$ selective with no membrane stabilizing activity, whereas 36 is 8-fold more potent and 6-fold more $ß_2$ selective than propranolol.[64,65] Carvedilol (37), bufuralol (38) and N-696 (39) combine vasodilation with ß-blockade.[66-68] The pharmacological activity of 39, 40, and 41 (pA_2) has been shown to correlate with relative receptor binding (K_i) ability.[69] A metabolically unstable and thus "short acting" ß-blocker (42) has been prepared by replacing the usual ether side chain link with an ester function.[70] Ester 43 represents a second approach to this kind of ß-blocker.[71] Compound 44 incorporates an amine substituent that results in a 10-fold increase in potency compared to the usual *t*-butyl function.[72] Finally, replacement of the usual aromatic ring with a dicyclopropylimine function (45) resulted in a ß-blocker equivalent to propranolol in activity and selectivity.[73]

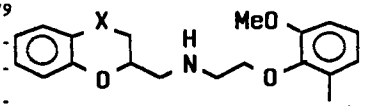


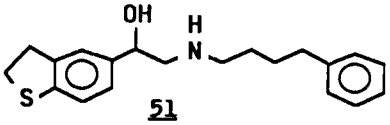


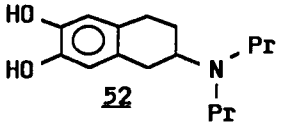


α-Adrenoceptor Agonists and Antagonists - Two reviews have appeared recently on this subject.[74,75] Isolation of $_2$ receptor proteins from pig, guinea pig, hampster and rat tissue has been achieved.[76] Reconstruction studies with this material resulted in a preparation that synthesized cGMP on stimulation by α-agonists.[77] Quantitative correlations between α_1 (^{3}H-prazosin) and α_2 (^{3}H-clonidine) binding site affinity and α_1/α_2 agonist/antagonist pharmacology *in vitro* and *in vivo* have been established.[78] The α_2/α_1 selectivity ratio of idazoxan (46) can be increased from 5 to 20-fold greater than yohimbine by substitution of the 2-position with an ethyl or propyl group (47).[79] RS-21,361 (48) is less potent but also more selective than 46.[80] In a related series, the α_1/α_2 selectivity ratio is increased to 1000 (49) from 347 (50) by substitution of sulfur for oxygen.[81] Tibalosine (51) is an α_1 antagonist that lowers blood pressure without increasing heart rate by antagonizing both central α_1 (BP↓, HR↓) and peripheral α_1 (BP↓, HR↑) receptors.[82] The aminotetralin 52 is as potent and selective an α_2 agonist as M-7 without any β adrenoceptor activity and thus a superior pharmacological tool.[83]

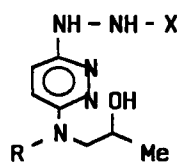


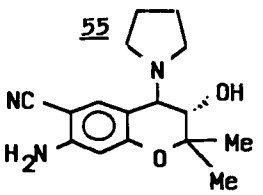




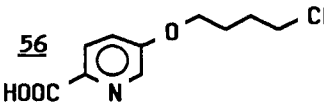


Vasodilators - Cardralazine (53) and pidralazine (54) are both structural analogs of hydralazine.[84,85] Pidralazine given daily to salt-loaded SHR at 1 mg/kg lowered blood pressure and prevented vascular lesions. Cardralazine was given to conscious dogs at 1 mg/kg *i.v.* and caused hypotension and reflex tachycardia. Regional vascular resistances were reduced for up to 4 hrs.



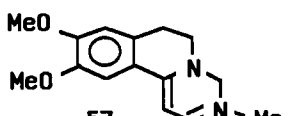


Compound 55 was the most active antihypertensive agent in a series of substituted benzopyranols.[86] Doses from 0.03 mg/kg *p.o.* lowered blood pressure in SHR by 40-115 mm Hg. Antihypertensive activity was greatest for compounds having strong electron withdrawing groups at position 6. Compound 56, a derivative of fusaric acid, lowered blood pressure in SHR, and in DOCA and renal hypertensive rats the activity of 56 was greater than

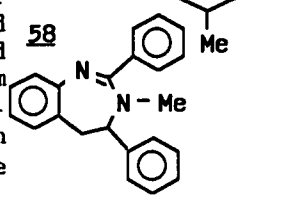


56

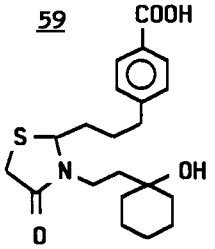
fusaric acid at doses of 10-100 mg/kg *p.o.*[87]. Trequinsin (57) was modelled after the natural plant alkaloid stepharine.[88] Trequinsin lowered blood pressure in spontaneously, renal and DOCA hypertensive rats as well as normotensive dogs. Trequinsin also increased cardiac rate. These effects are apparently a result of cyclic AMP phosphodiesterase inhibition. Trequinsin also inhibited platelet aggregation. The benzodiazepine 58 lowered blood pressure in SHR by 56 mm Hg when given in daily doses from 5-50 mg/kg for 3 days.[89] The prostaglandin analog 59 increased renal blood flow and decreased blood pressure in anesthetized dogs.[90] Compound 59 was more potent than the dopamine agonists SKF 82526 or ibopamine.



57



58



59

Atrial Peptides - During the past year there has been an explosive growth of knowledge concerning natriuretic and vasorelaxant peptides contained in mammalian atria. The peptides apparently derive from a 152 amino acid precursor[91]. The amino acid sequences of several atrial peptides are shown below.

All peptides contain a 17 amino acid central sequence enclosed by a cystinyl disulfide bridge which is necessary for biological activity.[92] Peptides 60, 61, 63, 64, 65, 66, and 67 have been iso-

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*- Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gly-Ser-Gly-Leu-Gly-Cys

Leu-Ala-Gly-Pro-Arg-Ser-Leu-Arg-Arg-Ser-Ser--*--Asn-Ser-Phe-Arg-Tyr      60

          Ser-Leu-Arg-Arg-Ser-Ser--*--Asn-Ser-Phe-Arg-Tyr                 61

                Arg-Ser-Ser--*--Asn-Ser-Phe-Arg-Tyr                       62

           Arg-Arg-Ser-Ser--*--Asn-Ser-Phe-Arg-Tyr                        63

                Arg-Ser-Ser--*--Asn-Ser-Phe-Arg                           64

                     Ser-Ser--*--Asn-Ser                                  65

                     Ser-Ser--*--Asn-Ser-Phe-Arg                          66

                     Ser-Ser--*--Asn-Ser-Phe-Arg-Tyr                      67

Ser-Leu-Arg-Ser-Ser--*(Met  for first Ile)-Asn-Ser-Phe-Arg-Tyr            68

Asn-Pro-Val-Tyr-Gly-Ser-Val-Ser-Asn-Ala-Asp-Leu-Met-Asp-Phe-
Cys-Asn-Leu-Leu-Asp-His-Leu-Glu-Asp-Lys-Met-Pro-Leu-Glu-Asp              69
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lated from rat atria. Peptide 62 has been synthesized and has natriuretic and vasorelaxant potency similar to 60.[93,94] Radioiodinated 62 binds with high affinity to membranes from rabbit aorta and kidney.[95] The amino acids on the N-terminal side of the cystinyl disulfide bridge of 62 are less important determinants of natriuretic and vasorelaxant potency than the amino acids on the C-terminal side.[96,97] Peptides 65, 66, and 67 show similar natriuretic activity in rats.[98,99] Peptide 65 is a potent relaxant of chick rectum but not rabbit aorta whereas 66 and 67 are potent relaxants of both tissues.[100] Atrial peptide 64 is natriuretic in isolated kidneys and, due to the presence of the C-terminal Phe-Arg, has been proposed as a substrate for ACE.[101] A metallo-dipeptidase has been found in bovine atria which converts 66 to 65 and is inhibited by captopril.[102] Peptide 68 has been isolated from human atrium and is similar to 61.[103,104] A peptide containing the sequence 69 has been isolated from pig atrium and is vasorelaxant but not natriuretic.[105] The vasorelaxant mechanisms of atrial peptides are not completely understood. Peptide 62 causes vasorelaxation which is associated with increases in cGMP.[106-108] Vasorelaxation is not dependent upon an intact vascular endothelium.[109] Peptide 62 resembled sodium nitroprusside

in its pattern of vasorelaxation.[110] Natriuretic rat atrial extracts did not inhibit Na^+-K^+ ATPase in toad bladder.[111] Peptides 62, 64, and 68 have been infused in anesthetized dogs. Peptides 62 and 68 produced natriuresis in the kidney near the site of infusion but not the contralateral kidney.[112,113] Peptides 62 and 64 produced natriuresis and hypotension and decreased plasma renin activity.[114,115] Rat atrial extracts or 67 were found to decrease blood pressure and heart rate in anesthetized rats.[116,117] Natriuretic peptides 63 and 66 produced selective renal vasodilatation in rats.[118,119] Peptide 62 was given to renal hypertensive rats at a rate of 1 µg/hr and reduced blood pressure and plasma renin activity to normal levels after 5 days of infusion.[120] Natriuretic peptides 62 and 64 have been shown to decrease aldosterone production by the adrenal cortex.[121-125] Radioimmunoassays have been developed for 62 and 63. In rats 63 was found in the right atrium at higher concentrations than in the left and also in plasma and hypothalmus.[126] A high sodium diet increased cardiac and plasma 63. Peptide 62 was also found in heart and plasma.[127,128] The detection of natriuretic peptides in the circulation and the demonstration of their release from isolated hearts establishes an endocrine role for the heart.[129,130] Salt-sensitive hypertensive rats have a high level of atrial natriuretic activity whereas cardiomyopathic hamsters have a decreased level.[131,132] Atrial peptides could influence blood pressure by their direct natriuretic and vasorelaxant properties as well as by decreasing renin and aldosterone production. This new regulatory system may represent a new site for therapeutic manipulation of blood pressure.

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Chapter 8. Pulmonary and Antiallergy Agents

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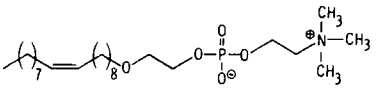
Introduction - Several encouraging results in the development of new pulmonary and antiallergic agents were reported in 1984. These included the discovery of orally active leukotriene (LT) antagonists, specific inhibitors of 5-lipoxygenase (LO) and site specific inhibitors of phospholipase A_2 (PLA_2). Of particular significance was the finding that asthmatics were hyperreactive to LTD_4 compared to individuals without a history of atopy.[1] The role of platelet activating factor (PAF) as a mediator of asthma and inflammation continues to be of importance;[2,3] for a comprehensive review on PAF see chapter 20 of this volume.

Phospholipase A_2 (PLA_2) Inhibitors - Work with bilayers containing lysophosphatidylcholine indicated that many inhibitors of PLA_2 modify the quality of substrate interface rather than interacting directly with enzyme.[4] Recently, however, specific active site inhibitors (IC_{50} 1-10μM)(e.g. 1) of porcine-pancreatic PLA_2 were described.[5] Although these compounds did not possess in vivo activity, it is encouraging that the design of specific inhibitors based on conformation and active site is possible. Also of interest are analogs of arachidonic acid (AA) methylated at C-7 (2) which inhibited PLA_2 activity from rat mast cells (RMC) and snake venom.[6,7]



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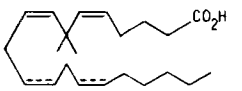

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Corticosteroids inhibit PLA_2 through the induction of antiphospholipase proteins. Despite the disparity in molecular weight, macrocortin (15 k) found in rat macrophages, lipomodulin (40 k) found in rabbit neutrophils, and renocortin (15 + 30 k) found in rat renomedullary interstitial cells are thought to be functionally similar molecules.[8] It was proposed that antiphospholipase proteins be known as lipocortins.[9] In addition, it was shown that normal human serum contains an inhibitory factor, presumably a protein, which blocks PLA_2 activity.[10] The synthesis of lipocortins by recombinant DNA technology may provide valuable corticosteroid-like agents.

Corticosteroids continue to play an important role in asthma therapy. Inhaled beclomethasone, after a decade of use in asthma, shows no evidence of tracheobronchial lining damage and potential side effects have not materialized except for oral thrush, sore throat and hoarseness which seldom necessitates discontinuation of treatment.[11]

Lipoxygenase (LO) Inhibitors - During irreversible inhibition of 5-LO from RBL-1 cells by 5,6-dehydro AA, it was postulated that a 4,5-dehydro AA hydroperoxide intermediate could undergo facile oxygen-oxygen bond

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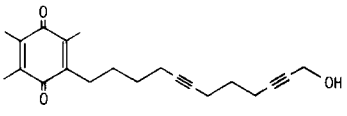
homolysis to radicals which inactivate the enzyme. Evidence for this intermediate was provided by Lineweaver-Burke analysis which demonstrated that deactivation is subject to a primary kinetic isotope effect when the inhibitor carries a deuterium 7(R) label.[12] Detailed kinetic studies with soybean LO suggested that oxygenation (presumably by hydroxyl radical) of a methionine residue at the catalytic site is the reason for deactivation by acetylenic fatty acids.[13] A single methionine is oxidized to methionine sulfoxide under conditions leading to self-inactivation with reticulocyte LO.[14]

Many of the reported 5-LO inhibitors are antioxidants or free radical scavengers and as such are generally non-specific; for example, NDGA, ETYA, BW 755c, phenidone, quercetin, and propylgallate were reported to inhibit 15-LO, 12-LO or cyclooxygenase (CO) in addition to 5-LO.[15-17] However, caution must be exercised in categorizing inhibitors as non-selective based on structure. Depending on the source of enzyme, qualitative and quantitative differences were noted with the above compounds. Furthermore, apparent antioxidants or free radical scavengers, such as AA-861 (3),[18] cirsiliol (4),[19] 6,7,4'-trihydroxyisoflavin (5),[20] and caffeic acid (6)[21] are reported to be selective inhibitors of 5-LO.



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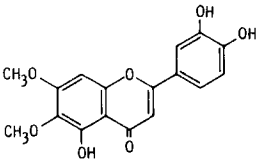

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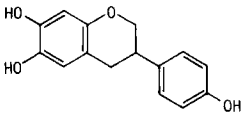

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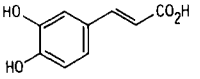

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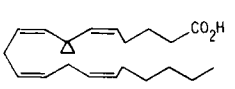
Substrate analogs continue to be of interest as an approach to the design of novel 5-LO inhibitors. The 7-ethano AA derivative 7 was reported to inhibit (86% at 100 μM) the production of 5-HETE from RBL-1 cells.[22] Several 7,13-bridged AA analogs (8) were selective 5-LO inhibitors.[23] A series of hydroxamate AA derivatives were designed as potential iron chelators; the most potent analog (9) had an IC_{50} of 0.03 μM as an inhibitor of 5-LO from RBL-1 cells.[24]



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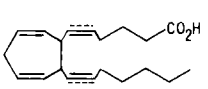

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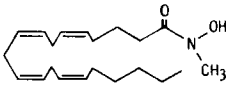

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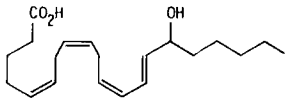
A series of aromatic ring stabilized analogs of 15-hydroxyeicosatetraenoic acid (HETE) (10) was announced as potent and selective inhibitors of rat PMN 5-LO.[25,26] Interest in 15-HETE analogs is based on reports that 15-HETE is an endogenous inhibitor of 5-LO; these reports were recently confirmed in RBL-1 cytosol.[27] The SAR of the 15-HETE series is informative and can be appreciated in terms of structures 11 and 12. In general, esters of 11 were more active than the corresponding carboxylic acids. The most potent positional isomer of 11 (IC_{50} = 0.6 μM) had a methyl ester moiety at C-2. Blocking the side chain hydroxyl group with acetyl, methyl or tetrahydropyranyl lowered activity. Other modifications, such as substituting functions

for the carboalkoxy in the A ring, reversing the ether linkage and eliminating water from the alkyl side chain also reduced activity. The incorporation of nitrogen (12) within the A ring increased 5-LO inhibitory activity. It is significant that like the carboalkoxy phenyl analogs (11), the pyridyl isomer (12) with nitrogen in the 2 position was more active than the corresponding 3 or 4 positional isomers. The most potent compound of structure 12 (R = OH, CH_3; IC_{50} = 0.1 μM) contained a quinoline ring. The in vitro profile of 12 (R = OH, H; 2-pyridyl) was indicative of the selectivity of this series. It inhibited 5-LO from rat PMNs (IC_{50} = 0.5 μM) and from human PMNs (IC_{50} = 8 μM) but was completely inactive against 12-LO from rat platelets and CO from sheep seminal vesicles.[26] Compound 12 (R = OH, H; 2-quinolinyl) antagonized LTC_4-induced contraction of GP lung in vitro and it inhibited antigen induced increase in airway resistance (73% at 100 mg/kg) when orally administered to GPs.[28]



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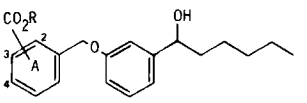

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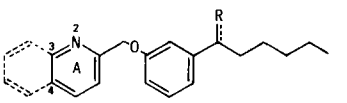
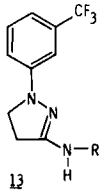


12

The primary thrust in the development of mixed CO/LO inhibitors has been the discovery of novel antiinflammatory agents. However, compounds which inhibit both enzymes can also be considered as potential anti-allergy agents. The activity of the CO/LO inhibitor BW 755c (13 R = H) was enhanced by alkylation of the exocyclic nitrogen atom, the n-Pr analog (13) being 10 fold more active.[29] CBS-1108 (14) inhibits 5-LO activity in rabbit PMNs and inhibits human platelet CO (IC_{50} for both is 2.0 μM).[30] A series of quinoline carboxamides (15) also possess potent CO/LO inhibitory activity.[31]



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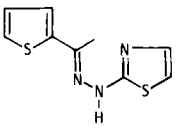

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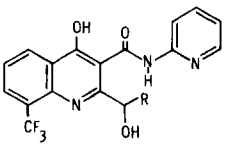


15

Leukotrienes (LT) - Comprehensive reviews on the biosynthesis, metabolism, receptors, functions, pharmacology, and clinical significance of LTs have appeared.[32-36] In addition, prospects for the inhibition of LT biosynthesis was discussed.[37] The synthesis of mono- and poly-HETEs including 8- and 9-HETEs,[38] 12-HETE,[39] LTB_4,[40] 5,15- and 8,15-diHETE,[41] and 5,6,15- and 5,14,15-triHETE (lipoxins A and B, respectively)[42] was reported. Other oxidized metabolites of AA have been prepared, such as 14,15-EPETE and LTA_4,[43] 11,12- and 14,15-EPETE,[44] and 20-OH and 20-COOH LTD_4.[45]

Several articles involving LTA_4 biosynthesis and metabolism have appeared. Studies with LO from potato tubers indicated that both 5-LO and LTA_4 synthetase activities resided in the same protein and that the formation of LTA_4 from 5-HPETE was catalyzed by the 8-LO activity of the enzyme.[46] Contrary to earlier reports, erythrocytes are capable of transforming LTA_4 into LTB_4.[47] Studies in both RBL cell homogenates and rat liver cytosol indicated that LTC synthetase is a new and distinct glutathione S-transferase (GST).[48,49] Thus, it seems possible that specific inhibitors of LTC synthetase can be discovered which do not

interfere with the GST detoxification pathway. New HETEs and their corresponding biological activities have been the subject of recent reports. Lipoxin A, stimulated superoxide anion generation (equipotent with LTB_4) and degranulation (2 orders of magnitude less potent than LTB_4) in human neutrophils without providing a substantial aggregation response.[50] In contrast, 8,15-diHETE possessed chemotactic activity for human neutrophils (comparable with LTB_4) but was inactive with respect to degranulation or generation of superoxide anion radicals.[51] However, the chemokinetic activity of 8,15-diHETE has been questioned.[52] One of the steps in the metabolic deactivation of LTs may be C-20 oxidation; the C-20 alcohol and carboxylic acid of both LTD_4 and LTB_4 were less potent in contracting GP ileum and attracting leukocytes than the unoxidized C-20 parent molecules.[53]

The heterogeneity of LT receptors was reviewed.[54] Specific binding sites for LTC_4 were found in human fetal and rat lung,[55,56] hamster and GP ileal smooth muscle,[57,58] and specific binding sites for LTD_4 were found in GP lung[59] and human alveolar macrophages.[60] A LTE_4 receptor different from either LTC_4 and LTD_4 receptors was described in GP tracheal smooth muscle and it was suggested that LTE_4 may account for airway hyperirritability.[61] LTB_4 binds to human PMNs[62] and two classes of receptors specific for LTB_4 were also described for human PMNs.[63] A receptor for the 20-hydroxy derivative of LTB_4 was also demonstrated with human PMNs.[64] The heterogeneity demonstrated for LT receptors may be exploited in the discovery of new antiasthma drugs much in the same way as the study of different adrenergic receptors has benefited cardiovascular drug development.

Evidence for the role of LTs in the pathophysiology of asthma and allergic diseases has continued to accumulate.[65,66] There are indications that human eosinophils produce a substantial amount of LTC_4 and that these cells contribute to the amplification of the allergic response.[67,68] Activation of human lung mast cells with anti-IgE leads to phospholipid turnover and to release of AA metabolites from both LO (LTC_4, LTB_4 and 5-HETE) and CO (PGD_2) pathways.[69] Inhaled LTC_4, LTD_4 are more potent bronchoconstrictors than histamine or methacholine in normal subjects.[70] LTB_4, LTC_4 and LTD_4 were found in the sputum of patients with severe asthma.[71] LTB_4 was found in the circulation of asthmatic patients[72] and elevated concentrations of LTD_4 were found in the pulmonary edema fluid of patients with adult respiratory distress syndrome.[73] Allergic subjects when challenged with antigen showed increases in LTC_4 and LTD_4 extracted from nasal secretions.[74]

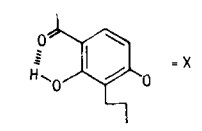
Interactions of LO and CO products were reviewed.[75,76] In GP lung strips it was shown that LTC_4 and LTD_4 stimulate production of TxA_2 and PGI_2.[77,78] In contrast, PGI_2 was reported to antagonize smooth muscle reactivity to LTC_4 and LTD_4.[79] It is significant that exposure of normal monkey airways to subthreshold doses of LTD_4 or PAF altered the airway reactivity so that a second challenge of the same or alternative agonist generates an airway response.[80]

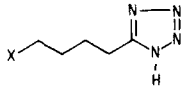
Leukotriene Antagonists - Despite limited progress in the development of LTB_4 antagonists, there has been a flurry of activity in the area of sulfidopeptide LT antagonists culminating in the development of the orally active LTD_4 antagonist, LY 171,883 (16) (Note the commonality of the moiety X in 16 - 22 based on FPL 55,712). Compound 16 is a competitive antagonist of LTD_4 on GP ilea and parenchyma (pK_B = 7.19 and 6.51, respectively); however, it is a noncompetitive LTD_4 antagonist on GP trachea and did not antagonize the effects of LTC_4 on GP ilea.[81] In contrast, it was effective against both LTC_4 and LTD_4- induced bronchospasm in vivo (GP, 30 mg/kg p.o.). The bronchospasm produced by

ovalbumin in sensitized GPs (pretreated with pyrilamine, propranolol and indomethacin) was also blocked by 16 at 30 mg/kg p.o. In addition, this compound also possesses bronchodilator activity (IC_{50} = 22.6 μM for PDE inhibition in human peripheral PMNs).

Another compound, Wy-44,329 (17), competitively antagonized LTD_4 on GP ilea (pK_B = 9.4) and inhibited the bronchoconstriction in GP induced by LTD_4, LTC_4 or ovalbumin (ED_{50} = 0.11 mg/kg, 0.17 mg/kg and 0.47 mg/kg, respectively).[82] It possesses a potency comparable to the prototype LT antagonist FPL 55,712 (18) but has a much longer biological half life (17 at 10 mg/kg given i.v. 40 min before LTC_4 challenge in GP was still effective in reducing bronchoconstriction by >50% whereas 18 was inactive). In addition 17 also was active as a mediator release inhibitor (ED_{50} = 0.26 mg/kg i.v. in rat PCA).

Several other compounds structurally related to FPL 55,712 or LTs were recently described. The tetrazole 19 gave 93% inhibition of LTD_4 -induced bronchoconstriction in GPs (5 mg/kg i.v.).[83] The bronchoconstriction induced by LTE_4 was inhibited in GPs by the carboxylic acid 20.[84] The hydroxy-acid 21 inhibited the LTD_4-induced bronchoconstriction in GPs (ED_{50} = 0.21 mg/kg i.v.).[85] The naphthotriazole 22 at 4 x 10^{-7}M gave 50% inhibition of SRS-A induced contraction of GP ilea.[86] The desamino-2-nor LT analog 23 antagonized both LTD_4 and LTE_4-induced contractions of GP trachea in an apparently competitive fashion (K_B = 0.29 μM vs LTD_4, pA_2 = 5.9 vs LTE_4).[87]





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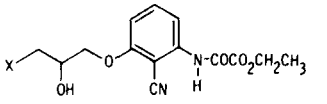

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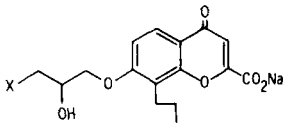

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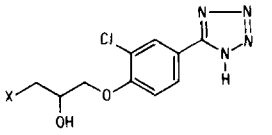

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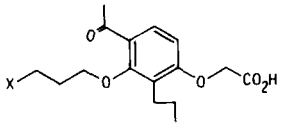

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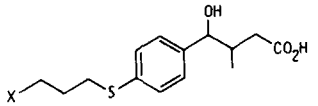

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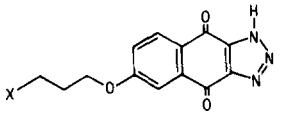

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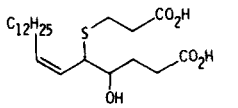

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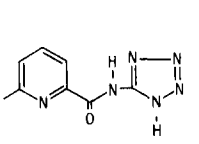
Mediator Release Inhibitors (MRI's) - Comprehensive reviews dealing with mediator release from mast cells and basophils and its pharmacological modulation have appeared.[88-90] The action of flavonoids on mediator release has been the subject of considerable study.[91-93] The mode of action of the prototype MRI, disodium cromoglycate (DSCG) remains controversial.[94] Evidence has recently appeared that the DSCG-binding protein on basophils constitutes the calcium channel which opens upon immunological stimulus.[95]

A recent review of the second generation MRIs has summarized the problems encountered in the development of an orally active analog of

DSCG.[96] Paramount among these problems is the poor correlation of activity in the rat PCA test with clinical efficacy. This has lead to a search for newer models of mediator release and for compounds that exhibit novel profiles of activity relative to DSCG, especially compounds that influence the release of LTs. Among the more traditional MRIs with novel structures are TA 5707 (24), RHC 3164 (25) and RHC 3988 (26), all of which are orally active and have activity profiles similar to DSCG.[97-99] MRIs which have a more unique biological profile include Ly-83583 (27) which preferentially inhibited antigen-induced release of LTs relative to histamine both *in vitro* (GP lung) and *in vivo* (rat peritoneal cavity).[100] By contrast, in the latter system DSCG greatly reduced histamine release relative to LT release. Unlike DSCG, tiacrilast inhibited the release of histamine, SRS-A and Tx from antigen challenged GP lung fragments, and doqualast significantly inhibited LT release in both human and GP lung fragments.[101,102] Traxanox showed a dose-related inhibition of SRS-A release in rats and GPs unlike DSCG.[103] CI-922 (28) not only inhibited the LT-mediated contraction of GP lung strips with an activity 6x proxicromil (DSCG was inactive in this model) but also the synthesis of 5-HETE.[104,105] In clinical trials, aerosolized nedocromil sodium (at 4 mg, qid) had a significant therapeutic effect on bronchial asthmatics relative to placebo.[106]



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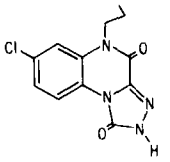

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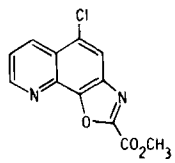

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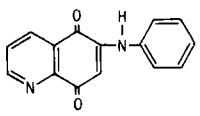

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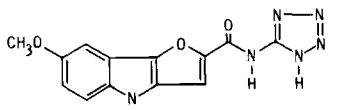


28

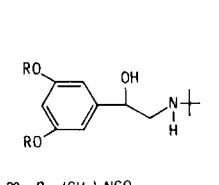
β-Adrenoceptors and Agonists - A symposium on β-adrenoceptors in asthma was published[107] and the purification and characterization of the mammalian β_2-adrenoceptor was described.[108] The presence of auto-antibodies to β_2-adrenoceptors in asthmatics was reviewed;[109] 5% of juvenile asthmatics produce these autoantibodies.[110] The concomitant use of β-adrenergic agonists, especially oral formulations, and methylxanthines remains of concern because of cardiotoxicity.[111] Bitolterol mesylate was approved by the FDA for use in asthma as an aerosol inhaler. Two lipophilic terbutaline ester prodrugs, bambuterol (29) and D2438 (30), were effective bronchodilators with a longer duration of effect than terbutaline.[112] Chemical modification of clenbuterol lead to the identification of mabuterol, a compound with long lasting β_2-agonist properties after oral administration in animals and man.[113]

Xanthines - The pharmacology and therapeutic use of theophylline was reviewed[114] and the elusive mechanism of the bronchodilator action of methylxanthines continued to be a subject of interest.[115-117] Orally administered, caffeine is an effective bronchodilator in juvenile

asthmatics.[118] Some piperazine and piperidine derivatives of theophylline, flufylline (Sgd 19578, 31) and fluprophylline (Sgd 14480, 32) possess bronchodilator and hypotensive properties with low toxicity.[119]

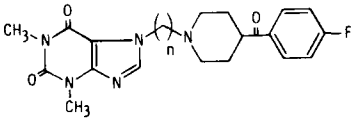
Rhinitis and its therapy - The understanding of the pathophysiology of allergic nasal disease has improved greatly[120] and there has been recent direct proof that mast cell mediators, including histamine, PGD_2 and leukotrienes, and kinins, are released during an allergic reaction.[121,122] Current therapy of rhinitis consists of H_1-receptor antihistamines, decongestants and topical antiinflammatory steroids. The H_2-antagonist, cimetidine, added to the H_1-antagonist, chlorpheniramine, was of additive benefit in the treatment of allergic rhinitis,[123] confirming earlier observations on an additive effect of locally applied H_1- and H_2-antihistamines on histamine-induced nasal airway resistance.[124] The problem of sedation limits the use of many H_1-antihistamines; however, several H_1-antihistamines with reduced sedative potential may provide advantages over current antiallergic therapies especially in the treatment of rhinitis. Astemizole possesses a long duration of action permitting once daily oral administration and is efficacious in allergic rhinitis.[125] Terfenadine is another non-sedative H_1-antihistamine but is less efficacious than astemizole for the treatment of hay fever.[126] Both astemizole[127] and terfenadine[128] offer protection against exercise-induced asthma. AHR-11325 (33),[129,130] BW825C (34),[131] SKF 93933 (35)[132] and SCH 29851 (36)[133] are also orally effective, non-sedative H_1-antihistamines in animal models. Reviews of the mechanism of action and therapeutic efficacy of ketotifen[134] and oxatamide,[135] two of the newer but sedative H_1-antihistamines, also appeared.

Intranasal fenoterol[136] was not effective in allergic rhinitis whereas double-blind trials demonstrated topical safety[137] and efficacy for ipratropium bromide in perennial rhinitis and paroxysmal rhinorrhea.[138,139] The cyclooxygenase inhibitor flurbiprofen was almost as effective as chlorpheniramine in reducing the severity of allergen-induced rhinitis.[140]



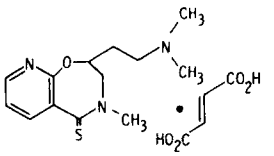
29 R = $(CH_3)_2NCO$

30 R = t-$BuCO_2$-C$_6$H$_4$-CO



31 n = 2

32 n = 3



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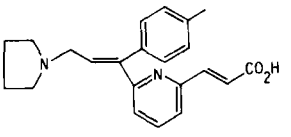

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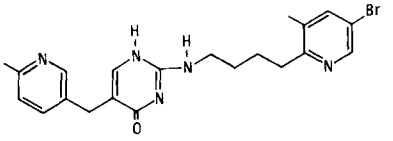

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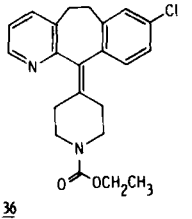
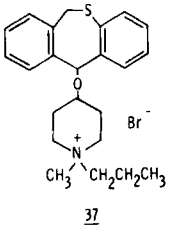


36

Anticholinergic bronchodilators - The controversial role of anticholinergic bronchodilators in airways disease was reviewed.[141] Inhaled atropine methonitrate produced bronchodilation in patients with chronic emphysema demonstrating the importance of vagal nerve pathways rather than locally released mediators in this disease.[142] AA28-263 (37), a new anticholinergic drug, was as effective as inhaled terbutaline in patients with intrinsic asthma.[143] The time to maximal effect was longer but it possessed a longer duration of action than terbutaline. Several salts of thiazinamium administered by aerosol possessed equipotent anticholinergic activity in guinea pigs but the duration of this effect varied ($Cl>Br>MeSO_4$). Changes in the alkyl side chain also affected aerosol anticholinergic potency and duration.[144] The sulfoxide metabolite of thiazinamium was equipotent with the parent but had a shorter duration of action.[145]

Miscellaneous - L-640,035 (38) is an orally effective antagonist of contractile prostanoids (PGD_2, $PGF_{2\alpha}$, PG endoperoxide) on airway smooth muscle.[146] It also inhibits allergen-induced bronchoconstriction in a strain of "hyperreactive" rats and in conscious squirrel monkeys.[147] A number of 5-thia 8,10,12-triaza prostaglandin analogs displayed aerosol bronchodilator activity in guinea pigs but only 39 and 40 showed potent oral bronchodilator activity.[148]



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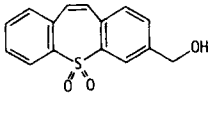

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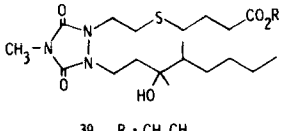

39 R = CH_2CH_3

40 R = H

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Chapter 9. Antiglaucoma Agents

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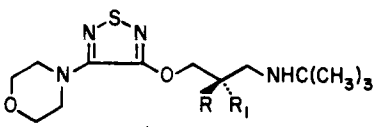
Introduction - Glaucoma, the leading cause of irreversible blindness in the Western world, is characterized by optic nerve damage associated with visual field loss and/or abnormally high intraocular pressure (IOP). Two million Americans are estimated to have glaucoma.[1] As many as ten million additional Americans are suspected to have ocular hypertension, i.e., elevated IOP without optic nerve or visual field damage.[2] Unfortunately, no means presently exists for reliably predicting which ocular hypertensive individuals will develop glaucoma. Intraocular pressure is controlled primarily by aqueous humor (AH) dynamics which, in turn, are determined by the difference in the rates at which AH enters and leaves the eye. Aqueous humor enters the posterior chamber by three physiological mechanisms - predominantly active secretion by the nonpigmented epithelial cells of the ciliary process and, to a lesser extent, ultrafiltration of blood plasma and diffusion. Newly formed AH flows from the posterior chamber through the pupil into the anterior chamber; it supplies the nutritional needs of the avascular lens and cornea and ultimately leaves the eye via filtration through the trabecular meshwork (TM) situated at the juncture of the cornea and iris and, in primate eyes, via the uveoscleral outflow pathway as well.[3] Blockade of these outflow pathways, in both the absence (primary open-angle glaucoma, the most prevalent disease form) and the presence (primary closed-angle glaucoma) of anatomical obstruction of the TM by the iris, invariably is responsible for the elevated IOP associated with glaucoma. All known antiglaucoma drugs lower IOP by decreasing AH formation and/or increasing AH elimination and, accordingly, are ocular hypotensive agents. Recent advances in the development of antiglaucoma drugs are presented below.

Adrenergic Antagonists - The ability of systemically administered β-adrenoceptor antagonists to lower IOP in man was demonstrated first with propranolol in 1967.[4] The potent local anesthetic activity of propranolol precluded its topical use. In 1977, topically instilled timolol (Ia) was reported to decrease IOP in both experimental animals[5] and glaucomatous patients.[6] Timolol is a nonspecific β-blocker which neither causes ocular irritation nor displays local anesthetic activity.[7] Topical timolol has been tolerated well by glaucomatous patients treated continuously for periods up to and including four years; no tachyphylaxis developed.[8] As noted in a recent review,[9] the clinical success of timolol has provided impetus for evaluating a large number of β-blockers for ocular hypotensive activity. Timolol is no longer the sole β-blocker used to treat glaucoma. Befunolol (2a) and carteolol (2b) were introduced recently into the Japanese market and metipranolol (2c) is marketed now in Germany. Befunolol is a nonselective β-adrenoceptor antagonist.[10] Carteolol is also a nonselective β-blocker but, unlike befunolol, possesses intrinsic sympathomimetic activity.[11] Metipranolol is a potent, nonselective β-blocker; however, unlike timolol, meti-



I

a, R=H ; R_1=OH

b, R=OH; R_1=H

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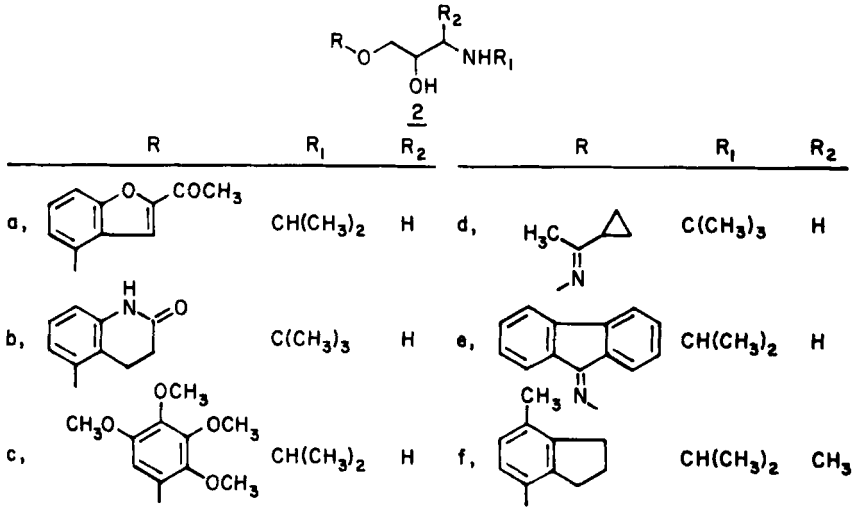
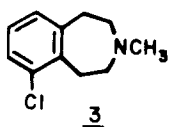
pranolol is a local anesthetic when applied topically to the rabbit cornea.[12] Other compounds at various stages of development include l-bunolol, betaxolol, celiprolol, cetamolol, pindolol, l-moprolol and the recently disclosed oxime ether falintolol (2d).[13] Of these β-blockers, only betaxolol,[14] celiprolol[15] and cetamolol[16] possess notable degrees of β_1-selectivity. The rationale for their development stems from the fact that timolol, by virtue of its ability to block β_2-adrenoceptors, must be used prudently in patients afflicted with respiratory problems. In a recent study,[17] topically instilled betaxolol did not compromise the respiratory function of asthmatics who were shown to be susceptible to topical timolol. That selective β_1-blockers lower IOP is seemingly at variance with the concept that the ocular hypotension elicited by β-adrenoceptor antagonists is a consequence of β_2-adrenoceptor blockade (vide infra). It is feasible that the ocular penetration of these β_1-selective compounds is of a magnitude which allows them to reach the target tissue(s) in concentrations needed to block β_2-adrenoceptors. The IOP of normotensive rabbits was decreased by the topical administration of the selective β_2-blockers IPS-339 (2e)[18,19] and ICI-118,551 (2f).[19,20]



Animal[21] and human[22] studies have revealed that the ocular hypotensive action of timolol is due to decreased AH inflow. Radioligand binding techniques have been used to demonstrate that the rabbit iris and ciliary body are rich in β-adrenoceptors[23] as are isolated ciliary processes.[24] Recent reports indicate that the vast majority of these β-adrenoceptors belong to the β_2-subtype.[25,26] Additional support for this receptor subtype assignment has emerged from catecholamine-stimulated adenylate cylase studies.[27-29] Human ciliary processes also are rich in β_2-adrenoceptors.[30] The IOP lowering action of β-blockers generally is attributed to their ability to block ocular β_2-adrenoceptors. This may, in fact, be an oversimplification.[9,31] For example, the ability of topically administered timolol to decrease blood flow to the ciliary process[32] may contribute to its pharmacological effect. When invoking the non-involvement of β-adrenoceptor blockade in the ocular hypotensive action of timolol, a frequently used argument is the fact that R-timolol (1b) reduces IOP in both animals[33,34] and man.[35] However, the assumption that R-timolol is appreciably less potent in blocking ocular β-adrenoceptors than is timolol, which bears a (S)-center of asymmetry, is incorrect. The affinity of R-timolol for rabbit iris plus ciliary binding sites is one-third that of timolol.[36] The ocular hypotensive effect of timolol in cats is reduced by sympathectomy[37] and, if the assumption is made that β_2-adrenoceptors are involved in timolol's mode of action, this observation would imply that their location may not be postsynaptic. As expected, the repeated instillation of timolol increased the number of β-binding sites in rabbit iris plus ciliary body.[38]

Blockade of either α_1- or α_2-adrenoceptors can lower IOP. With regard to α_1-blockers, the ocular hypotensive effect of topical prazosin in rabbits[39,40] was shown to result from decreased AH formation[41,42] rather than from changes in systemic blood pressure.[39,40] In contrast, the IOP lowering action of corynanthine in both rabbits and monkeys was not due to reduced AH formation. Increased uveoscleral outflow was proposed as a possible mechanism to explain this result.[43] Concentrations of dapiprazole which decreased IOP in both conscious rabbits[44] and human volunteers[45] also proved to elicit miosis. Neither AH inflow nor IOP was reduced in human volunteers treated with topically instilled thymoxamine.[46]

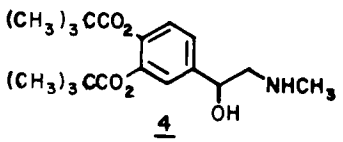
The IOP lowering action of the α_2-adrenoceptor antagonist SKF-86466 (3) in rabbits is not secondary to a reduction in blood pressure. This action appears to be local since the unilateral instillation of ocular hypotensive doses of 3 failed to lower IOP in the contralateral, untreated eye.[47] Topically administered yohimbine reduced AH formation in the cat.[42] Radioligand binding experiments have demonstrated the presence of both α_1-and α_2-binding sites in rabbit iris plus ciliary body.[48]



3

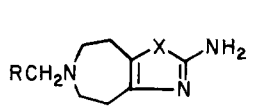
Adrenergic Agonists - Adrenergic antagonists clearly lower IOP. Paradoxically, adrenergic agonists also lower IOP as evidenced by the ocular hypotensive activity of topically administered epinephrine, norepinephrine and isoproterenol. Attempts to relate this activity to the effects of adrenergic agonists on AH inflow and outflow in several species of experimental animals have generated a welter of contradictory data.[49,50] The fact that the anatomy of the eye varies from one species to another undoubtedly has contributed to this confusion.

Dipivefrin (4), a marketed product, is a prodrug of epinephrine and, when administered topically, lowers the elevated IOP of the human glaucomatous eye at a concentration approximately one-tenth that of epinephrine.[51] As a consequence of the enhanced lipophilicity imparted by its O-pivaloyl substituents, the ocular penetration of dipivefrin in the rabbit is about ten-fold better than that of epinephrine.[52] The major site of dipivefrin hydrolysis is the cornea.[53] Intraocularly liberated pivalic acid is not metabolized in the eye, apparently is not sequestered in ocular tissues and is eliminated rapidly from the eye.[54]



4

The α_2-adrenoceptor agonist clonidine lowers IOP in both humans[55] and experimental animals.[56] In man, this effect may be secondary to a systemic hypotensive effect.[57] Moreover, the unilateral, topical instillation of clonidine in cats is associated with a reduction in the IOP of the contralateral, untreated eye.[56] This result indicates that clonidine's site of action is not local. A clonidine-induced decrease in AH formation has been observed in both humans[58] and cats.[42] Other α_2-agonists which lower IOP in rabbits are B-HT933 (5a) and B-HT920 (5b); their unilateral, topical administration diminished IOP in both eyes.[59]

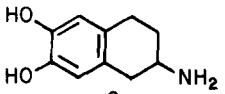


5

a, R = CH_3 ; X = O
b, R = $CH{=}CH_2$; X = S

The ocular hypotensive effect of l-isoproterenol is limited by ocular hyperemia and the development of tolerance to the pressure-lowering effect.[60] A surprising observation was the finding that d-isoproterenol lowered the IOP of rabbits more than did the racemate.[61] Subsequently, ocular hypotensive activity was shown in the rabbit for d-salbutamol and d-soterenol.[62] However, this effect may be species dependent since d-isoproterenol failed to lower the IOP in both humans and monkeys.[62] The effects elicited by d-isomers of adrenergic drugs in the rabbit eye have been proposed to reflect the partial agonist behavior of these discrete enantiomers.[63]

Dopaminergic Agents - Bromocriptine, given either orally or topically, decreased IOP in normal volunteers without altering either pupil diameter or plasma prolactin levels.[64,65] Animal studies have shown that bromocriptine has an ocular hypotensive action in rabbits but not in monkeys.[66] In contrast, lergotrile and pergolide were active in both species.[66]. Their site of action may not be local since the IOP of both eyes of rabbits was lowered following unilateral instillation.[67] The IOP-lowering action of lergotrile and pergolide in the rabbit is decreased by either sympathetic ganglionectomy or pretreatment with domperidone.[67] These findings suggest that the most likely site of action is the DA_2-receptor located on sympathetic nerve endings or ganglia. Based on studies of the ocular hypotensive effects elicited by dopamine and three methylated homologs, it has been proposed that, as larger alkyl moieties are substituted on the amino group, the mode of action changes from a direct (postjunctional) to an indirect (prejunctional) action.[68] The IOP-lowering action of aminotetralins, such as A-6,7-DTN (6), in rabbits is antagonized by sulpiride, thus implicating DA_2-receptors.[69] Surprisingly, topically applied haloperidol lowered IOP in normotensive rabbits by reducing AH inflow.[70,71]

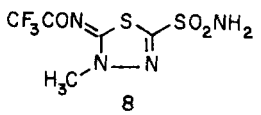


6

Cholinergic Agents - The traditional therapy for the treatment of glaucoma is pilocarpine, which reduces IOP by increasing AH elimination. Inherent in its mechanism of action are the undesirable side effects of miosis and problems of accommodation.[72,73] Added to this is the need to instill the drug four times daily. The latter problem can be overcome by instilling pilocarpine in the form either of a gel[74] or a polymeric salt.[75] N-Demethylated carbachol (7) is claimed both to lower IOP in glaucomatous patients and to be devoid of the side effects (vide supra) elicited by pilocarpine.[76] Another study in glaucomatous patients failed to confirm these claims.[77]

7

Carbonic Anhydrase Inhibitors - Systemically administered carbonic anhydrase inhibitors (CAIs), of which acetazolamide is the prototype, have been used to treat glaucoma for over three decades. By inhibiting CA in the ciliary process, CAIs reduce AH formation and, hence, lower IOP.[78,79] However, as a consequence of the undesirable side effects (e.g., altered taste, paresthesia, malaise, fatigue, depression, anorexia, nausea, weight loss and diminished libido) attending their systemic administration, patient compliance is frequently poor. Numerous attempts to circumvent these side effects by administering CAIs topically have proved generally futile. Exposure of the cornea of the anesthetized rabbit to a 3% solution of trifluormethazolamide (8) for ten to thirty minutes elicited a fall in IOP[80] by virtue of a reduction in AH formation.[81] The topical administration of a gel formulation containing the 6-(2-hydroxyethoxy) analog (9a) of ethoxzolamide (9b) recently was reported to lower IOP in conscious, ocular normotensive and hypertensive rabbits.[82] That it is possible to develop an oculohypotensive CAI which is active when topically instilled in a conventional manner is illustrated by the preclinical pharmacology of L-645,151 (9c), the O-pivaloyl derivative of L-643,799 (9d).[83,84] Acting as a prodrug of L-643,799, L-645,151 was shown to undergo corneal esterase-mediated hydrolysis to L-643,799 and to deliver the latter through the isolated rabbit cornea at a rate which was 46-fold higher than that from L-643,799.[83] Single drop (50 µl), topical instillations of 0.25% L-645,151 and 2% L-643,799 lowered the elevated IOP of the α-chymotrypsinized rabbit eye. The site of action of topically applied L-645,151 was demonstrated to be local and no diuresis was observed.[84] In an independent study, topically administered, divided doses



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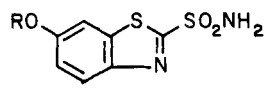
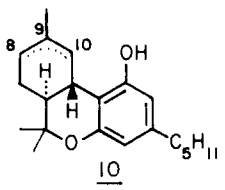


9

a, R = $(CH_2)_2OH$
b, R = CH_2CH_3
c, R = $COC(CH_3)_3$
d, R = H

of L-643,799 were found to elicit a small, but significant, IOP reduction in conscious, normotensive rabbits.[85]

Cannabinoids - Delta-9-tetrahydrocannabinol (Δ^9-THC, 10a) lowers IOP in several species including man[86-88] but leads to ocular toxicity in both experimental animals[88] and man.[89] Topically instilled Δ^8-THC (10b) has been reported to reduce rabbit IOP at nontoxic concentrations.[90] Numerous cannabinoids and cannabinoid derivatives have been evaluated in the rabbit for IOP lowering activity.[91-94] Cannabigerol (11), a monocyclic analog of Δ^9-THC, recently was demonstrated to lower IOP in cats and, unlike other cannabinoids, was claimed not to evoke ocular toxicity.[95]



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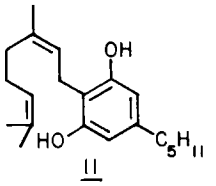
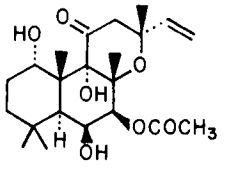


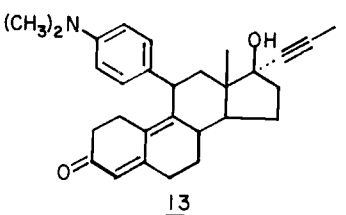
11

Miscellaneous Agents - Forskolin (12) is a naturally occurring diterpene which directly activates the catalytic subunit of adenylate cyclase.[96] Topically administered forskolin lowers IOP in both experimental animals and man.[97-101] This effect generally is attributed to a reduction in AH formation,[97-99] although an increase in outflow facility was observed in one study.[100] Forskolin is sparingly soluble in aqueous media and is administered either as a suspension or as a solution in ethanol. In suspension, forskolin has been observed in some,[97-99] but not in all, experiments[102] to be topically active. The intraocular injection of forskolin decreased IOP.[98,100,102] Tolerance to the repeated instillation of forskolin has been observed in monkeys[99] but not in rabbits.[98] In order to explain the decrease in AH formation following forskolin-evoked adenylate cyclase activation, it has been proposed that the stimulation of the enzyme promotes the reabsorption of fluid from the posterior chamber and its secretion into the ciliary stroma and thereby decreases net aqueous inflow.[97,98] This concept has been contested.[103] The effects of intravenously administered acetazolamide and topical forskolin on AH secretion in the rabbit are additive.[104] The irreversible stimulation of adenylate cyclase by the direct injection of cholera toxin into the rabbit eye dramatically lowered IOP.[105]



12

The long-term topical administration of the glucocorticoid antagonist RU 486-6 (13) lowered IOP in normotensive rabbits.[106] This observation is of interest in view of the strong evidence implicating glucocorticoids in the regulation of IOP. Certain patients on glucocorticoid therapy develop an elevated IOP.[107] This is thought to result from a reduction in AH outflow.[108] Dexamethasone has been shown by autoradiography to localize in the outflow pathway region cells of human tissue samples.[109] The presence of high affinity binding sites for dexamethasone has been confirmed in both rabbit iris plus ciliary body[110,111] and cultured human trabecular cells[112] by means of radioligand binding studies. Cortisol metabolism is altered in cells cultured from TM specimens obtained from glaucomatous patients. These cells accumulate 5β-dihydrocortisol and, to a lesser extent, 5α-dihydrocortisol. These cortisol metabolites are not found in similar cells obtained from nonglaucomatous patients.[113] The IOP-raising effect of topical dexamethasone in young rabbits is potentiated by the co-administration of 5β-dihydrocortisol.[114]



13

The topical administration of colchicine to rabbits elicits a prolonged (greater than 24 hours) fall in IOP.[115] Colchicine appears to lower IOP by disrupting microtubules, which results in reduced AH formation by the ciliary processes.[116] The diuretic triamterene has been observed to lower IOP in glaucomatous patients. The mechanism of action of topical triamterene is puzzling, because a diminution in the IOP of the untreated, contralateral eye was recorded, yet the systemic administration of the compound was ineffective.[117] The oral administration of the calcium-channel blocker nitrendipine elicited a slight, but significant, reduction in the IOP of nonglaucomatous individuals.[118] In contrast, the IOP of normotensive rabbits was increased after the topical administration of verapamil, diltiazem or nifedipine, the effect being attributed to local vascular changes. Topical verapamil also raised IOP in normotensive volunteers.[119]

Applied topically in low concentrations, prostaglandin $PGF_{2\alpha}$ reduced IOP in rabbits.[120] However, tachyphylaxis to the ocular hypotensive effect of $PGF_{2\alpha}$ developed rapidly in this species.[121] Moreover, increasing the dose of $PGF_{2\alpha}$ elevated, rather than lowered, IOP in rabbits.[120] In contrast, the repeated topical administration of $PGF_{2\alpha}$ to cats or monkeys elicits a persistent reduction in IOP.[121] PGE_2 acts in a similar manner.[122] However, neither agent may be suitable for clinical use since PGE_2 is unstable in aqueous media and the ocular penetration of $PGF_{2\alpha}$ is limited by its hydrophilic nature.[123] The ocular delivery of PGE_2 in cats is enhanced by the instillation of its methyl, ethyl or isopropyl ester derivatives. A four-day, topical treatment with these derivatives did not result in tachyphylaxis in the cat. The topical instillation of analogous $PGF_{2\alpha}$ esters was not associated with ocular discomfort in cats.[124] Topically administered $PGF_{2\alpha}$ increased outflow facility in both cats and monkeys. Whether increased outflow facility completely explains the ocular hypotensive effect of $PGF_{2\alpha}$ is not clear.[125] The IOP of human volunteers was lowered by topically applied $PGF_{2\alpha}$; however, side effects, including ocular smarting, headache and conjunctival hyperemia, were observed[126]. The intraocular injection of leukotriene C_4 or D_4 marginally decreased IOP in cats.[127]

In summary, the traditional treatment for glaucoma has been topically administered pilocarpine or systemic CAIs. Neither drug is ideal and the armamentarium of the physician has been enormously enhanced in the last decade by the introduction of new topical therapeutics such as timolol and dipivefrin. The current preclinical interest in compounds possessing a variety of mechanisms of action, e.g., topical CAIs, direct activators of adenylate cyclase and glucocorticoid antagonists, suggests that new and exciting antiglaucoma agents will be introduced within the next decade.

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Chapter 10. Agents for the Treatment of Peptic Ulcer Disease

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Introduction - Although H_2-receptor antagonists continue to play a dominant role in the treatment of peptic ulcers[1], the current trend is toward an increase in the choice of drug types available to the clinician. Much recent effort has been focused on the prevention of ulcer recurrence following the cessation of treatment or after long term maintenance therapy with antisecretory agents. These studies have been reviewed for H_2-receptor blockers[1] and for sucralfate, colloidal bismuth, pirenzepine, tricyclic antidepressants and omeprazole.[2] The treatment of ulcers with antacids has been refined.[3] The therapeutic indications for H_2-receptor antagonists have expanded[4-6] and a move to a once-a-day evening dosage form for these agents is apparent. Much research has centered on the defensive factors which contribute to the maintenance of the gastric mucosal barrier and the proceedings of a major symposium on this subject have been published.[7] The current consensus of opinion is that ulcer disease is thought to occur because of a lack of balance between aggressive factors (i.e., acid secretion) and defensive factors which restore the integrity of the mucosal barrier. The pharmacological control of acid secretion has been reviewed by Konturek.[8] The quest for new agents to treat peptic ulcers continues unabated, a fact which is amply illustrated by the 40-50 compounds which are currently in the advanced clinical or preclinical stages of development.

Histamine H_2-Antagonists - A shift in emphasis is taking place with regard to the manner in which cimetidine (1) and ranitidine (2) are being used to control gastric acid output in peptic ulcer patients. In general, it is felt that total suppression of acid secretion is not desirable, and that a more efficient reduction of nocturnal acid secretion is of therapeutic importance. Thus, a single nighttime dose of cimetidine[9,10] (600 or 400 mg) or ranitidine[11] (300 or 150 mg) was as effective in suppressing 24 h acid output as the usual multiple dose/day regimen. The duodenal ulcer healing rates after 4 weeks treatment with nocte cimetidine (600 mg)[10] or ranitidine (300 mg)[12] were 63% and 84%, respectively. A once nightly dose of cimetidine (400 mg) or ranitidine (150 mg) was effective in maintenance therapy (1 year).[13] Whether the frequency of recurrence might be reduced without maintenance therapy after ulcer healing with H_2-receptor blockers must await further clinical studies, but the initial data available suggest that cimetidine[14] and ranitidine[15] can diminish the rate of relapse during the first 6-12 months.

Gastric ulcer healing was significantly better with ranitidine (300 mg/day for 6 weeks) than placebo,[16] whereas cimetidine (1 g/day) and ranitidine (300 mg/day) were equally effective for this purpose.[4,17,18] Patients healed with cimetidine and maintained thereon for 1 year (400 mg, nocte) had a much lower (14%) ulcer recurrence rate than the placebo group (55%)[19], and ranitidine (150 mg, nocte) was similarly effective after a 6 month maintenance program.[17]

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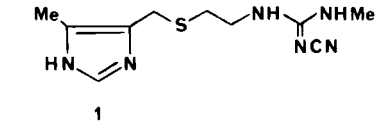
In general, cimetidine was ineffective in the prevention of acute upper gastrointestinal bleeding.[20] It is suggested that H_2-receptor antagonists might still be useful for preventing rebleeding.[21] Recent studies suggest that both cimetidine and ranitidine are effective in ameliorating the symptoms of gastroesophageal reflux disease.[6] A multicenter study has established that cimetidine is effective in healing stress related (e.g., burn injuries, renal failure, etc.) gastroduodenal mucosal lesions.[5]

The effects of H_2-receptor antagonists on the absorption and the hepatic and renal metabolism of commonly used drugs continue to be of considerable concern. Liver blood flow was not altered in healthy volunteers after 4 weeks of cimetidine (1 g/day) but hepatic microsomal enzyme activity was reduced.[22] Both cimetidine and ranitidine blocked rat liver ethylmorphine demethylase, an enzyme which is more sensitive to inhibition than the cytochrome P-450 mixed function oxidase.[23] In rats, cimetidine reduced acetaminophen hepatoxicity while ranitidine unexpectedly enhanced it.[24]

It is anticipated[25] that famotidine (3) will be launched in Japan and several European countries in 1985, and thus will be the third H_2-antagonist to enter the market for the treatment of peptic ulcer disease. The results of recent clinical studies have demonstrated that this agent is highly effective for healing duodenal and gastric ulcers and for maintenance therapy of patients so afflicted. Thus, famotidine (20 mg bid, 40 mg bid or 40 mg at night) and ranitidine (150 mg bid) were equi-effective (>90%) for healing duodenal ulcers after eight weeks of treatment, and 40 mg nocte famotidine was significantly better (91%) than placebo (62%) for gastric ulcer healing over the same time period. A single nighttime dose of famotidine shows remarkable efficacy for the relief of gastric ulcer pain and for promoting healing in such patients.[25] These results are not unexpected in view of the impressive suppression of basal and stimulated gastric acid output both in healthy[26] and duodenal ulcer[27] subjects. Famotidine has also been utilized for the maintenance (up to seven months) of Zollinger-Ellison syndrome patients.[28] Repeated administration of famotidine (40 mg, 28 days) has no effect on basal hormone levels and, in contrast to cimetidine, it does not block liver cytochrome P-450 enzymes (40 mg, 5 days).[26]

The cyanoamidine and carbamoylamidine moieties are bioisosteric with the cyanoguanidine and 1,1-diamino-2-nitroethylene units found in several H_2-antagonists. The carbamoyl analog (4) of famotidine was 30 times more effective as an H_2-antagonist in the isolated right atria of guinea pigs and 50 times more potent as an inhibitor of histamine stimulated gastric acid secretion in the anesthetized dog than cimetidine.[29] Rats treated with very high doses of tiotidine (5) developed various gastric mucosal abnormalities including carcinomas, and trials with this agent have been dropped.[30] Loxtidine (6), an H_2-antagonist of the insurmountable type, when taken in doses of 20, 40, and 80 mg in the evening by healthy volunteers, reduced nocturnal acid and pepsin secretion by 91-97% and 86-90% respectively.[31] A dose regimen of 40 mg bid raised the median 24 h intragastric pH from 1.6 (controls) to 6.0! Long term (27 months) oral administration of loxtidine to rats produced gastric adenocarcinomas and development of this agent, as well as the close structural analog lamtidine (7) has been discontinued.[32] The putative link between the prolonged hypochlorhydria produced by these and related compounds, such as tiotidine, and the induction of gastric carcinomas has not gone unnoticed.[30] L-643,441 (BMY-25260, 8), a

non-competitive H_2-antagonist, gave long lasting inhibition (67% at 23-25 h) of pentagastrin stimulated gastric acid secretion in normal subjects.[33] Two more recently described long acting compounds are BMY-25368 (9) and BMY-25405 (10). In dimaprit stimulated guinea pig atrial tissue, 9 was an insurmountable antagonist whereas 10 was not. In the Heidenhain pouch dog (p.o.), greater and more prolonged inhibition of histamine stimulated gastric acid secretion was elicited by 10, which was bound more tightly to the H_2-receptor.[34] The 1,2-benzoisothiazol-1, 1-dioxide WY-45087 (11) is a potent H_2-antagonist *in vitro* (pA_2 = 8.1, guinea pig atria) which inhibited basal acid secretion in the pylorus ligated rat and the Pavlov pouch dog.[35] The closely related thiophene compound WY-45662 (12) inhibited the output of basal acid in the Shay rat and food stimulated gastric acid secretion in the Pavlov pouch dog more potently than ranitidine.[35] The pyridine N-oxide derivative SR-58042 (13) and ranitidine were equally effective in the competitive inhibition of histamine induced gastric acid secretion in isolated guinea pig gastric mucosa and in the prevention of histamine and pentagastrin stimulated acid secretion in the anesthetized rat (i.v.).[32]



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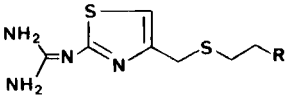
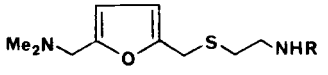


R

3 H_2N, NSO_2NH_2

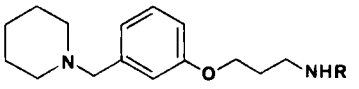
4 H_2N, $NCONH_2$

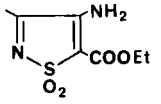
5 $-NH$, NCN, $NHMe$

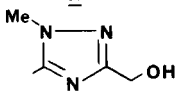


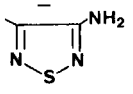
R

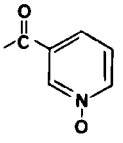
2 $NHMe$, $CHNO_2$

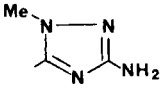


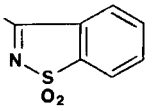
16 

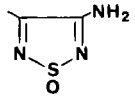
6 

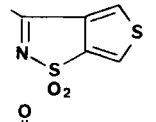
10 

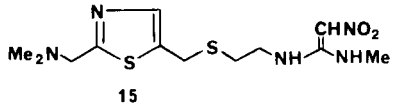
17 

7 

11 

8 

12 



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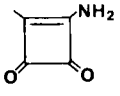
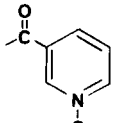
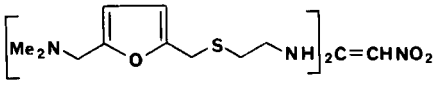
9 

13 



18

14 $-COCH_2OAc$

TZU-0460 (14) is 4-6 times more potent than cimetidine in animals, has no antiandrogenic action, and does not prolong hexobarbital sleeping time in

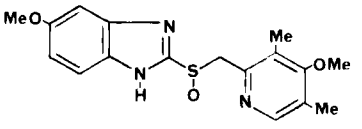
rats. Clinical studies have shown that a 50 mg dose inhibits basal acid by ca. 90% for 3 h.[36] Nizatidine (15) is more potent than cimetidine as a competitive H_2-antagonist *in vitro*[37] and as an inhibitor of pentagastrin stimulated acid secretion in humans.[36] *In vitro*, the isothiazole-1,1-dioxide (16) is an irreversible competitive H_2-antagonist which does not inhibit the binding of tritiated 5α-DHT to the androgen receptor. *In vivo*, it is similar to ranitidine with regard to antisecretory potency, and like ranitidine, has no effect on hexobarbital sleeping time.[38] CM-57755 (17) is a comparatively weak (0.06 - 0.17 times cimetidine) H_2-antagonist *in vitro*. In conscious, gastric fistula cats, however, it was as potent as, but of longer duration than, cimetidine in the inhibition of basal or dimaprit stimulated gastric acid secretion.[39] A number of symmetrical and unsymmetrical N,N'-bisheteroaryl substituted cyanoguanidines and 2-nitro-1,1-ethendiamines were synthesised, some of which were equal or greater in potency than ranitidine. For example, the symmetrical compound (18) was 141 times as potent as ranitidine as an H_2-antagonist in guinea pig atria and 8.7 times stronger as an inhibitor of histamine stimulated gastric acid secretion in the anesthetized rat.[40]

Inhibitors of H^+/K^+-ATPase - Brief accounts of the pre-1984 information on the mechanism of action, animal pharmacology and the results of early clinical trials on omeprazole (19) have been published.[2,41] Further studies have shown that this compound is effective in preventing stress-induced gastric lesions in the rat[42] and have confirmed[43] its cytoprotective activity in the same species.[44] In gastric fistula dogs, i.v. omeprazole was much longer acting (>24 h) and about eight times more potent than i.v. cimetidine in the inhibition of pentagastrin stimulated gastric acid secretion.[45] Omeprazole was a much more effective inhibitor of the H^+/K^+-ATPase (IC_{50} = 1.84 μmol/l) than of the phosphodiesterase (IC_{50} = 573 μmol/l) of enriched guinea pig parietal cells.[46] Long term oral administration (40 μmol/kg bid, 60 days) to rats caused a two-fold increase in the enterochromaffin like (ECL) cells, gastrin cell volume and number were increased and D cells were decreased. These changes were, however, fully reversed eight weeks post-treatment.[47]

The results of several clinical studies on omeprazole have now appeared in print and the data reinforces and expands on earlier preliminary observations. In normal subjects, acute or chronic oral administration causes a rapid, profound and long lasting inhibition of both basal[48-51] and pentagastrin stimulated[48,50,52] gastric acid secretion. Nocturnal acid secretion was very well controlled in these subjects.[48-51] Whereas a single 80 mg oral dose of omeprazole was reported to obliterate pepsin output (and acid secretion)[48] in pentagastrin stimulated normal males, and a bolus i.v. injection (0.35 μg/kg) reduced pepsin levels (basal and pentagastrin stimulated),[53] chronic oral daily dosing (30 or 60 mg, two weeks) seemed to have no significant effect on pepsin production.[50] There is no doubt, however, that upon repeated oral dosing with omeprazole, the strongly decreased acid production is paralleled by substantially elevated (up to four-fold) serum gastrin levels in fasted, meal or pentagastrin stimulated healthy males. Both of these parameters rapidly return to normal on cessation of treatment[52,54,55] and rebound effects seem to be absent or minimal.[51] Finally, the long term elevation of the gastric juice pH resulting from prolonged omeprazole use is associated with a higher than normal bacterial count and elevated intragastric nitrite and nitroso compound concentrations.[56] These changes also disappear shortly after the

medication is withdrawn. The results of several clinical trials,[57-60] including two multicenter ones[59,60] demonstrate that omeprazole is a remarkably effective, side effect free[61] agent, for the treatment of duodenal ulcers. Most patients are symptom free after one week on an oral 30-60 mg daily dose regimen and after four weeks endoscopically confirmed healing rates routinely exceed 90%. This agent also shows excellent promise in healing ulcerative peptic esophagitis.[62] Clinical trials on omeprazole have been suspended because of the occurrence of "tumor-like" changes in the gastric mucosa of rats receiving 50-5000 times the normal human dose.[63]

An imidazo[1,2-a]pyrazine derivative (20, SCH 32651) inhibited gastric acid secretion in pylorus ligated rats and in histamine, dimaprit, pentagastrin or food stimulated dogs (p.o.). In isolated guinea pig fundic musosa, this compound elicits several responses analogous to those observed with omeprazole, and consequently it is probable that it possesses antisecretory activity by virtue of the inhibition of H^+/K^+-ATPase.[64] It has recently been reported[65] that picartamide (RP 40749, 21) is an H^+/K^+-ATPase antagonist which causes long lasting inhibition of basal and histamine stimulated gastric acid secretion in pylorus ligated rats (p.o.) with a potency and duration much greater than found for cimetidine.[66] In male subjects, a 50 or 100 mg dose was more efficacious than cimetidine (1g) in the induction and maintenance of an elevated intragastric pH.[65] The inhibition of pentagastrin stimulated gastric acid secretion in healthy males by oral picartamide (1 or 2 mg/kg) was effective (50 and 71%, respectively) and prolonged (> 6h).[67] In patients with a history of duodenal ulcers, repeated daily doses (150 mg, oral) caused a highly significant reduction in gastric acidity, an elevation of serum gastrin levels and a decrease in pepsin concentration.[68] In a four week double blind study in patients with acute duodenal ulcers, daily evening doses (100 or 150 mg) effected complete healing in 95% of the cases,[69] several of which had been resistant to cimetidine, ranitidine, sucralfate or bismuth salts. In general, the patients were symptom free three days after dosing commenced. The development of this agent has been suspended.[70] Fenoctimine (22) was more potent than cimetidine in the reduction of basal acid secretion in the gastric fistula rat and inhibited the production of gastric acid stimulated by histamine, gastrin tetrapeptide or bethanechol in the chronic gastric fistula dog.[71] This compound is not an H_2-antagonist but does inhibit the H^+/K^+-ATPase of hog gastric mucosa.[72] The development of fenoctimine has been discontinued for unspecified reasons.[63]



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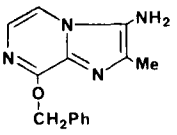

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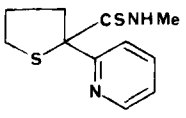

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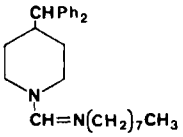


22

Gastroduodenal Mucosal Defense - Whereas the effect of aggressive factors of endogenous (e.g., acid, pepsin, bile) and environmental (e.g., alcohol, aspirin) origin has long been studied, the investigation of factors involved in the protection of the gastric mucosa has only recently commenced. Cytoprotection, originally associated only with prostaglandins, is now thought to be induced also by antacids[73], mucoprotectants (e.g., sucralfate,)[74] anticholinergic agents (e.g.,

pirenzepine, 36),[75] omeprazole (19),[44] certain histidine decarboxylase inhibitors,[76] epidermal growth factor,[77] sulfhydryl compounds,[78] and the gastrin antagonist proglumide.[79] Some of these agents are thought to act by stimulating the biosynthesis of endogenous prostaglandins (adaptive cytoprotection), whereas others elicit unknown defensive responses. A brief summary[80] and a comprehensive article[81] on the current status of gastroduodenal mucosal defense have been published.

Natural, as well as certain synthetic PG's, enhance gastric mucus output. Administration of PGE_2 or 16,16-dimethyl PGE_2 to rats, at doses which did not alter the thickness of the mucus layer, nevertheless protected the animals against ethanol induced lesions, even though penetration of the necrotic agent was the same as in the absence of the PG's.[82] This suggests that cytoprotection by PG's is a consequence of changes effected at the cellular level.

Duodenal bicarbonate secretion, which may be under the control of endogenous prostaglandins,[83] provides a second line of defense behind the "unstirred" layer of mucus. Increased vascular permeability preceded the development of grossly visible lesions after ethanol exposure, and prostaglandins blocked this damage.[84] Other elements of the gastroduodenal mucosal defense believed to be linked to prostaglandins are the rapid epithelial cell migration and mucosal surface restitution after damage,[85] the maintenance of gastric surface hydrophobicity,[86] lysosomal membrane stabilization[87] and the activation of endogenous mucosal sulfhydryl containing compounds.[78]

Prostaglandins - Misoprostol (23) has been approved for marketing in Mexico.[88] In two clinical studies,[89] one of them[90] multicenter, oral misoprostol (200 µg, qid) was similar in efficacy to cimetidine (300 mg, qid) in healing duodenal ulcers after 4 weeks of treatment. In another multicenter trial,[91] misoprostol (200 µg, qid, 4 weeks) was considerably more effective (76.6%) than placebo (51%) in healing duodenal ulcers. Diarrhea was the major side effect observed in 10-13% of the subjects, usually in the early stages of the trials. Misoprostol has also been used to successfully treat two patients with hemorrhagic gastritis refractory to conventional management (surgery, cimetidine).[92] CI 115,574 (24) and rioprostil (25) bear obvious structural resemblence to misoprostol. The maximally effective dose of 24 for the inhibition of pentagastrin stimulated gastric acid secretion in healthy individuals was 750 µg. At this dose, there was a substantial and sustained stimulation of mucus secretion into the gastric juice.[93] The development of this agent has been suspended, although apparently not for reasons of toxicity.[94] Rioprostil has both antisecretory and cytoprotective activity. In rats, the cytoprotective effect against ethanol is associated with decreased mucus loss from the gastric mucosa and not elevated mucus synthesis.[95] In normal subjects, rioprostil (300 µg, qid) completely protected against the endoscopically detectable injurious effects of aspirin (975 mg, qid),[96] and a single 600 µg dose inhibited peptone stimulated gastric acid secretion[97] by 74%. Pentagastrin stimulated acid and pepsin secretion were decreased by 51% and 67% respectively with the same dose.[98] Enisoprost (26) is a more potent and longer acting antisecretory agent in histamine stimulated Heidenhain pouch and gastric fistula dogs and is considerably less diarrheagenic in rats than misoprostol.[99] The 11α-methyl PGE_2 derivative trimeprostil (27), given qid (750 µg), was less effective (62%) than cimetidine (200 mg tid and 400 mg nocte; 90%) in healing

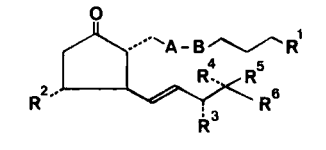
duodenal ulcers after 4 weeks treatment, and its use was associated with more side effects (nausea, vomiting) and a greater relapse rate (1 year post-treatment).[100] MDL 646 (28) inhibited basal and stimulated (histamine, pentagastrin) gastric acid secretion in rats (i.v., p.o.); it was more effective p.o. than i.v. as a cytoprotective agent[101] and less potent as a leuteolytic agent than the natural PGE's in hamsters (p.o.).[102] In healthy individuals a single oral 1000 µg dose reduced gastric acid output by 36% 2.5 h post-dose.[103]

The 11-deoxy-16-phenoxy PGE_1 derivative M&B 28,767 (29) is 60 times more potent than 16,16-dimethyl PGE_2 with regard to the inhibition of pentagastrin stimulated acid secretion, but less effective (0.3 times) in preventing indomethacin induced ulcers in the rat (p.o.).[104] Its propensity to cause uterine stimulation (rat, s.c.) and diarrhea (mouse, p.o.) is considerably less than observed for 16,16-dimethyl PGE_2.[105] Enprostil (30), an allenic PGE derivative, is an antisecretory and cytoprotective agent in animals and man. The inhibitory activity on stimulated acid secretion in the rat, cat, dog and rhesus monkey is profound (e.g., in dimaprit stimulated, gastric fistula cats, ID_{50} = 2.5 µg/kg) and persistent (up to 14 h in rats).[106,107] In the rat, it was 200-400 times more potent than PGE_2 in blocking experimentally induced gastric and duodenal ulcers,[107] and much more potent than the natural PG's in stimulating mucus secretion.[108] Both the rate of onset and the degree of acid inhibition in the rat was greater on oral than on intraduodenal (i.d.) administration.[106] Furthermore, in the Heidenhain pouch dog, food stimulated acid secretion was inhibited at a much lower dose when enprostil was deposited in the pouch (ID_{50} = 0.9 µg/kg) rather than the stomach (ID_{50} = 7 µg/kg).[106] This data indicates that at least a part of the antisecretory effect is topical, suggesting a direct, local action on the parietal cells of the gastric mucosa. A single oral dose of enprostil (35 or 70 µg) or cimetidine (600 mg) showed similar efficacy for the suppression of food stimulated gastric acid secretion in healthy subjects, but the duration of inhibition was much longer for the PG. Thus, acid production was reduced 83-94% for 8 h by enprostil and significant inhibition was still evident at 11h.[109,110] In the same individuals, gastrin levels were depressed (30-40%) by enprostil whereas cimetidine augmented gastrin release. A seven day study involving healthy volunteers demonstrated that doses as low as 3.5 µg bid conferred significant protection against aspirin induced (650 mg qid) gastric mucosal damage.[111] In two, 4 week clinical trials,[112,113] enprostil (35 or 70 µg bid) was clearly superior to placebo in healing duodenal ulcers. The only side effects were transitory diarrhea or loose stools in a low percentage of the patients. Enprostil has been approved for marketing in Mexico.

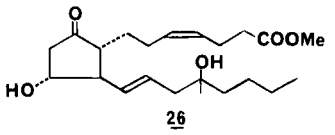
FCE 20,700 (31)[114] is a 13,14-dehydro prostaglandin which is reported to be a cytoprotective agent with little or no antisecretory activity. In the rat, orally administered 31 inhibited cold stress, indomethacin and ethanol induced gastric ulcers with ED_{50}'s of 262, 37.8 and 9.2 µg/kg, respectively, but the ED_{50} for the inhibition of acid secretion in the pylorus ligated animal was 2385 µg/kg.[114] For rosaprostol (32), a truncated F type prostaglandin, the antisecretory activity has been controversial,[115] but it does appear to have both antisecretory and cytoprotective properties in duodenal ulcer patients. Indeed, oral rosaprostol was almost as effective (78.9%) as cimetidine (90%) in healing duodenal ulcers, although the doses administered were enormous (500 mg, qid for 6 weeks) by prostaglandin standards.[116] Nocloprost (33), a 9R-chloro PGF derivative, has antisecretory and

cytoprotective activity in rats comparable to that of 16,16-dimethyl PGE_2,[117] but unlike the latter compound, the diarrhea induction potential is low. As expected, the long term stability problems associated with PGE derivatives are not found in nocloprost. The presence of PGE like pharmacological properties in 9β-substituted PGF derivatives is not without precedent.[118]

The thia-imino prostacyclin analog HOE 892 (34) has gastric antisecretory and cytoprotective properties in rats which are very similar to those of PGI_2.[119] In common with PGI_2, 34 is highly effective by s.c. but not p.o. administration. U-68215 (35), is an orally active benzindane prostacyclin mimic which has antisecretory (ED_{50} = 30 μg/kg) and cytoprotective activity (ED_{50} = 1 μg/kg) and inhibits aspirin induced ulcers (ED_{50} = 5 μg/kg) in rats. It is neither enteropooling nor uterotonic but it does lower mean arterial blood pressure at 150 μg/kg.[120]



	R^1	A-B	R^2	R^3	R^4	R^5	R^6
23	COOMe	CH_2CH_2	OH	H	Me/OH		n-Bu
24	$COCH_2OH$	CH_2CH_2	OH	H	Me/H		n-Bu
25	CH_2OH	CH_2CH_2	OH	H	Me/H		n-Bu
27	COOH	CH=CH	Me	OH	Me	Me	n-Bu
28	COOMe	CH_2CH_2	OH	OH	MeO	Me	n-Bu
29	COOH	CH_2CH_2	H	OH	H	H	OPh



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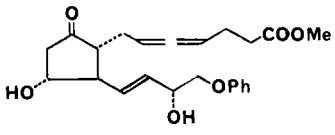

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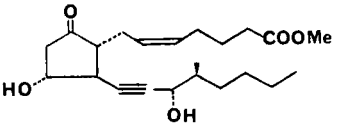

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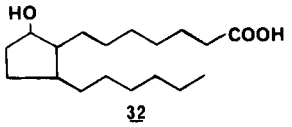

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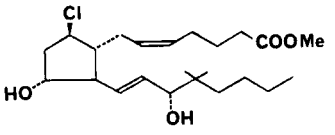

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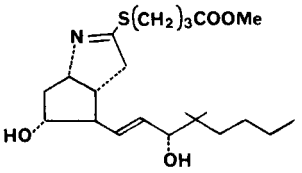

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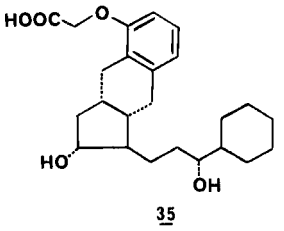

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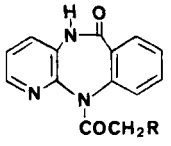
Anticholinergic Agents - The investigation of the inhibition of gastric acid secretion by the anticholinergic agent pirenzepine (36) led to the classification of muscarinic receptors as M_1 and M_2 subtypes.[121] Pirenzepine, a selective M_1-antagonist, inhibits acid secretion but has relatively little effect on gastrointestinal smooth muscle, the pupil, urinary bladder, or the heart. It is further proposed that pirenzepine inhibits acid secretion primarily by acting at the level of the parasympathetic (enteric) ganglion, whereas atropine, an M_1 and M_2 antagonist, acts both on the parietal cell receptor and the enteric ganglion. Pirenzepine (100 mg/day) is equal to cimetidine (1000 mg/day)[122] with regard to the rapidity and efficacy of duodenal ulcer healing and the relief of ulcer related pain. It is claimed to be effective, and superior to ranitidine, in the prevention of ulcer relapse.[123] Pirenzepine reduced food stimulated acid secretion in

healthy subjects[124] but repeatedly failed to suppress 24 h acid secretion in duodenal ulcer patients.[125,126] Pirenzepine and atropine are cytoprotective in animals at doses which have no effect on acid secretion.[127]

Timepidium bromide (37) is a potent antiulcer muscarinic antagonist with some anticholinergic side effects.[128] Darenzepine (38) is said to be more potent and to elicit less anticholinergic side effects than pirenzepine.[129] Telenzepine (39) has a six-fold greater affinity *in vitro* for M_1 over M_2 receptors (heart, smooth muscle).[130] In the rat (i.v.) it was 5 times as potent as pirenzepine in suppressing gastric secretion but its effect on salivation and mydriasis was similar to 36. A phase I clinical study showed that telenzepine (3 mg) was as potent as, but of longer duration than, pirenzepine (50 mg), for the inhibition of acid secretion. Side effects, such as dry mouth, were noted, however.[131]



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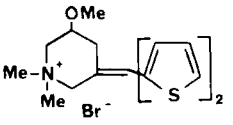

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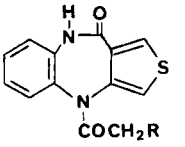

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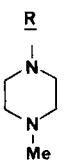


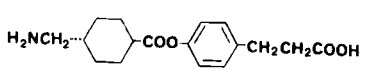
Mucosal Protectants - The pharmacology of sucralfate and its efficacy in the treatment of duodenal ulcers has been reviewed.[2,132] After 4 weeks of treatment, it was as effective as cimetidine in healing duodenal[133] and gastric[134] ulcers. Sucralfate is useful for the treatment of stress ulcers[135] and for the long term maintenance of duodenal ulcer patients.[136] It is claimed to be cytoprotective, to enhance mucus output[137] and increase mucosal PGE_2 generation[138], but in a clinical study it did not provide protection against aspirin induced gastric injury.[139]

Tripotassium dicitratobismuthane (Denol) healed duodenal[140] and gastric[141] ulcers as effectively as cimetidine. In a most intriguing study[140], it is claimed that the one year relapse rate for Denol-healed duodenal ulcer patients, not on maintenance therapy, is no different from those who received nightly maintenance doses of cimetidine. Denol was cytoprotective and increased mucosal PGI_2 biosynthesis in rats.[142]

Cetraxate (40), an anti-gastric ulcer agent widely used in Asia, was shown to be effective in the treatment of acute gastritis and acute aggravation of chronic gastritis in a double blind multicenter study.[143] Mezolidon (41), has no effect on acid secretion evoked by histamine or cholinergic agents but does inhibit basal and gastrin stimulated acid secretion in rodents. It blocks the formation of gastric and duodenal ulcers in various animal models[144] and inhibits gastric mucosal damage induced by ethanol or 0.6N HCl.[145] Meciadanol (42), a histidine decarboxylase inhibitor in gastric mucosa, did not affect basal or pentagastrin induced acid secretion in healthy subjects, but aspirin induced microbleeding was prevented on oral administration (250 mg, bid, 1 week).[146]

Gut Active Peptides - Two reviews[147,148] on gut peptides and the proceedings of a symposium on gastrointestinal hormones[149] were published. Proglumide (43), a gastrin and cholecystokinin[150] receptor antagonist, inhibits gastric acid secretion, has cytoprotective properties, and stimulates gastric mucus in rats (200 mg/kg) when administered p.o., i.p. or i.d.[79] It (2.4 g/day, i.v.) has been claimed to be superior to cimetidine in the clinical treatment of stress ulcers.

Gastrin and somatostatin were found to be elevated in the gastric antral mucosa of individuals afflicted with duodenal ulcers,[151] but these patients do not have reduced sensitivity to the inhibitory effects of somatostatin (200-800 pmol/kg/hr., i.v.) on gastric acid secretion.[152] Somatostatin appears to be particularly effective in the control of upper gastrointestinal bleeding in peptic ulcer patients,[153] a condition for which H_2-receptor antagonists usually are ineffective.



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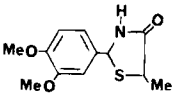

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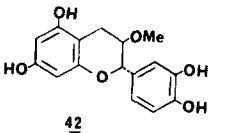

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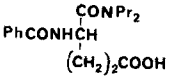

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Chapter 11. Plasminogen Activators

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Introduction - Thrombo-occlusive disease of the great and small vessels is the major cause of morbidity and mortality in the Western World. The majority of acute myocardial infarctions as well as cerebrovascular accidents are due to arterial occlusion; thrombosis of the deep veins of the leg with consequent pulmonary embolism is a major cause of morbidity as well. At present it is possible to induce thrombolysis of vascular occlusions by proteolysis and dissolution of fibrin, the prime constituent of the thrombus. Fibrinolysis is induced when the rate of plasmin generated from its inactive precursor plasminogen, an important plasma protein, is increased. Undesirable side effects including hypofibrinogenemia, reduced levels of Factors V and VIII and elevated levels of fibrinogen degradation products, can however result from systemic plasminogen activation. This can increase the risk of spontaneous hemorrhage. Recent efforts have focused on attempting to achieve "clot-specific" lysis, thrombolysis without extensive fibrinogenolysis.

The Biology of Plasminogen and Plasminogen Activation

The central step in the fibrinolytic system is conversion of the proenzyme plasminogen to the active enzyme plasmin, a relatively non-specific serine protease with trypsin-like specificity. Plasminogen is normally included in thrombi as they are formed; thus if activated *in situ*, the plasmin dissolves the fibrin without systemic fibrinogen breakdown.

Plasminogen, is a single-chain glycoprotein zymogen of molecular weight 92,000 daltons, which is highly disulfide crosslinked. Plasminogen, as isolated, exists as a heterogeneous family of molecules as post-translationally modified to yield molecules with various degrees of glycosylation[1] and proteolytic processing.[2] The best studied occurring variants of plasminogen are the lys- (intact molecule) and glu- (shortened amino terminus) plasminogens.

Plasminogen is made up of two functional domains, a protease domain and a fibrin binding domain. The fibrin binding domain is made up of five homologous triple loop structures known as "kringles;"[3] kringles are also observed in other blood serum proteases such as prothrombin, urokinase and tissue-type plasminogen activator. These kringle regions contain lysine binding sites which are thought to be related to the fibrin binding.[4] NMR studies[5] chemical and immunological mapping[6] and modification[7] have established that these epsilon-amino carboxylic acid binding sites exist in a tightly folded structure. X-ray crystallographic analysis of isolated kringle structures is now underway.

Activated plasmin is generated by two proteolytic events, the change of an Arg-Val bond[8] near the carboxyl-terminus of the molecule resulting in a two chain molecule held together by a single disulfide bond and the

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removal of an amino-terminal peptide to yield the more active lys-plasmin.[9] Once the active moiety, plasmin, has been generated it becomes a relatively nonspecific enzyme with high catalytic activity. Systemic plasminogen activation leads to the degradation of fibrinogen, Factor V, Factor VIII and is accompanied by the appearance of fibrinogen breakdown products with their own anticoagulant properties.[10]

The main inhibitor of plasmin in blood is alpha-2 anti-plasmin which is known to compete for the fibrin binding sites of plasmin.[11] Alpha-2 antiplasmin is a single chain glycoprotein of molecular weight 70,000 daltons which forms a one-to-one stoichiometric complex with plasmin [12] while undergoing proteolysis[13] and is the body's primary defense against systemic degradation of the hemostatic system.[10] During thrombolytic therapy the free inhibitor disappears due to its reaction with plasmin. A less specific inhibitor with less affinity, alpha-2 macroglobulin, provides the hemostatic system with a second line of defense.[14] Plasmin generated at the site of a clot (and therefore bound to fibrin) reacts far more slowly with inhibitors than plasmin in circulation[15] since the fibrin binding sites of bound plasmin are relatively unavailable.

Intrinsic Activation of Plasminogen - Fibrinolysis may be surface mediated in a manner analogous to the surface mediated intrinsic pathway of coagulation. This hypothesis is based on the observation that fibrinolytic activity develops in normal plasma incubated with an activating surface such as kaolin.[16 17] Surface mediated fibrinolysis is lacking in plasma with deficiencies of Hageman (Factor XII),[18] Fletcher (pre-kallikrein),[19] and Fitzgerald (high molecular weight kininogen)[20] factors. Thus, Factor XII, pre-kallikrein and HMW kininogen, along with plasminogen are required for this pathway. The precise molecule or molecular interactions that activate plasminogen are unclear, and theories have been presented suggesting that kallikrein is a direct plasminogen activator[21] while others suggest an additional proactivator.[22] Kluft[23] distinguishes between a Factor XII-dependent and a Factor XII-independent activity. The biological and clinical significance of the intrinsic pathway in activating plasminogen is not well established.

Exogenous Activation of Plasminogen - So called exogenous activation of plasminogen (due to added nonplasma components) is accomplished by two basic mechanisms, the proteolytic processing of plasminogen to plasmin by urokinase and the conformationally based activation of the zymogen plasminogen by nonenzyme proteins such as streptokinase. Though originally characterized as an exogenous activator (found in urine),[24 25] recent evidence indicates that urokinase is an endogenous activator produced in the vasculature[26] and found in the plasma.[27] The single-chain zymogen, pro-urokinase (pro-UK) is a true zymogen which will not activate plasminogen directly before the pro-UK is cleaved; however, it has been postulated that pro-UK might also activate plasminogen via the intrinsic pathway in the presence of fibrin.[28] Pro-urokinase is presumed to be converted at the site of the clot (presumably by trace amounts of plasmin) to two-chain urokinase which generates sufficient plasmin locally to induce thrombolysis, but does not activate plasminogen in the general circulation.

Streptokinase is a non-enzyme protein with a molecular weight of 47K-48K daltons produced by Lancefield group C strains of beta-hemolytic streptococci, the filtrates of which were observed (1933)[29] to cause the liquefaction of human plasma clots. Streptokinase activates the fibrinolytic system indirectly by initially forming a one-to-one stoichiometric complex with plasminogen, which produces a conformation change in the

latter without peptide bond cleavage of the zymogen.[30 31 32] This conformation change leads to the development of an active site in the plasminogen. The complex converts plasminogen to plasmin.[32 33]

Extrinsic Activation of Plasminogen - A plasminogen activator associated with vascular endothelial tissue was demonstrated when extracts were shown to lyse fibrin which contained plasminogen.[34] This tissue-type plasminogen activator (t-PA) was first partially purified from uterine tissue,[35] but it was the purification of t-PA from a melanoma cell line that allowed sufficient quantities to be obtained for significant biological and biochemical characterization.[36] Tissue-type plasminogen activator, like urokinase, is a protease which activates plasminogen to plasmin, but due to its affinity for and activation by fibrin, t-PA is known as a clot specific drug.[37 38 39]

Agents Used in Fibrinolytic Therapy

Inducers of Fibrinolytic Enzymes - Changes in the physiological state such as stress and exercise[40] are known to induce release of t-PA. Thus, some effort has been focused at finding inducers of the fibrinolytic state which would be clinically useful. *In vitro* and animal model studies have led to the testing of several of these compounds in human trials:[41] prostaglandins,[42] nicotinic acid,[43] anabolic steroids[41] and 1-desamino -8-D arginine Vasopressin (DDAVP).[44 45] None of these agents has yet shown the ability to induce sufficient levels of t-PA to achieve thrombolysis of major clots, but some have been used with partial success prophylactically.

Streptokinase- Streptokinase is the most widely used thrombolytic agent and has been shown to be effective in the lysis of venous thrombi in the leg, pulmonary emboli,[46] and coronary thrombi in acute myocardial infarctions.[47-50] The use of streptokinase in all studied clinical settings is associated with systemic fibrinogenolysis and resulting hypofibrinogenemia, elevation of fibrinogen degradation products, and depression of Factors V and VIII, which can lead to a high risk of clinically significant bleeding diathesis.

Human plasma contains antibodies to streptokinase, presumably from previous exposure to streptococci in the environment. The levels of antibody are variable[49] and are capable, if high enough, of neutralizing administered streptokinase. Thus, therapy with streptokinase requires a preliminary "*in vitro* resistance test",[51] with subsequent infusion of sufficient amount of material to neutralize the antibodies before fibrinolytic activation can be obtained.[52]

The pharmacokinetics,[53] mechanism of action[32] and clinical pharmacology[33] of streptokinase have been reported, and these studies have concluded that the streptokinase-plasminogen complex which appears following streptokinase infusion has a half-life of hours and is not fibrin specific.

Acyl-Enzymes - Active site titrants such as p-nitrophenyl p'-guanidinobenzoate could specifically acylate the active center of trypsin-like enzymes.[54] Several such acyl derivatives of streptokinase-plasminogen or streptokinase-plasmin complexes and plasminogen activator enzymes have been prepared. The theoretical advantage of such a derivative is that the drug would bind to fibrin in a clot while acylated, and deacylate *in situ* thus generating active plasminogen activators only at the site of a clot.[55] Such acyl-enzymes would theoretically provide advantages in efficacy,

pharmacokinetics, and reduced toxicity attributable to hyperplasminemia.

The activity of a number of other acylated derivatives of streptokinase complexes with various plasminogen species,[55-58] derivatives of a streptokinase-plasmin complex[57] and derivatives of a complex of 38K dalton proteolytic fragment of plasminogen (mini plasminogen) with streptokinase[56] have been studied. Two of the most promising agents, a p-anisoyl-lys-plasmin complex (BRL-26920) and a p-anisoyl-plasminogen-streptokinase complex (BRL 26921) were studied in dog and rabbit models of venous thrombosis.[56 57] Both agents demonstrated thrombolysis (release of radiolabeled fibrinogen) without fibrinogenolysis (preservation of serum fibrinogen levels). Though initial studies suggested that BRL 26291 was the most active and clot-specific of the derivatives, it was suggested from studies with human plasma in vitro, that BRL 26921 was not fibrinolytic without being fibrinogenolytic. Subsequent studies in patients with acute myocardial infarction have shown little or no clot specificity.[60] Thus, despite considerable clot-specific fibrinolysis in animals, acyl-enzymes have yet to yield clot-selective fibrinolysis in humans.

Urokinase - Active urokinase is a glycosylated serine protease composed of two polypeptide chains connected by a single disulfide bond. As with plasmin, the molecule has a fibrin binding domain which contains a kringle structure which is very similar to those found on plasminogen and a protease domain which is very homologous to other serine proteases;[61] the activation site of pro-UK lies between the two domains. As isolated from urine[62 63] or tissue culture,[26 64] urokinase occurs as a high (HUK) or low (LUK) molecular weight species. The LUK is a proteolysis product of HUK[65] which has all but 21 amino acids of the fibrin binding region cleaved from the amino terminus of the molecule.[66] Both forms have a single N-linked sugar attachment site on the protease domain. The enzymatic activity,[67 68] and the biochemical[69] and biophysical characteristics[70] of LUK and HUK have been compared extensively. In spite of the presence of a putative fibrin binding region on HUK, neither HUK nor LUK has demonstrated any in vitro[71 72] clot specificity or in animal models.[73-75] The primary structures of LUK[76] and HUK[77] isolated from human urine have been determined to have led to the cloning of the gene for the enzyme.[78 79]

Urokinase has been successfully used for thrombolytic therapy in venous thrombosis,[80] pulmonary embolism[81] and coronary thrombosis,[82] but both HUK and LUK suffer from many of the same drawbacks as streptokinase with regard to systemic fibrinogenolysis.[51 81] Studies with varying infusion rates and dosage of both HUK and LUK suggest a mean half-clearance of about 15 minutes[83] in man. Various inhibitors which react with both HUK and LUK have been isolated from blood[84] though the route clearance of UK has not been proved to involve these complexes.[85]

Urokinase has been produced by recombinant DNA technology as LUK[86] and this nonglycosylated form of the enzyme has equivalent enzymatic activity[87] and half-life to the urokinase isolated from mammalian sources as measured in in vitro and in vivo animal models.[88]

Derivatives of urokinase have been prepared by the addition of polyalkyleneglycols such as methoxypolyethylene glycol to the protein[89] The activity of the activator is retained in a dog model, however the in vivo half-life of the molecule is dramatically extended.[90]

Pro-UK has been isolated from urine,[91 92] tissue culture cells[93 94] and plasma.[27] Pro-UK has recently been demonstrated in studies in vitro

and *in vivo* (rabbits, dogs)[95] to be relatively clot specific[28 73 74] with specificity and activity equivalent to tissue-type plasminogen activator.[96] The half-life of pro-UK in *in vitro* human plasma is reported to be as long as 50 hours as contrasted to three to five hours for t-PA or active urokinase; this is probably due to the inability of urokinase inhibitors to interact with pro-urokinase.[93] However, the short blood lifetimes in rabbits of three to five minutes[28] are similar to t-PA (see below); clinical trials with tissue culture derived pro-UK are expected to begin in 1985.

Studies with a nonglycosylated (recombinant) form of pro-UK has demonstrated it to be more active and clot specific than recombinant urokinase (which is biologically indistinguishable from natural urokinase),[73 88] but less potent and specific than t-PA derived from melanoma cells,[88] an effect which may be an artifact of the rabbit model.[95] These studies showed a rapid (three to five minute) blood half-life of this nonglycosylated pro-UK, identical to the glycosylated form synthesized in tissue culture.

t-PA - Native t-PA as isolated from a melanoma cell line is a single-chain glycoprotein protease of molecular weight 65K daltons. The single-chain molecule is susceptible to enzymatic digestion to a two-chain molecule in which the two chains remain conveniently linked by a disulfide bond.[36 98] The melanoma derived t-PA is immunologically and chemically similar to the uterine form.[36 99] Slight differences in the amino terminal sequence, presumably due to proteolysis, have been reported.[100 101] Cloning of the cDNA from melanoma cells has led to the deduction of an amino acid sequence[102] (527 residues). The heavy or A chain (of the two chain form) contains the fibrin binding region. The light (or B) chain contains the catalytic site, with homology to other serine proteases.[103] There is considerable homology between native t-PA and high molecular weight urokinase in both the protease and fibrin binding domains. However there exist two kringles in t-PA and on additional amino terminal region that is homologous to the so-called finger domain region[104] associated with the fibrin affinity of fibronectin (cold-insoluble globulin).[105] There is also no immunological cross-reactivity of UK and t-PA.

Another source of microheterogeneity (Type I and Type II t-PA) leads to an apparent molecular weight difference of 3000 in the A chain or the single chain molecule.[103 106] This difference has been ascribed to the presence or absence of carbohydrate moieties at position 184 on the A chain of t-PA.[107 108] The activator has three other potential N-linked glycosylation sites, two of which are known to contain carbohydrate residues.[107]

The one-chain and two-chain forms have virtually the same fibrinolytic and plasminogen activating properties,[98 109] and it has been suggested that conversion of the one-chain to the two-chain form occurs rapidly (as a result of traces of plasmin) at the fibrin surface, such that physiologic fibrinolysis is mainly a result of two-chain t-PA.[109] Tissue plasminogen activator appears to have considerable clot specificity as evidenced by a striking fibrin-specific enhancement of plasminogen activation by t-PA.[37 39] One group found that fibrin increased the affinity of t-PA for plasminogen, but not the catalytic efficiency of the enzyme,[110] while another group found a significantly increased substrate turnover rate.[111]

Recombinant t-PA (rt-PA) has been produced in E. coli[102] and tissue culture cells. The material produced in tissue culture has been shown to

be biochemically[112] and biologically equivalent to melanoma t-PA.[111] Both rt-PA and melanoma t-PA have been shown to be capable of inducing thrombolysis without significant fibrinogenolysis in a wide range of animal studies including: rabbit jugular vein thrombosis (both),[111] dog femoral vein thrombosis (melanoma t-PA,[113] rt-PA),[114] dog coronary artery thrombosis (melanoma,[115] rt-PA),[116,117] and in primate coronary artery occlusion.[118] Pharmacokinetic analyses of rt-PA and melanoma t-PA in rabbits[119] and cynomolgus monkeys[120] show a short half-life of two to five minutes. Tissue-type plasminogen activator has been shown to react with a fast inhibitor[121] as well as with alpha-2 antiplasmin and alpha-2 macroglobulin at slower rates. However, the rapid clearance of t-PA seems to occur in the liver[122] in rats and be independent of its ability to bind inhibitors.

Human studies with melanoma t-PA have been limited due to scarce supply, but pilot studies in venous thrombosis[123] and coronary thrombosis[124] demonstrated the feasibility of clot-specific fibrinolysis in humans. More extensive trials with rt-PA are underway. They demonstrate a high rate of coronary thrombolysis with little fibrinogen breakdown.[125] The pharmacokinetics of rt-PA in humans appear to demonstrate a biphasic elimination with a rapid (five to ten minutes) early phase followed by a more prolonged second phase.[126,127] Two, large randomized trials comparing rt-PA to streptokinase in acute myocardial infarction have definitively shown rt-PA to be both more effective and less fibrinogenolytic.[128,129]

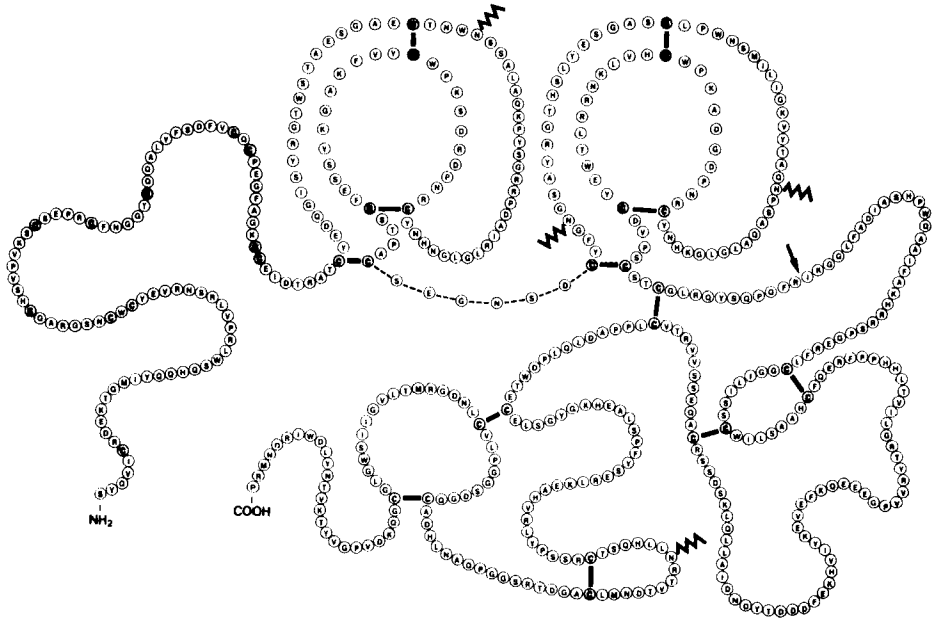

Figure 1

Primary sequence of t-PA. The fibrin binding domain is in the upper half of the picture and the serine protease domain in the lower half. All possible N-linked glycosylation sites are shown with jagged lines. The disulfides shown in straight lines are assigned by homology to other serine protease or plasminogen Kringle regions. The one/two chain cleavage site is indicated with an arrow. The two Kringle structures have a characteristic loop structure with three disulfide bands. The two domains of t-PA are covalently attached by a disulfide bond even if in two chain form. (Reprinted from Nature)

Methodological issues in the measurement of fibrinogen have been raised by studies with rt-PA. An in vitro fibrinogenolysis has been observed when plasma rt-PA levels are high,[117 126] and samples measured by the Clauss method[130] suggest fibrinogen concentrations lower than paired samples measured by sulfite precipitation, indicating that slowly coagulable fragments x and y are produced when fibrinogenolysis does occur in the setting of prolonged infusions of high doses of rt-PA.[126]

Partially carbohydrate depleted t-PA has been prepared by enzymatic digestion of melanoma t-PA.[131] Many of the biochemical characteristics of the deglycosylated t-PA remain intact.

Tissue-type plasminogen activator derivatives which are designed to modify its activity, half-life, or specificity are yet to be described; however developments in this area are expected in the next several years.

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Chapter 12. Gastrointestinal Motility Enhancing Agents

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Introduction - Alterations in the motility of the alimentary canal are associated with many symptoms of gastrointestinal disease. Examples of the digestive diseases which are manifested by a disturbance in motor activity are dysphagia, achalasia, diffuse esophageal spasms, gastric stasis, retching, vomiting, crampy abdominal pain, intestinal obstruction, paralytic ileus, and constipation.

The majority of these motility disorders have been treated with muscarinic agonists and cholinesterase inhibitors.[1,2] The development of synthetic motility enhancing agents has been slow, because the precise physiological abnormality is understood in only a small number of patients with symptoms of gastrointestinal motor dysfunction. In recent years there has been a rapid development in the understanding of gastrointestinal motor function. It has been demonstrated that motor function is controlled by myogenic smooth muscle mechanisms, by enteric and extrinsic autonomic nerves, and by regulatory peptides which may function as neurotransmitters or hormones.[3-5] Several excellent reviews on the physiological mechanisms which control motility of the esophagus[6], stomach[7], small bowel[8,9], and colon[10] are available. Advances in gastrointestinal pharmacology have also resulted in significant progress in the understanding of the receptor mechanisms which exert a profound influence on gastrointestinal motor function.[3,11] Progress in these areas of cellular and organ physiology has stimulated an interest in synthesizing a new generation of drugs to enhance gastrointestinal motility.

Gastrointestinal motility enhancing agents have not been reviewed in depth in the Annual Reports In Medicinal Chemistry. We have taken, therefore, the liberty of including background information on classical motility enhancing agents. For those who are neither gastrointestinal physiologists nor pharmacologists, we have included a brief outline on (a) the neuroanatomical organization of the gastrointestinal tract[12-14] and on (b) the methods employed to study motility and its disorders. Because the gastrointestinal tract is an endocrine organ, we have also included information on peptides and hormones which are directly or indirectly implicated in enhancing gastrointestinal motility. Detailed information on neuroanatomy and methodology is available in recent reviews.

Neuroanatomical Organization of the Gastrointestinal Tract - Both ends of the gastrointestinal tract (oro-pharynx and part of the cervical esophagus; anal canal) are composed of skeletal muscle. The remainder of the alimentary tract is composed of smooth muscle which is organized into several layers.[1,12] The muscularis externa is composed of a thick inner layer of smooth muscle cells in which the long axis of the cells

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is oriented in a circular direction and an outer thinner layer in which the long axis of the cells is oriented in the longitudinal direction. The outer longitudinal and inner circular muscle are attached to each other by connective tissue. The mucosa, the innermost layer of the gastrointestinal tract, contains a band of organized smooth muscle cells called the muscularis mucosa. The function of this muscle layer is unclear. In between the muscularis mucosa and circular muscle is the submucosa which is composed of connective, lymphatic, and vascular tissue. The general arrangement of muscle layers is the same throughout the gastrointestinal tract with minor variations. In the stomach, for example, the smooth muscle cells are also arranged obliquely. The smooth muscle cells within a given layer make various types of contacts which allow for electrical coupling between the cells.[17] Whether there is any coupling between the cells of longitudinal and circular muscle layers is controversial.[17]

Between the muscle layers resides a network of neural tissue, the enteric nervous system.[18-20] This peripheral nervous system consists of five networks or plexuses. The two main ganglionated plexuses are the myenteric (Auerbach's plexus) and the submucous (Meissner's plexus).

The myenteric plexus resides between the longitudinal and circular muscle layers and consists of a primary network of unmyelinated fibers which are arranged in bundles and connect the various ganglia. The secondary network is continuous with the primary network and contains small fibers and fewer neurons. The secondary network, in turn, is connected to the tertiary network which ramifies within the circular and longitudinal muscle layers. Meissner's plexus is located in the submucosa of the gut wall. The ganglia of this plexus are absent in the esophagus and anal canal. In addition to the two major plexuses are: a variably developed subserous plexus situated beneath the serosa; a mucosal plexus situated in the mucosa; and a deep muscular plexus situated in the deep layers of circular muscle.

The morphology of the enteric ganglia bears a close resemblance to the central nervous system , so much so that a layer analogous to the blood brain barrier has also been shown to envelop the ganglia.[22] A detailed account on the morphology, electrophysiology, and pharmacology of the enteric nervous system is available in several recent reviews.[18,23-25]

The central nervous system (parasympathetic) sends preganglionic fibers through the vagus and pelvic splanchnic nerves to the gut. The vagal preganglionics arise from the dorsal motor nucleus in the brain stem. These fibers synapse on the intramural myenteric ganglia which contain the postganglionic neurons. The sacral parasympathetic fibers accompany the roots of sacral segments 2, 3 and 4. The sympathetic preganglionic fibers synapse on the prevertebrael ganglia (celiac, superior, inferior mesenteric). The postganglionic neurons from the sympathetic ganglia travel along the blood vessels to the gut wall. All neurons in the gastrointestinal tract are not efferent. The preganglionics contain efferent (motor) and afferent (sensory) fibers whereas the intramural plexus contains cell bodies and processes of afferent, efferent, and interneurons. All components of the enteric and central nervous system are essential for coordination of gastrointestinal motility.

Methodology - Over the past twenty years gastrointestinal motility has been monitored using several different techniques. Motility is a dynamic term and consists of two distinct components, the movement of the gut wall and the movement of ingesta through the gastrointestinal tract. The two components are related but are independent variables. A single method cannot, therefore, be used to evaluate motility. The movements of the gut wall can be measured by (a) recording intraluminal pressure and by (b) attaching electromechanical sensors to the gut wall. The measurement of transit is complex and is usually performed by placing markers in the lumen of the bowel and following their progress. Each method has advantages as well as disadvantages. Methodology of gastrointestinal motility has been described in detail in several books and review articles.[15-17,26]

Normal and Abnormal Motility - Different parts of the gastrointestinal tract have diverse functions, and hence the motility of each organ is different. For example, since the major function of the esophagus is to carry the ingesta from the pharynx to the stomach, this function is performed adequately by peristaltic movements which travel in the aboral direction. The speed of peristalsis and amplitude of contractions are regulated at three different levels, i.e. by the central nervous system, the enteric nervous system, and by the smooth muscle. Pathology at any of these centers results in disordered motility and can produce such entities as diffuse esophageal spasms, achalasia, etc. The small bowel, on the other hand, is involved in bringing nutrients in contact with the luminal surface for ultimate absorption and for propelling unabsorbed material in an aboral direction. These functions are performed by peristaltic movements.

In the stomach and small bowel large size indigestible particles along with digestible ones are transported aborally by a cyclically occurring process which is called the migrating myoelectrical complex (MMC),[8] the so-called "interdigestive housekeeper."[9] The MMC in humans has an average cycle length of 100 minutes and is disrupted when food is ingested. In the interdigestive stage MMC can be divided into four phases. Phase III is the most prominent phase which is marked by high amplitude contractions. The mechanisms which initiate or control MMC cycles are not known. For a detailed account on the origin and regulation of MMCs, readers are referred to many recent reviews.[8,9]

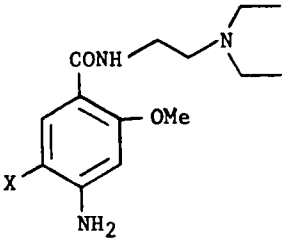
Motility Enhancing Agents

(a) Muscarinic Agonists and Cholinesterase Inhibitors - Acetylcholine is an excitatory neurotransmitter and in the gastrointestinal tract evokes contractions.[1,2,27,28] Other muscarinic agonists such as bethanechol, methacholine or carbachol also increase intestinal motility. Therapeutically bethanechol is the preferred muscarinic agonist since it is devoid of nicotinic effects and acts directly on the muscle.[1] Bethanechol increases the amplitude of contractions in the esophagus, increases lower esophageal sphincter (LES) tone,[6,25] and increases motility of the stomach, small bowel, and colon.[29,30] It also has been traditionally used in patients suffering from gastroparesis, postoperative paralytic ileus, and intestinal obstruction.[1,31-33]

Drugs which are cholinesterase inhibitors act as muscarinic agonists, enhancing the effect of released acetylcholine by inhibiting its degradation.[34] An example of such a drug is neostigmine which has been used clinically to enhance tone and peristaltic contractions in both the small and large bowel.[35-37]

(b) Benzamides

Metoclopramide (1) - Since its introduction, metoclopramide has been used therapeutically to accelerate bowel transit in fluoroscopic examinations, to facilitate tube placement into the small bowel, to relieve diabetic gastroparesis, and to treat gastroesophageal reflux.[39,40] Several excellent reviews on metoclopramide are available.[39-43] Although the literature is often conflicting, metoclopramide has been shown to increase the resting tone of the LES, enhance gastric peristalsis, increase gastroduodenal coordination, and decrease small bowel transit time.[44] Metoclopramide is a centrally acting dopamine-D_2 antagonist which also possesses cholinergic, quinidine-like, and local anesthetic properties.[1,45] In the smooth muscle where it stimulates contractions, the effect is exerted via cholinergic nerves.[46] However, recently metoclopramide has also been shown to act directly via non-cholinergic mechanisms.[47]



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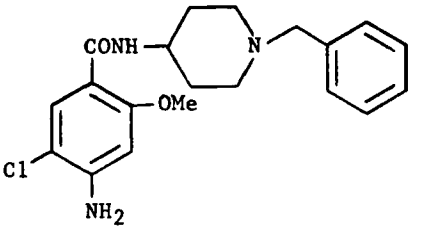

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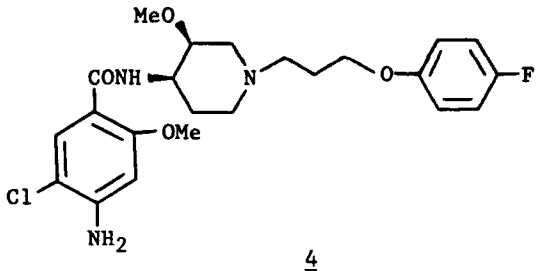
Bromopride (2) - In clinical studies it has been demonstrated that bromopride increases esophageal motility, increases LES tone, and accelerates small bowel transit.[48] Studies on field-stimulated guinea pig ileum strips suggest that bromopride, like metoclopramide, increases the amplitude of contractions by acting on cholinergic nerves.[49]

Clebopride (3) - This substituted benzamide has been shown to facilitate gastric emptying and increase lower esophageal sphincter pressure.[24,43,50-54]

Cisapride (4) - This is a gastroprokinetic drug which has been shown to have no antidopaminergic effects.[55] In guinea pigs and dogs cisapride stimulated the motility of the stomach and small intestine and improved antroduodenal coordination.[55,56] Cisapride has also been shown to: improve gastric emptying; induce premature MMC in the ileum and colon; increase propulsive activity; accelerate small bowel transit time; and shorten colonic transit time in dogs and humans.[57-60] In humans, cisapride increases the esophageal motility.[61] The exact mechanism(s) by which cisapride exerts its prokinetic effect is not known.[55] It has been suggested that the colonic effects of cisapride are mediated via a noncholinergic system whereas in the upper gastrointestinal tract the effects are mediated through facilitation of cholinergic transmission.[61]

Dazopride (5) - This analog of metoclopramide is in an early stage of clinical development. It has been suggested that dazopride does not cross the blood brain barrier and, thus, is devoid of central nervous system side effects observed with metoclopramide.[62] In anesthetized cats dazopride increases the tone of the lower esophageal sphincter[63]; in dogs dazopride increases antral contractions.[64]

Effects of dazopride on other parts of the alimentary tract are unknown.



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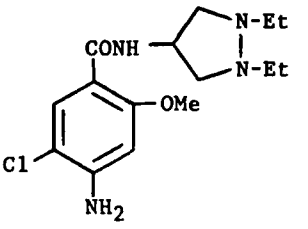

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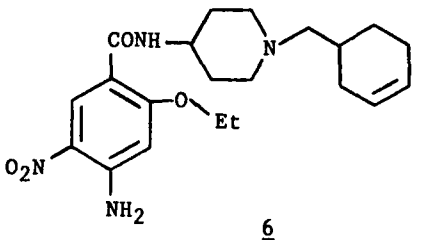
Cinitapride (6) and BRL-20627 (7) - These are two other gastroprokinetic agents reported to be devoid of dopamine antagonist activity.[46] Cinitapride increases the amplitude of field-stimulated guinea pig ileum contractions whereas BRL-20627 augments basal stomach activity.[65] It has been suggested that these compounds may act on prejunctional sites. Whether they act at a specific receptor site is unknown.[46]



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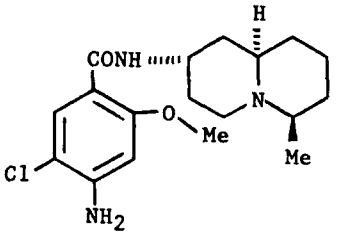

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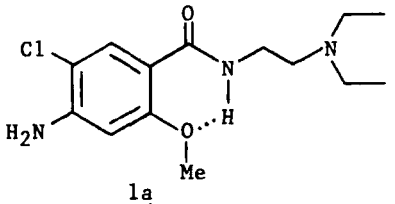
Mechanism of Action of Benzamides - In this group of compounds, also known as o-anisamides and orthopramides, metoclopramide is the oldest and most extensively studied. Until recently, metoclopramide was assumed to act through its antidopaminergic activity. A considerable body of evidence against the existence of specific dopamine receptors in the gut has accumulated,[51,66] and it is generally accepted that benzamides do not affect the gut system via their antidopaminergic activity.[46]

Experimental evidence for the existence of two kinds of 5-hydroxytryptamine (5-HT) receptors on the cholinergic nerves of the guinea pig myenteric plexus has been obtained: stimulatory, whose excitation leads to stimulation of acetylcholine release, and inhibitory with an opposite effect.[67] Both metoclopramide and cisapride are postulated to act as partial agonists on the excitatory 5-HT receptor based on the stimulation of acetylcholine release.[67] Additional evidence for the role of 5-HT in enhancing gastric motility was obtained in dogs.[68] Evidence for cisapride's acting as a 5-HT antagonist on myenteric neurons of guinea pig ileum preparations was presented recently.[69] In summary, metoclopramide and congeners appear to exert their effect upon the gastrointestinal tract via 5-HT receptors, although the exact mechanism remains to be elucidated.

Structure-Activity Relationships - With the exception of cinitapride, all of the benzamides are derived from 4-amino-5-chloro-2-methoxybenzamide in which the basic side chain nitrogen is separated by either two or three carbon atoms from the amide nitrogen atom. In an attempt

to examine topographical properties of substituted benzamides which are responsible for selective receptor binding, a quantum mechanical PCILO method was applied to model compounds of metoclopramide,[70] and clebopride[71]. From these studies it was concluded that metoclopramide and congeners should have a limited conformational freedom due to the presence of an intramolecular hydrogen bond between the amide hydrogen and methoxy oxygen acting as a conformational lock, as illustrated by 1a for metoclopramide. It was thus postulated that the pseudo cycle resulting from this hydrogen bonding is a key factor in receptor recognition[71].



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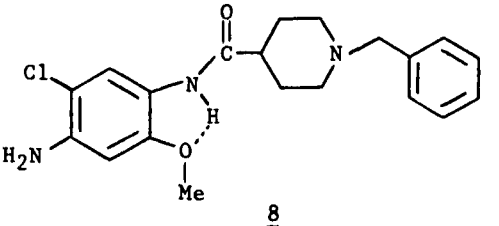

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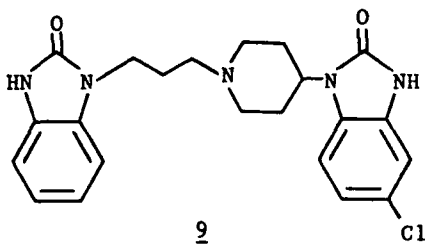
Subsequent work using ^{1}H NMR studies showed that hydrogen bonding, while important in $CDCl_3$, does not occur in D_2O. Nevertheless, its existence was postulated in the lipophilic environment of membranes and receptors.

IR spectroscopy indicated stronger hydrogen bonding in clebopride compared to the reversed amide analog 8.[73] This finding was used to rationalize the lack of gastroprokinetic activity noted with 8 even though it was essentially equipotent to clebopride in antagonism of apomorphine-induced climbing activity in mice. Whether or not hydrogen bonding is an essential structural feature in the benzamide series remains to be confirmed; however, to date no prototype compound deviating from the above described general structure has been found to be active in enhancing gastrointestinal motility.

(c) Domperidone (9) - Domperidone differs from the benzamides both structurally and by mode of action. Structurally it is a benzimidazole analog of pimozide (10), and pharmacologically it is a potent peripheral dopamine antagonist.[74] Its mode of action upon the gastrointestinal tract is unclear. Suggestions have been advanced that domperidone's action is mediated via α_1- and α_2-adrenergic receptors, as well as dopaminergic receptors.[46,75,76] In humans, domperidone has been shown to stimulate esophageal motility and increase lower esophageal sphincter tone.[73-84] In animal experiments, domperidone produced inconsistent effects on gastric tone and gastric emptying. In the field-stimulated in vitro preparations domperidone increased the amplitude of gastric contractions in the guinea pig, dog and humans. In the intact dog, however, while domperidone did not affect the propagation velocity of antral contractions it did reduce the frequency of contractions in the antrum. Domperidone has been shown to improve gastroduodenal coordination and thus improve gastric emptying.[85] The effect of domperidone on the small and large intestine is not known.

(d) Peptides and Hormones - The role of peptides in gastrointestinal motility is becoming very important since various peptides have been localized immunocytochemically, and their role as neurotransmitters or neuromodulators is becoming apparent.[3-5] Secretin was the first peptide hormone discovered, and now about 20 more peptides which are



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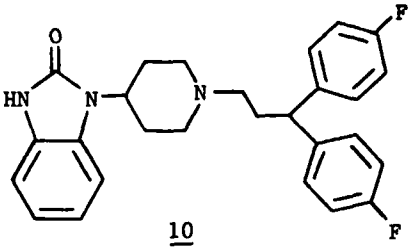

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biologically active have been identified.[86] Our purpose here is to briefly describe those bioactive peptides which enhance gastrointestinal motility. Detailed information as to their structure and function can be found in several excellent reviews.[3-5,86,87]

The influence of a particular peptide on gut motility may be different in vivo and in vitro.[3] For example, cholecystokinin in in vitro preparations causes contractions of the LES, stomach, small and large bowel in various animal species.[7,9] In vivo, however, cholecystokinin produces contractions only in the colon.[87]

Gastrin, another peptide, increases sphincter tone and produces contractions in the stomach, small and large bowel.[4,5] Motilin also produces contractions in the gastrointestinal tract; the effect, however, is pronounced only in conscious animals and in the interdigestive stage of the MMC pattern.[8,11] Several other peptides such as vasoactive intestinal peptide, secretin, and glucagon influence gastrointestinal motility by inhibiting or relaxing smooth muscle. Substance P caused contractions in the LES of opossums.[86] We are unaware of any peptides which are currently being used as therapeutic agents in patients with disorders of gastrointestinal motility.

In summary, the currently available prokinetic compounds have therapeutic usefulness in treating some gastrointestinal motor disorders. Their lack of specificity, potency, and the side effects associated with their use limit their benefit. There is a need to synthesize and identify prokinetic compounds which will enhance gastrointestinal motility and be devoid of side effects. This goal will be achieved only when further advances are made in our understanding of the physiology and pharmacology of gastrointestinal motility.

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SECTION III-Chemotherapeutic Agents

Editor: Frank C. Sciavolino, Pfizer Central Research
Groton, Connecticut 06340

Chapter 13. ß-Lactam Antibiotics

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Introduction - Though the ß-lactam antibiotics are probably the most thoroughly studied single class of therapeutic agents, new discoveries continue to emerge. Notable among the newer developments in 1984 was the discovery by three industrial research groups of a new class of naturally-occurring 7α-substituted cephalosporins. In addition, a new semisynthetic 7α-substituted penicillin having increased stability to ß-lactamases was described. An emerging problem of potential significance to the therapeutic use of the newer ß-lactam antibiotics is the development of resistance to these drugs during therapy by pathogens which produce an inducible, chromosomally-mediated ß-lactamase.[1] The most frequent causative organisms are Enterobacter, Serratia and Pseudomonas species. This type of resistance is expressed across ß-lactam classes. Reviews have appeared on ß-lactams and resistance,[2] clinical perspectives on the 3rd generation cephalosporins,[3-5] and broad-spectrum penicillins.[6,7] Reviews also appeared discussing the relationship between ß-lactam structure and ß-lactamase stability,[8,9] properties of the newer cephalosporins,[10] historical development of the cephalosporins,[11] chemistry of monobactams,[12] synthesis and SAR of aminothiazole cephalosporins,[13] chiral syntheses of ß-lactams,[14] X-ray studies on penicillins and cephalosporins,[15] and penicillin binding proteins (PBPs).[16] A review of the properties, biosynthesis and fermentation of carbapenems was published[17] as was a comprehensive two part set of books devoted to the discovery, fermentation, biosynthesis, SAR and clinical use of the ß-lactams.[18,19] Several chapters of another book also described the properties, biosynthesis and fermentation of the ß-lactams.[20] The proceedings of an international FEMS symposium on the architecture and growth of bacterial cell walls was published in book form.[21] One review[22] and several publications[23-24] reported studies on the mechanisms of ß-lactam ring opening. Several studies attempting to correlate spectral,[25-27] electronic[28,29] and X-ray[30,31] data with biological activity were published.

Cephalosporins - A new class of cephalosporins containing a 7α-formamido group, typified by chitinovorin A (1), was isolated from the fermentations of three different genera of bacteria. Research groups from Squibb,[32] Shionogi[33] and Takeda[34-37] reported the discovery almost simultaneously. These compounds, named chitinovorins or cephabacins, also have a unique peptide-containing substituent at the 3-position. Though the derivatives reported had weak antibacterial activity, the 7α-formamido group imparted good ß-lactamase stability. The focus of semisynthetic modifications in the ß-lactam antibiotics has been directed toward derivatives having broad-spectrum activity with increased potency against both gram-positive organisms and Pseudomonas or derivatives with improved pharmacokinetics. Several new analogs containing 7-heterocyclyl-α-oximino-acetamido side chains having broad-spectrum activity and improved pharmacokinetics were reported. Extensive SAR studies[38-40] led to the new parenteral compound FR17126 (2) whose spectrum of activity

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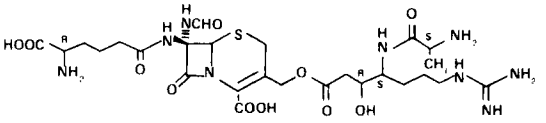

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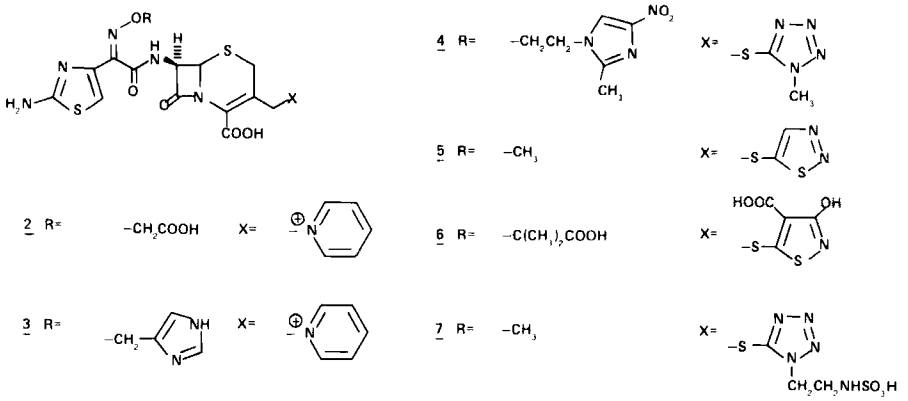
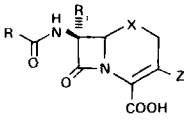
is comparable to ceftazidime's but is 2-4 times more potent versus some Pseudomonas, Proteus and Enterobacter species.[41,42] Two 7-[aminothiazolyl-oximino]cephalosporins with unique substituents attached to the oxime were reported. DN9550 (3) is reported to have a spectrum and potency similar to ceftazidime's[43,44] while YM14408 (4) has increased Gram-positive activity but is weakly active against Pseudomonas.[45] L-105 (5), a structural analog of cefotaxime with a similar spectrum of activity, exhibited increased potency versus Gram-positive bacteria compared to other third generation cephalosporins.[46-48] Two other analogs, YM-13115 (6)[49] and SKF 88070 (7),[50-53] were reported to show antibacterial and pharmacokinetic profiles similar to those of ceftriaxone. The synthesis and SAR studies of analogs in which the 2-aminothiazole moiety has been oxidized to the N-oxide led to FCE 20635 (8)



which is similar to cefotaxime in its in vitro and in vivo antibacterial properties.[54] CGP 31523A (9) is reported to have broad-spectrum activity similar to HR810 but with superior activity versus S. aureus.[55] CGP17520 (10), a parenteral cephalosporin with asecond generation spectrum of activity, showed therapeutic efficacy against S. aureus and several other Gram-positive bacterial infections in mice superior to that of cefuroxime or cefoxitin even though in some instances its in vitro activity was inferior.[56-58] An oxacephem, 2355-S (11), showed potent broad-spectrum activity similar to that of 6315-S (12) but had a superior pharmacokinetic profile.[59,60] The synthesis and SAR study of a series of



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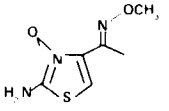
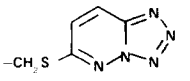
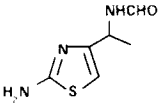
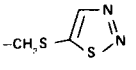
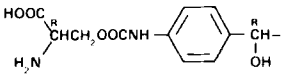
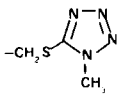
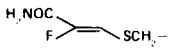
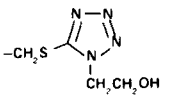
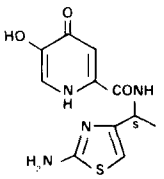
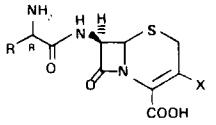
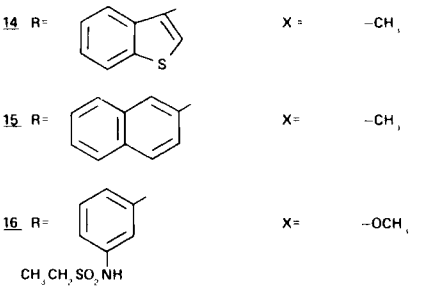
8		-H		S
9		-H		S
10		-H		S
11		$-OCH_3$		O
12	F_2CHSCH_2	$-OCH_3$	"	O
13		-H	-H	CH_2

carbacephems also was reported.[61] The most active compound from the series, KT-4697 (13), retained broad-spectrum activity against Gram-negative bacteria including Pseudomonas, but in vivo activity was weak. Penicillins andcephalosporins with 7α-substituents other than methoxy generally show reduced antibacterial activities. However, a research group from Beecham has synthesized a series of 7α-hydroxymethyl-cephalosporins having the piperacillin/-cefoperazone-type of 7-acyl side chain. These agents exhibit activity versus Gram-negative bacteria superior to cefoperazone's, but they have little activity against Gram-positive organisms.[62] Researchers at Eli Lilly carried out extensive SAR studies on 7-arylglycyl cephalosporins in searching for an oral agent with a long duration of action. Two analogs were selected for more extensive evaluation.[63,64] The in vitro activities of LY164846 (14) and LY171217 (15) are limited to Gram-positive bacteria and H. influenzae with potencies similar to those of cephalexin and cefaclor.[65,66] Both were well-absorbed orally in laboratory animals, but their routes of elimination varied dramatically among the species tested.[67,68] CGP19359 (16), another oral cephalosporin with a spectrum of activity like cephalexin and cefaclor, was well-absorbed orally in man and produced prolonged serum concentrations.[69] Interest in pro-drug forms of parenteral cephalosporins has continued with further studies on T-2588,[70]

cefuroxime axetil[71-74] and water soluble esters of aminothiazole derivatives.[75] Publications also appeared describing SAR studies on the cephamycin MT-141,[76,77] and laboratory and/or clinical studies on cefpimizole (U-63196E),[78,79] cefodizime,[80-83], BMY-28142,[84-86] cefpirome (HR-810)[87,88] and cefonicid.[89]



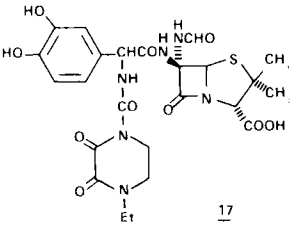


Penicillins - Researchers at Beecham synthesized the 6α-formamidopenicillins and 7 α-formamidocephalosporins from the corresponding 6α or 7 α-methylthio derivatives by a single, mercury-catalyzed, reaction with N,N-bis(trimethylsilyl)-formamide.[90] This work paralleled the discovery of the naturally-occurring 7α-formamidocephalosporins. BRL36650 (17), the parenteral 7α-formamidopencillin having a piperacillin-like 7-acyl function, displays potent, broad-spectrum activity with MIC values below 1.0 μg/ml against most Gram-negative bacteria, including Pseudomonas. It is essentially devoid of activity against Gram-positive bacteria comparing well both in vitro and in vivo with ceftazidime against Pseudomonas species and with cefotaxime and aztreonam versus the Enterobacteriacae.[91-94] BRL36650 is stable to bacterial ß-lactamases, even the chromosomally-mediated Type 1 ß-lactamases produced by Enterobacter and Pseudomonas species. Preliminary pharmacokinetic studies in man show it to be well tolerated and to exhibit a favorable pharmacokinetic profile.[95] The synthesis, antibacterial activity and ß-lactamase stability of a group of 6α-hydroxymethylpenicillins was reported.[96] Penicillin analogs also were reported in which the 2-position gem-dimethyl groups have been removed[97], the 6-amide NH group was replaced by O, C and S,[98] and the 6-amide function was replaced by a vinyl group.[99] Additional papers were published on pharmacokinetic studies in laboratory animals and man of the orally-absorbed pro-drugs lenampicillin (KBT-1585)[100,101] and bacmecillinam.[102]

Penems - The synthesis and biological evaluation of a series of 2-alkyl(aryl)oxy and 2-alkylthiopenems were reported.[103-105] Extensive in vitro and in vivo laboratory evaluations of the parenteral penem SCH34343 (18) have shown it to have potent activity against a broad-spectrum of Gram-positive and Gram-negative bacteria including Bacteroides species but not Pseudomonas aeruginosa.[106-109] SCH34343 is stable to a variety of plasmid and chromosomal ß-lactamases.[110] Following parenteral dosing in rats and dogs 41-45% of unchanged drug was excreted in the urine.[111] Like they are with thienamycin, PBPs 2, 1a and 1b appear to be the primary targets for 18.[112] The syntheses and in vitro antibacterial activities of a series of 2-heterocyclylthiomethylpenems, some of which displayed potent activity, were published.[113]

Carbapenems - The total synthesis of L646,591 (19) and a comparison of its essential chemical and biological properties with those of thienamycin was published.[114] 19 is chemically stable, highly resistant to renal dipeptidase-I and retains high in vitro antibacterial activity. A SAR study of a group of 5,6-cis-carbapenems related to epithienamycin concluded that relative affinities for essential PBPs are more important in determining antibacterial activity than

are permeability effects and ß-lactamase production.[115] The syntheses of 2-ß-alanyloxymethyl, 2-glycyloxymethyl and 2-methyl analogs of thienamycin were described.[116] These analogs showed only weak activity against P. aeruginosa though they displayed potent, broad-spectrum activity toward Gram-positive and other Gram-negative bacteria. Analogs of thienamycin and compound 19 in which the 3-carboxyl group was replaced by a tetrazole ring were ten times less active in vitro than their carboxyl counterparts.[117] A stereocontrolled, total synthesis of (+) thienamycin was published.[118] Carbapenams OA-6129D and -E were isolated from fermentation of a Streptomyces and postulated to be intermediates in the biosynthesis of the corresponding carbapenems.[119]



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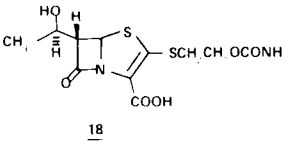

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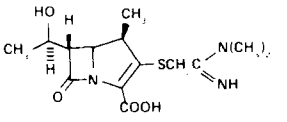
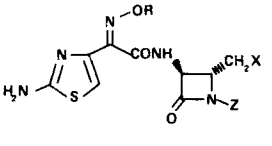


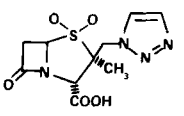
19

Monocyclic ß-Lactams - SQ82,531 (20), an orally-adsorbed pro-drug ester of the parenteral monobactam SQ82,291 (21), was reported.[120] In these compounds the N-1 sulfonate group present in aztreonam was replaced by an oxyacetic acid moiety. Here also, methylation at C-4 improves antibacterial activity and ß-lactamase stability.[121] Compound 21 exhibited poor activity against Gram-positive and anaerobic bacteria but, with the exception of Pseudomonas species, displayed potent activity against the Enterobacteriaceae comparing favorably with amoxicillin, cephalexin, cefaclor and cefuroxime against susceptible organisms. Like aztreonam, 21 has highest affinity for PBP-3. After oral administration of 20 to mice, 18% was recovered as 21 in the urine.[122] The carboxyl group of the N-1-oxyacetic acids was replaced with tetrazole, sulfonate, phosphonate and phosphinate groups; in vitro activity against susceptible Gram-negative bacteria decreased in the order $COOH = SO_3H > \text{tetrazole} = P(R)O_2H$.[123] Comprehensive reviews were published on SAR[124,125] and on the influence of ring-activating substituents at the N-1 position of monocyclic ß-lactams.[126] Substitution of tetrazole or tetrazole-N-acetic acid moieties for the N-1 sulfonate group produced derivatives having only moderate to poor activities.[127,128] The 4-α-fluoromethyl derivative BO-1165 (22) is more potent in vitro than aztreonam against the Enterobacteriaceae, is similar against Pseudomonas, and is stable to most ß-lactamases.[129] Papers were published on the synthesis and in vitro activities of 4-hydroxymethyl, 4-acyloxymethyl[130] and 4-alkoxycarbonyl analogs of aztreonam.[131] Human pharmacokinetics of Ro17-2301 (AMA-1080) were reported.[132] A chlorinated analog of nocardicin A was isolated from a Streptomyces species.[133,134]



	R	X	Z
20	CH_3	H	$-OCH_2COOCH_2COOC(CH_3)_3$
21	CH_3	H	$-OCH_2COOH$
22		F	$-SO_3H$

ß-Lactamase Inhibitors - A novel penicillanic acid sulfone, YTR-830 (23), was shown to be more effective than sulbactam in reducing the MICs of ampicillin or amoxicillin against Gram-positive and Gram-negative organisms and compared favorably with or, in some cases, was superior to clavulanic acid.[135,137] A brief overview of the ß-lactamases and their inhibition was published.[138] Several publications described studies on the nature of inactivation of ß-lactamases by various inhibitors. Penicillanic acid sulfone appears to inhibit irreversibly the RTEM ß-lactamase from E. coli by forming a crosslink within the enzyme. The crosslink is a ß-aminoacrylate fragment derived from C-5, C-6, C-7 of the inhibitor.[139] The novel inhibitor 24 also appears to inactivate the RTEM ß-lactamase by the same kind of mechanism.[140] The E-isomer of 24 is not a substrate, inhibitor or inactivator of the enzyme. Easton and Knowles[141] studied the effectiveness of five different ß-lactams at inhibiting the TEM-2 ß-lactamase. They correlated inhibition of the isolated enzyme with the ability of the inhibitors to reduce the MICs of ampicillin in intact cells which produce this ß-lactamase. The best inhibitors in whole cells were those that required the fewest number of molar equivalents to inhibit the purified enzyme. By this criteria, the effectiveness of inhibitors studied decreased in the order carbapenems > clavulanic acid > quinacillin sulfone > sulbactam. Unlike clavulanic acid and sulbactam, which are irreversible inhibitors of ß-lactamases, the carbapenem SF2103A was found to act as a tight-binding, competitive inhibitor.[142]



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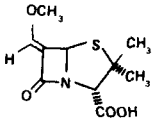

24

Enzymatic Synthesis of Novel ß-Lactams - The progress made in recent years in unraveling the biosynthetic pathway that converts the Arnstein tripeptide, L-α-aminoadipyl-L-cysteinyl-D-valine (ACV), into penicillins and cephalosporins has opened a new avenue to synthesizing novel ß-lactams. The research of Baldwin and Demain and Wolfe has demonstrated that tripeptides related to ACV can act as substrates for the normal biosynthetic enzymes and thereby produce modified ß-lactams. Thus, using purified enzyme preparations AC-(D-allylglycine), AC-(D-isoleucine) and AC-(D-norvaline) have been converted by this method into 2-allyl and 2-ethyl penicillins accompanied by varying proportions of cephams, homocephams and homocephems.[143,144] More recently, four pathway enzymes from Streptomyces clavuligerus have been immobilized on an ion-exchange resin, and, in the presence of appropriate cofactors, selected tripeptides were converted into modified penicillins or cephalosporins.[145] Enzyme specificity requirements dictate that L-α-aminoadipyl-L-cysteinyl moieties comprise the first two amino acids of the tripeptide. Modified penicillins thus were produced by substituting the appropriate D-aminoacid for D-valine. Using this approach the nucleus of ceftizoxime, containing a D-α-aminoadipyl side chain on the 7-amino group, was synthesized.

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Chapter 14. Determinants of Microbial Resistance to Beta-lactam Antibiotics

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Introduction—Until recently, the medicinal chemist has had little opportunity to take advantage of the biological research that described the organization and function of the envelope of Gram-negative bacteria. Rational design of effective antibiotics was limited in scope to increasing beta-lactamase stability or designing inhibitors of this enzyme. The trend toward development of very broad spectrum beta-lactams has encouraged the pharmaceutical industry to take advantage of recent basic scientific discoveries aimed at elucidating the organization and function of the bacterial envelope. Since an effective, broad spectrum agent must penetrate the outer membrane (OM), survive diffusion among the periplasmic beta-lactamases, and bind avidly to penicillin-binding proteins (PBPs), an understanding of the interactions of these resistance determinants becomes vital for the design of new agents. Many excellent reviews have described in detail these individual determinants.[1-10] The scope of the present report will be to give a more unified picture of how the OM, beta-lactamase, and PBPs interact to influence the effectiveness of broad spectrum beta-lactams.

Penetration Across the OM—The initial barrier to beta-lactams in Gram-negative bacteria is the outer surface of the cell, which consists of capsular material and the OM. Capsules and external slime layers usually play a minor role in reducing the rate of diffusion of anionic beta-lactams into the cell.[11,12] A more relevant barrier limiting free diffusion of beta-lactams into the bacterial cell is found in the OM, located exterior to the peptidoglycan (Figure 1).

The OM is an asymmetric membrane characteristic of Gram-negative organisms only and is composed of lipopolysaccharide (LPS), phospholipids, lipoproteins, and proteins.[3,13-15] The outer half of the lipid bilayer contains mostly LPS and protein with only a small amount of phospholipid, while the inner layer contains the majority of the phospholipid, additional protein, and no LPS.[13-15] No active transport processes exist in the OM, so the degree of free diffusion of antibiotics across the phospholipid bilayer plays a major role in determining their rate of entry into the periplasmic space. Two general mechanisms have been described to explain the diffusion of antibiotics and other molecules across the OM. The first applies to hydrophobic antibiotics that must first pass the hydrophilic LPS side chains, then partition into the hydrophobic interior of the OM, reaching the aqueous phase on the other side.[16] This process is highly influenced by temperature and the hydrophobicity of the compound.[16,17] The OM of most enteric bacteria is highly resistant to diffusion of very hydrophobic molecules, which ensures their survival in the presence of bile salts and free fatty acids in the intestinal tract.[16] The second mechanism of diffusion is employed by most beta-lactams and involves water-filled porin channels that traverse the OM.[2,13,16] That porins are the major diffusion path for beta-lactams across the OM, was supported by studies with porin-deficient mutants of *Escherichia coli* and *Salmonella typhimurium* that were resistant to these agents.[18-20] This was also supported by *in vitro* studies using purified membrane components in liposome reconstitution experiments.[21-23] The OM of *E. coli* K-12 contains approximately 1×10^5 porin channels per cell.[24] Each channel is about 1.2 nm in diameter and results from the association of a porin trimer.[24] Two major porins exist in *E. coli* K-12, Omp F (protein Ia) and Omp C (protein Ib), whose synthesis is regulated by the omp B gene.[25] Expression of the individual porin species is influenced by the environment of the cell. Thus, other minor porins such as Pho E (protein E) may be expressed under appropriate growth conditions.[24-26] Knowledge of porin regulation has proven useful, since differences in diffusion characteristics of various beta-lactams have been observed between Omp F and Omp C species,[24] with Omp F being the more efficient pore due to its slightly larger diameter.[25,27,28] Studies using oligosaccharides of varying size have defined the exclusion limit for porin channels in *E. coli* to be approximately 600 daltons.[2,16,29,30] This limit is expected to have only a minimal effect on the diffusion of beta-lactams, however, and only compounds with very bulky side groups may be marginally retarded in their movement through the channel by hitting the outer edge of the pore.[1,31]

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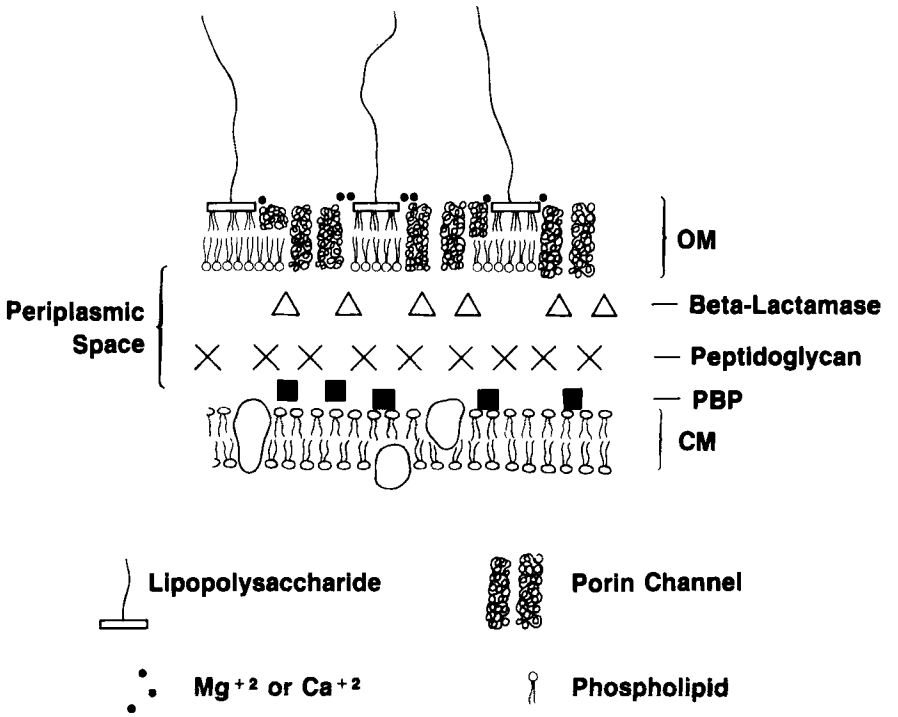
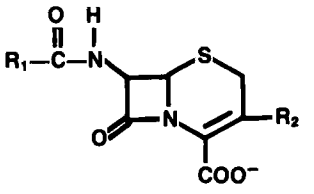
Figure 1



An important contribution to the study of beta-lactam diffusion through porin channels was made by Zimmermann and Rosselet who studied the hydrolysis of compounds by beta-lactamase, relating the degree of hydrolysis in broken and intact cells at multiple substrate concentrations.[32] Permeability measurements were taken using intact cells at steady state concentrations of substrate where the rate of antibiotic diffusion across the OM was equal to the rate of hydrolysis in the periplasmic space. This biochemical approach to permeability is more meaningful than the standard crypticity measurement taken at a single substrate concentration and has shed much light on the relationship of hydrophobicity and permeability. Using this assay, Nikaido *et al.* have been able to show that for a series of monoanionic compounds in *E. coli* K-12, a ten-fold increase in the octanol/water partition coefficient for the protonated form of a solute, resulted in five- to six-fold decrease in permeability.[1,26] When a series of monoanionic cephalosporins were compared in the Zimmermann and Rosselet assay, it was found that net charge also had a marked effect on the permeability coefficient (P) in *E. coli* containing the Omp F and Omp C porins.[1,26,31] As shown in Figure 2, those compounds having positive charges produced the highest diffusion rates within the class.[31] Compared to cefacetrile (**1**), the zwitterion cephaloridine (**2**) had an outstandingly high permeability value compared to other compounds tested.[26] The rapid diffusion of cephaloridine is likely due to the proximity of the positive charge to the 4-carboxyl group in this molecule.[26] The presence of additional negative charges, in contrast, functioned to decrease diffusion across the OM. Compounds with two negative charges like SCE-20 and cefsulodin (**3**) diffused an order of magnitude slower than cephaloridine.[26,31] Studies using proteoliposomes containing both Omp F and Omp C porins from *E. coli* were utilized to evaluate the diffusion characteristics of several newer beta-lactams.[31] Imipenem (**6**) demonstrated a relative diffusion rate of 216, the highest value of any compound analyzed, probably because of its compact structure and zwitterionic nature.[31,44] Compounds with very bulky side chains like mezlocillin, piperacillin, and cefoperazone (**4**) had ten-fold lower permeability rates through the Omp F porin compared to imipenem.[31,44] Compounds containing the oxime substituent like ceftizoxime (**5**), cefotaxime, and cefuroxime, showed permeability values significantly lower than those predicted from their hydrophobicity measurements. The oxime side chain has been shown to form a hydrogen bonded ring structure with the amide hydrogen on C-7 of these cephalosporins, and it is thought that this ring formation causes steric hindrance that interferes with diffusion through the porin channels.[31]

Porins have been identified in many other genera of Gram-negative bacilli, although their interactions with beta-lactams have not been characterized as fully.[33,34,44] The porins in *Pseudomonas aeruginosa* appear

Figure 2



	R_1	R_2	Relative Diffusion Rate
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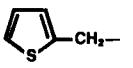
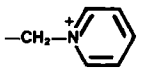
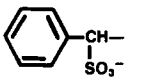
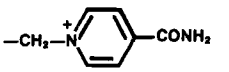
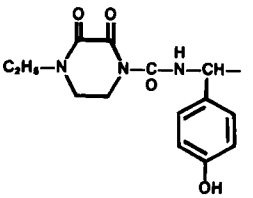
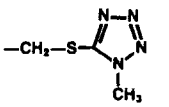
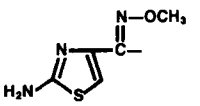
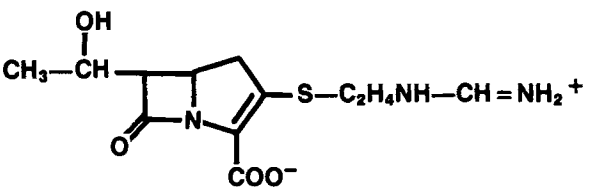
2			167
3			37
4			16
5		H	35

Relative diffusion rate of five cephalosporins through liposomes containing the Omp F porin.[31] Rates are normalized to that observed for cephacetrile. Diffusion is influenced by hydrophobicity, net charge, and size of substituents.



6

quite different than those in enteric organisms since their exclusion limit is several thousand daltons.[35,36] This larger pore size renders hydrophobicity and net charge less important in determining antibiotic activity against *Pseudomonas* than against enteric organisms. The relative ineffectiveness of many beta-lactams against *P. aeruginosa* comes from the non-functional nature of the porins in the OM. Although this organism has roughly the same number of porin channels as *E. coli*, it has been estimated that only 1 in 400 are maintained in a functional

state that would allow penetration of beta-lactams.[35-37] Thus, the regulation of porin function along with the LPS composition of the OM and beta-lactamase activity, all contribute to the resistance of *P. aeruginosa* to beta-lactams.[36-43]

In laboratory strains of bacteria, accumulated data indicate that rapid diffusion of beta-lactams through the OM can be correlated with susceptibility.[18-20,26] It is not surprising then, that beta-lactam resistant mutants with deficient levels of porins[33,45,46,48] or changes in OM phospholipid and LPS composition[43,47,49,50] have been isolated both in the laboratory and in the clinic. Mutants with pleiotrophic effects on porin expression have also been isolated *in vitro* with *E. coli* K-12 using beta-lactams of current clinical interest such as cefoxitin, cefotaxime, aztreonam, imipenem, mecillinam, and SCH-29,482.[25,51] In *E. coli* K-12, the production of Omp F is depressed at 37°C or in growth medium containing 0.9% NaCl.[52,53] This makes sense from a survival point of view since Gram-negative organisms would benefit from decreasing production of the more efficient Omp F pore *in vivo*, thereby avoiding diffusion of a variety of toxic substances into the cell. This appeared to explain the emergence of resistance during therapy with a mutant strain of *S. typhimurium* that totally lacked the Omp C porin.[37] When grown at 37°C, the mutant expressed decreased levels of Omp F porin as well, which led to the development of high level resistance to beta-lactams.[37] Therefore, regulation of the Omp F porin *in vivo* could play an important role in the development of resistance to some beta-lactams.

Despite the multiplicity of factors that determine the effectiveness of beta-lactams, it seems evident that OM permeability remains an important component in the puzzle. Some new compounds like imipenem appear to possess rapid diffusion characteristics in Gram-negative bacteria and thereby demonstrate an excellent spectrum of activity including strains resistant to the third generation cephalosporins. Preliminary studies suggest that carbapenems may also have an additional diffusion mechanism not observed with other beta-lactams.[51] In this sense, novel beta-lactams may still be designed that combine excellent permeability characteristics with stability to beta-lactamases. The interactions of beta-lactams with these enzymes in the periplasmic space will be considered in light of the unexpected emergence of resistance to some beta-lactamase stable compounds.

Interactions with Periplasmic Beta-lactamases—The second barrier to free diffusion of beta-lactams to the PBPs lies with beta-lactamases which in Gram-negative organisms are strategically located in the periplasmic space (Figure 1).[54] This location makes any consideration of their role in resistance linked to the OM permeability of beta-lactams. Thus, the net impact of beta-lactamase on a given substrate depends on (1) the concentration of substrate in the periplasmic space, (2) its affinity for beta-lactamase, and (3) its susceptibility to hydrolysis. Several reviews have outlined the various classification schemes for beta-lactamases.[4,6,55-57] A much simplified overview indicates that the plasmid encoded beta-lactamases are generally penicillinases that are produced constitutively and with exceptions, do not confer resistance to the newer cephalosporins, penems, carbapenems, or monocyclic beta-lactams. A broader variety of enzymes are encoded by chromosomal genes and these beta-lactamases are species or sub-species specific, inducible in some genera, and do not rapidly hydrolyze most of the third generation cephalosporins and novel beta-lactams mentioned above.[4,6]

The most predictable outcome of beta-lactamase expression has been seen for the plasmid encoded enzymes. Spread of the Richmond and Sykes Type III TEM beta-lactamase has severely limited the clinical utility of penicillin G and ampicillin[58] but has had little impact on the efficacy of the newer agents. Some of the more unexpected observations have involved the Richmond and Sykes Type I chromosomal cephalosporinases.[4,57] Sensitive isoelectric focusing methods have shown that practically all Gram-negative bacilli contain some type of chromosomally-mediated beta-lactamase,[59] although the level of expression of the enzymes varies greatly among individual isolates. Early observations indicated that these cephalosporinases were inducible in such genera as *Enterobacter, Citrobacter, Pseudomonas, Serratia,* and *Providencia.*[4,60] This simply means that the cell can reversibly increase the level of cephalosporinase as much as 500-fold upon exposure to beta-lactams. Essentially any beta-lactam can act as an inducer, provided its diffusion into the cell is adequate and it is relatively stable to the induced enzyme. The biochemical events leading to induction have not been elucidated, although the regulatory genes for cephalosporinase in *Enterobacter cloacae* have begun to be characterized.[61-66] Since true induction is a reversible process, it might be assumed that the most effective inducers are beta-lactams that are stable to the induced beta-lactamase yet are not highly active against the bacterium. In this regard, cefoxitin has received much attention as an excellent inducer of such beta-lactamases, particularly in *Enterobacter.*[66-70] Cefoxitin is relatively stable to hydrolysis by cephalosporinase and is not highly active against many *Enterobacter* strains;[66,67] however, many other beta-lactamase stable compounds are as good or better at inducing cephalosporinase as cefoxitin.[66,71] It is clear that reversible induction of beta-lactamase is a dynamic process.[66,68] Competition occurs between the induction of high levels of beta-lactamase and the diffusion of intact inducer to the lethal targets in the cell. Carbapenems, penems, and some third generation cephalosporins are excellent inducers of beta-lactamase, but they are also highly active against wild type *Enterobacter* strains or constitutive mutants that produce high levels of cephalosporinase in a stable manner.[66]

This is likely due to their high stability to cephalosporinase and excellent diffusion through the OM.[31,66,72,74] Carbapenems and penems also have a high affinity for PBP 2, and productive binding to this lethal target may partially explain the excellent *in vitro* activity of these agents against such organisms.[75] As single agents, carbapenems and penems should be highly effective against bacteria that contain inducible beta-lactamases, although antagonism has been observed with these drugs when used in combination with beta-lactams that are susceptible to inactivation by high levels of beta-lactamase.[76,77] The monobactams are also highly stable to cephalosporinase, yet are poor inducers of this enzyme.[6] This may suggest that the complete bicyclic molecule is required to sustain optimum induction.

The carbapenems and some third generation cephalosporins also seem to be less prone to clinical failure through emergence of resistant mutants during therapy than earlier compounds like cefamandole.[73,78-81] Constitutive mutants producing high levels of cephalosporinase have been responsible for such resistance emergence, and these mutants usually demonstrated cross-resistance to several beta-lactamase stable compounds, causing some concern about their spread throughout the clinical environment.[61-63,73,78,81,82] A non-hydrolytic barrier or "trapping" mechanism has been proposed to account for the cross-resistance to such compounds.[73,83,84] This mechanism is based upon the observation that compounds like carbenicillin, cefoxitin, cefotaxime, and moxalactam are poor substrates for many cephalosporinases yet are potent suicide inhibitors of these enzymes.[85,86] The theory states that even for non-substrate beta-lactams, the molecules may diffuse into the periplasmic space and become "trapped" after binding to unusually high numbers of derepressed beta-lactamase molecules, slowing their diffusion to the PBPs.[84] This has some merit in that compounds like moxalactam have Ki values in the nanomolar range for *Enterobacter* cephalosporinase.[87] The debate surrounding the "trapping" mechanism centers around whether or not sufficient numbers of beta-lactamase molecules could exist in the periplasmic space of derepressed organisms to completely bind all incoming beta-lactams, thereby rescuing the cell by the non-hydrolytic barrier. It has been calculated that for the constitutive mutant *E. cloacae* 55M,[62] approximately 2×10^5 beta-lactamase molecules are present per cell, with a turnover rate of 167 enzyme molecules per cell per second.[31,44] This substantial amount of beta-lactamase would be a significant barrier, severely impeding many third generation cephalosporins from reaching the PBPs on the cytoplasmic membrane.[31,44] It is likely, however, that additional mechanisms are involved to complete the efficiency of the barrier. Careful studies illustrate that so-called beta-lactamase stable compounds like cefotaxime and moxalactam are indeed hydrolyzed at low rates when tested at substrate concentrations representative of those found in the periplasmic space.[6,31,44] Relevant differences between beta-lactams can best be determined at these concentrations using the Kcat/km ratio for physiological efficiency.[6,88] When comparing the stabilities of beta-lactams in strains hyper-producing beta-lactamase, it becomes clear that a complete picture of permeability, drug binding by beta-lactamase (trapping), and hydrolysis is needed to explain the resistance observed.[6,44,84,89] From a structure-activity standpoint, those beta-lactams that diffuse rapidly across the OM, avoid hydrolysis, and achieve critical concentrations at the lethal targets will be most effective *in vitro* against strains that can regulate their beta-lactamase production. The final topic covered will consider the interactions at the PBPs that can decrease this effective inhibition.

Binding to the PBPs—The lethal targets in the bacterial cell for beta-lactams are the penicillin-binding proteins (PBPs) or penicillin-sensitive enzymes (PSEs) that can be identified in membrane preparations using radiolabeled penicillin G. Such studies indicate that *E. coli*, other enteric Gram-negative bacilli, and *P. aeruginosa* all contain seven major species of PBPs that migrate in similar patterns on SDS polyacrylamide gels.[7,90-92] The higher molecular weight PBPs (1a, 1b, 2, and 3) are essential to cell survival since covalent binding with beta-lactams or inactivation through temperature sensitive mutation has resulted in lethality.[8,9] Binding to PBP 1a or 1b results in rapid cell lysis while binding to PBPs 2 and 3 results in unstable ovoid and filamentous cells, respectively.[8] The lower molecular weight PBPs (4–6) bind the majority of labeled penicillin G in membrane preparations, yet binding is not associated with cell death.[8] Beta-lactams have variable specificity for binding to PBPs, and examples exist of compounds that kill at the MIC either by lysis, production of spherical cells, or filamentation.[8] Most beta-lactams bind to multiple PBPs at concentrations relevant to clinical therapy and therefore kill bacteria by a combination of these mechanisms.[8] More extensive detail of the structure and function of PBPs can be found in several current reviews.[7-9,93]

Since most beta-lactams bind to multiple PBPs, it was hypothesized that high level resistance through mutation at these targets would be extremely rare.[93] This prediction has not been totally correct, however, and resistant mutants with altered PBPs have been isolated both in the laboratory and in the clinic. Decreased affinities for beta-lactams may involve a single PBP[94-98] or multiple PBPs[99-105] in a given strain. Decreases in affinity of beta-lactams for PBPs can occur up to 400- to 500-fold in mutant strains compared to the wild type, and this has been associated with a significant rise in MICs to beta-lactams.[14] Studies using cell-free membrane preparations for binding of labeled compounds give some insight into alterations at the lethal targets,

but observed changes in affinity have not always correlated with decreases in the susceptibility of the whole cell.[9] More meaningful comparisons between changes in PBP affinity and resistance can be obtained when viable cells are subjected to labeled beta-lactam, a procedure made possible by the recent development of ^{3}H-labeled penicillin.[9] Such *in vivo* labeling procedures take into consideration permeability factors that influence access of beta-lactams to the PBPs.[9,106]

The mechanisms responsible for decreases in PBP affinities for beta-lactams in mutant strains are not known. Arguments for changes in the relative quantities of PBPs[101,103] or changes in the affinity of specific PBP components have been made.[103-105] It is interesting that multiple PBP changes in a single strain can occur, indicating that expression of these membrane proteins may be under coordinate control.[103-105] Evidence also exists that mutation at the PBPs may be a multi-step process that leads to strains with incremental increases in beta-lactam resistance.[103] The emergence of such multi-level, resistant mutants has been well documented during high dose beta-lactam therapy of *P. aeruginosa* infections in the clinic.[103] Thus, the unexpected appearance of resistant clinical isolates with multiple PBP changes may, in part, be a result of the increased selective pressure applied in the clinical environment through the increased use of beta-lactams.[93,103]

In light of the potential for strains to develop PBPs with decreased affinity for beta-lactams, it is of interest to study the structure-activity relationships that lead to compounds which have high affinity for these targets. It appears that PSEs have multiple recognition sites for beta-lactams in addition to the active site; therefore, analoging compounds for improved PSE affinity can produce more potent antibacterials.[9] General observations in *E. coli* have shown that PBP 1a has a high affinity for cephalosporins while PBP 2 is highly sensitive to mecillinam, imipenem, penems, and clavulanic acid.[7,9,75] PBP 2 is not highly sensitive to cephalosporins and monobactams.[7,9] PBP 3 is sensitive to penicillins, monobactams, and beta-lactamase stable cephalosporins like cefotaxime and ceftazidime.[7,9] The 7α-methoxy group on cephamycins has been shown to promote high beta-lactamase stability and increased affinity to PBPs 1, 3, and the non-lethal targets.[107,108] Penems containing a 6α-hydroxyethyl group were analogously highly active against many Gram-negative organisms, stable to beta-lactamase, and showed high affinity for PBP 1a, 1b, 2, and 4-6.[109] Side groups derived from penicillins and cephalosporins have also been evaluated extensively on monobactams in order to study the correlation between binding to PBPs and antibacterial spectrum.[110] Current approaches suggest that useful information is also being gained by studying the 3° structure of the essential PBPs by X-ray crystallography.[93] The genes encoding all seven of the PBPs of *E. coli* have been cloned on high copy plasmids, allowing acquisition of large quantities of purified PBPs using improved isolation methods.[93,111-113] The penicillin-binding domain of PBP 3 from *E. coli*, for example, has been cloned and the amino acid sequence at the active site studied.[112] Such advances could support the rational design of novel beta-lactams that have activity directed at the active site of the PSEs.

Interplay of OM, Beta-lactamase, and PBPs in Determining Resistance to Beta-lactams—Specific examples have been given illustrating changes in OM components, beta-lactamase expression, and affinity for PBPs, which significantly change the activity of beta-lactams against Gram-negative bacilli. The activity of earlier beta-lactams that were susceptible to beta-lactamase was highly influenced by their rate of penetration through the OM.[18-20] Compounds like cephaloridine are effective only if they penetrate rapidly across the OM, allowing sufficient numbers of molecules to escape degradation by beta-lactamase. The importance of rapid diffusion in designing new drugs for use in the clinic, however, can only be appreciated when considered with their beta-lactamase stability and affinity for the PBPs. The equilibration time for the periplasmic space concentration of most beta-lactams to reach one-half that outside the cell has been calculated to be only a matter of seconds in *E. coli*, regardless of the magnitude of the partition coefficient for the compound.[1] Thus, for new beta-lactams, consideration of the partition coefficient alone is insufficient to predict good antibacterial activity. The key to understanding the dynamics that influence the arrival of beta-lactams to the lethal targets rests with considering the rate of removal of drug from the periplasmic space by beta-lactamase, interaction with PBPs, and diffusion into the cytoplasm. Due to constant removal of intact drug from the periplasmic space, the concentration of drug available to the PBPs is never as high as that found outside the cell. Recent estimates indicate that for an extracellular concentration of beta-lactam equal to 100 μM, only about 1 μM is achieved in the periplasmic space of *E. coli* due to the permeability barrier of the OM.[31,44] Although this concentration should be more than adequate to saturate the PBPs, it is obvious that any decrease in diffusion, increase in hydrolysis, or loss of PBP affinity could significantly compromise the effectiveness of a beta-lactam. When considering the dynamics of beta-lactams in the periplasmic space of organisms with inducible beta-lactamases, the "trapping" phenomenon appears plausible for those compounds which demonstrate moderate permeability coefficients compared with beta-lactams like imipenem. It is difficult to predict for each drug-organism combination which of these parameters plays the most important role in determining the outcome of exposure of the bacterial cell to beta-lactams. It is clear that selection of resistant strains can occur *in vivo* to any of

these resistance determinants. More importantly, many resistant isolates appear to be altered at more than one of these determinants.[102,105,114] The design of new antimicrobial agents then should continue in a direction that optimizes diffusion across the OM, stability to degradation by beta-lactamase, and affinity for the PSEs in order to combat the wide range of resistance determinants encountered in Gram-negative organisms.

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Chapter 15. Quinolone Antibacterial Agents

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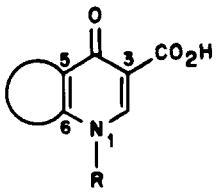
Introduction* - Quinolone antibacterials are generally defined as 1-substituted-1,4-dihydro-4-oxo-3-pyridinecarboxylic acids which have an additional ring fused at the 5,6-positions (1). Discovery of the first therapeutically useful antibacterial quinolone, nalidixic acid (2),[1] prompted the syntheses of a large number of structural variants. A comprehensive review has outlined the synthetic methods, microbiology, and structure-activity relationships of those derivatives reported prior to 1977.[2] This report will highlight the pertinent literature concerning quinolone antibacterials subsequent to that review with emphasis on recent developments in 6-fluoro-7-piperanzinylquinolones.



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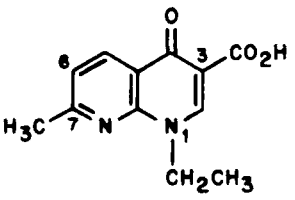

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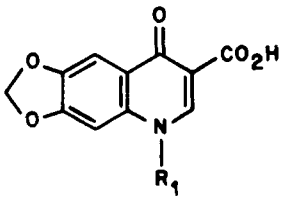

3, $R_1 = CH_2CH_3$
12, $R_1 = OCH_3$

General - An evolution of structural modifications of NAL has resulted in increased potency/spectrum such that the newest agents have excellent Gram-negative (including P. aeruginosa), increased Gram-positive, and in some instances, better activity against anaerobes. This increased potency has broadened the therapeutic potential of quinolones for parenteral and oral treatment of systemic infections other than UTI.[3] OXO (3), a dioxoloquinoline derivative, was the first NAL analog to demonstrate significantly better potency with a broadened spectrum to include Gram-postive organisms and greater antipseudomonal activity. The activity of NOR (4), a 6-fluoro-7-(1-piperazinyl)quinolone, represented the next significant increase over previous quinolones.[4] Modifications primarily at positions 1- and/or 8- have resulted in further improvement of the antimicrobial profile of NOR. Of particular significance is the increase in activity noted upon replacement of the 1-ethyl substituent of quinolones with cyclic substituents (e.g. cyclopropyl, 4-fluorophenyl) of larger steric bulk.

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*Abbreviations used in this report: AMI-amifloxacin; CIP-ciprofloxacin; ENO-enoxacin; FLU-flumequine; MIL-miloxacin; NAL-nalidixic acid; NOR-norfloxacin; OFL-ofloxacin; OXO-oxolinic acid; PEF-pefloxacin; ROS-rosoxacin; UTI-urinary tract infections.

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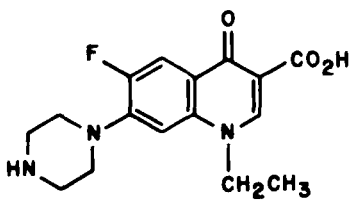
Mechanism of Action. NAL and its analogs act through inhibition of bacterial DNA synthesis.[5] The biochemical target of quinolones is the procaryotic enzyme DNA gyrase, a type II topoisomerase.[6] In an energy requiring process, bacterial DNA gyrase introduces negative supercoils into circular duplex DNA. Negative supercoilng relieves the torsional stress of unwinding helical DNA that is necessary for transcription and replication. Gyrase from *E. coli* and *Micrococcus luteus* has been studied and found to consist of A and B subunits.[7] Quinolones bind to the A subunit while novobiocin, coumermycin and related compounds interact with the B subunit.[6] Binding of NOR to single-stranded DNA has also been proposed as a mechanism of action.[8]

Resistance - Mutants selected *in vitro* for resistance to one quinolone generally show cross-resistance to other quinolones but not to other classes of antibiotics.[9] Quinolone resistance can also occur from reduced cellular permeability; mutants of this type can show cross-resistance to beta-lactam antibiotics.[10] Plasmid mediated resistance to quinolones has not been detected.[11] Quinoline resistance occurs with the same frequency as with other antibacterials and MICs 200-fold greater than the original MIC can occur. Urine drug levels that can be attained with the new more potent quinolones are greater than the MICs of these resistant strains, although MICs of resistant *P. aeruginosa* can be quite high (NOR resistant MIC = 256 μg/ml).[12]

Toxicity - Quinolone antibacterials are generally well tolerated. For example, in mice the acute LD_{50} for ENO is 330 mg/kg (iv) and >5000 mg/kg (po).[13] The most prominent toxic effect observed is erosion of cartilage in joints of immature animals. This effect has been reported for NAL, OXO, cinoxacin, pipemidic acid, and piromidic acid, and has been shown to occur in rabbits,[14] dogs,[15] and monkeys[16] and only in immature animals.[15,16] Clinical side effects can include dizziness, visual disturbance, hemolytic anemia, photosensitivity, and intracranial hypertension.[17]

The following marketed quinolones have been reviewed and will not be discussed in detail: NAL,[17] citrated NAL,[18] OXO,[19] cinoxacin,[20] pipemidic acid[21] and piromidic acid.[22]

Norfloxacin (AM-715, MK-366) - The *in vitro* spectrum of NOR (4) includes most Gram-negative pathogens with MIC_{90} values ca. 1 μg/ml[23,24] It is more potent *in vitro* than other oral agents used for GI infections[25] and for UTI[26] although its potency is reduced in the presence of acidic urine.[23] For sexually transmitted diseases, NOR is more potent than penicillin G, ampicillin, spectinomycin and doxycycline against *N. gonorrhoeae*.[27] It is less active than other quinolones against *Ureaplasma*[28] and shows only moderate activity against *Chlamydia trachomatis*.[29] Activity against Gram-positive cocci is less than that for the Gram-negative bacteria.[24,26,30] Against anaerobic bacteria NOR is less active than ampicillin or metronidazole.[30] NOR is active against *Legionella*[31] and acts synergistically with amphotericin B against fungi[32] and with coumermycin against *S. aureus*.[33]



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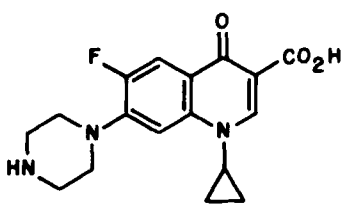
NOR is less well absorbed orally in animals[34] and man[35] than ENO[36] or CIP.[37] Tissue levels (lung, kidney) in rats and monkeys exceed serum

levels[38] as do prostatic tissue levels in man[39] while blister fluid levels in man are 70% of those in serum.[35] Peak serum levels (1.6 μg/ml) in man occur 1 hr after oral dosing with 400 mg.[40,41] About 30% of the dose was eliminated in the urine (0-24 hr) of which 80% was NOR.[40,41] Pharmacokinetics of NOR were significantly altered in patients with a glomerular filtration rate <10 ml/min[42] and urinary excretion was reduced by probenecid.[43] NOR administered orally at 400 mg twice daily for 10 days showed comparable efficacy to trimethoprim/sulfamethoxazole (TMP/SMZ) at 160/800 mg twice daily for 10 days in both hospitalized patients[44] and outpatients.[45] Equivalent efficacy to TMP/SMZ was also reported in acute pyelonephritis.[46] NOR was more effective than amoxicillin against complicated UTI.[47] Side effects (dizziness, nausea) were generally few[47] and no greater than with TMP/SMZ in controlled trials.[45,46] NOR administered orally at 600 mg twice daily was as efficacious as 2 gm of spectinomycin given im.[27] Both compounds gave 100% cures in treating gonococcal urethritis in males, including infections caused by penicillin-resistant N. gonorrhoeae. NOR is marketed in Europe and Japan and completing clinical trials in the U.S.

QSAR studies which led to the design of NOR involved multiphysicochemical parameter analysis of substituents at positions-6, 7, and 8.[48,49] NOR was more potent in vitro against S. aureus, E. coli and P. aeruginosa than 45 analogs with differing substitutions at these positions and position-1.[4] NOR and PEF have served as reference compounds for several SAR studies as outlined in other sections of this report.

Ciprofloxacin (Bay o 9867) - CIP (5) shows the same relative spectrum of activity as other fluoroquinolones,[50] but is generally the most potent member of this class. It is 2- to 8-fold more potent in vitro than NOR against members of the Enterobacteriaceae, P. aeruginosa and S. aureus.[51,52] CIP has shown greater potency against some Enterobacteriaceae than ceftazidime, gentamicin,[52] thienamycin and aztreonam.[53] Activity against the anaerobe B. fragilis was 4-fold greater than NOR and 16-fold greater than metronidazole.[54] CIP antibacterial activity decreased in acidic urine but was not affected by the presence of 50% serum.[52] In vitro CIP showed no antagonism, and in some cases synergy, with aminoglycosides and cephalosporins.[55] In various experimental P. aeruginosa infection models, CIP was more efficacious than azlocillin, tobramycin or ceftazidime[56] and equivalent to azlocillin plus tobramycin.[57] The efficacy of CIP was intermediate between ticarcillin and tobramycin against P. aeruginosa pneumonia in guinea pigs.[58]



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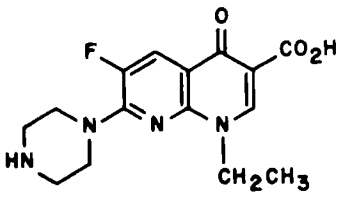
The Cmax of CIP increased with increasing oral doses to humans.[59,60] Oral CIP (500 mg) was rapidly absorbed showing peak serum levels up to 2.9 μg/ml 1 to 2 hr after dosing and a serum half-life of 4.9 hr, with 41% of the dose in the 0-24 hr urine.[59] Four microbiologically active urinary metabolites of CIP have been cited.[61] After oral dosing, drug concentrations in saliva,[59] and blister fluid,[37] were 50% and 60%, respectively, of serum levels. High concentrations of CIP in feces were attributed to incomplete GI absorption.[60] Intravenous CIP (100 mg, bolus) showed a rapid and large volume of distribution, a serum half-life of 4 hr, and 76% of the dose was excreted in the urine by 24 hr.[62] The drug rapidly penetrated peritoneal fluid,[63] blister fluid,[62] and prostatic tissue.[64] CIP is in

advanced clinical trials in Europe and the US.

Enoxacin (AT-2266, CI-919) - ENO (6) shows essentially the same spectrum *in vitro* as NOR with NOR being somewhat more potent.[65] It is distinguished from NOR by better oral absorption.[34,36,66] ENO was very active against *Neisseria* and *Haemophilus* spp., but showed reduced activity against streptococci.[65] Against aminoglycoside-resistant *P. aeruginosa* ENO was more potent *in vitro* than 11 other agents, including amikacin, piperacillin, imipenem and aztreonam.[67] Against systemic infections in mice, oral ENO was more efficacious than NOR but less efficacious than gentamicin.[66] In mice, rats, and dogs oral ENO showed greater drug concentrations in plasma, tissues, and urine than equal weight doses of oral NOR.[34] In human volunteers,[36] ENO (600 mg po) exhibited superior oral absorption relative to NOR, a higher Cmax (3.7 μg/ml), longer serum half-life (6.2 hr), greater urinary recovery (67% in 0-24 hr), and greater blister fluid penetration.[36] Five metabolites of ENO have been detected in human urine with the major metabolite, a 3-oxo-piperazinyl derivative, representing 8% of the dose.[68] Oral ENO (300 to 900 mg/day) was effective in human respiratory and genitourinary infections with minimal side effects.[69] ENO was efficacious in treating uncomplicated gonorrhea[70] and a systemic *P. aeruginosa* infection, the latter at 1200 mg/day.[71] ENO increases plasma theophylline levels whereas PEF and CIP do not.[72] ENO is expected to be marketed soon in Europe and Japan and is in clinical trials in the US.



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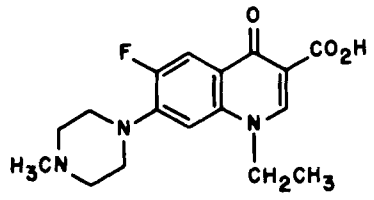
SAR studies showed that within several pairs of 7-(4-methyl-1-piperazinyl) and 7-(1-piperazinyl) analogs having different C-6 substituents, similar *in vitro* activity was generally noted.[73] Potency for those pairs followed the order 6-F>Cl≥CN>H. The 7-(4-methyl-1-piperazinyl) derivatives having 1-ethyl, vinyl, or $-CH_2CH_2F$ substitutions were found to have greater toxicity and, in some instances, greater activity than their piperazine counterparts. Substitution of the piperazine with 3-amino-1-pyrrolidinyl functionality increased *in vivo* activity relative to ENO.[74]

Pefloxacin (1589 RB) - The *in vitro* spectrum and potency of PEF (7) is similar to that of NOR but shows slightly better activity against Gram-positive cocci (MIC vs. *S. aureus* ≤ 0.5 μg/ml)[51,75] and *B. fragilis*.[51] PEF was more potent *in vitro* than its desfluoro analog.[76] It was as effective as CIP and more efficacious than tobramycin or ticarcillin in treating *Pseudomonas* pneumonia in neutropenic guinea pigs.[77] Against *S. aureus* endocarditis in rabbits, PEF was as effective as cephalothin or vancomycin.[78] Orally, PEF was more efficacious than NOR in experimental infections suggesting better oral bioavailability.[76] In human volunteers, peak plasma levels following a 400 mg oral dose were 3.8 μg/ml and the plasma half-life of 9 to 10 hr was much longer than for other quinolones.[79] Following a 800 mg oral dose of PEF, 16% was excreted into the 0-24 hr



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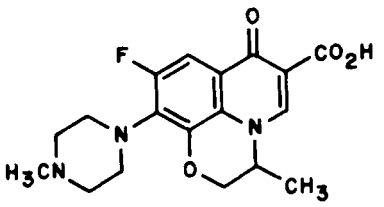
urine, 5% as PEF and 11% as NOR. PEF (7.5 and 15 mg/kg, iv or po) has been used to treat meningitis in humans.[80] Intravenous PEF penetrates human bone[81] and has been efficacious in the clinical treatment of chronic staphylococcal osteomyelitis both alone and with rifampicin.[82] PEF has been approved for marketing in France.

Ofloxacin (DL-8280) - The in vitro potency of OFL (8) is comparable to NOR against members of the Enterobacteriaceae.[51,83] OFL is somewhat less potent than NOR against P. aeruginosa and is generally less potent than CIP against Gram-negative bacteria.[51] Like PEF, OFL is more potent in vitro than NOR[51,83] and comparable to CIP[51] against staphylococci and streptococci. OFL was more potent against B. fragilis than any of these other fluoroquinolones.[51,83] OFL was less potent than erythromycin against Mycoplasma.[84] Against systemic bacterial infections in mice, OFL was 2- to 6-fold more efficacious than NOR by the oral route.[83] The oral bioavailability of OFL in human volunteers has been estimated by Cmax as 2.9-, 3.9-and 4.9-fold greater than ENO, CIP and NOR, respectively.[85] Following a single 600 mg oral dose of OFL, the mean Cmax was 10.7 μg/ml at 1.2 hr with a mean serum half-life of 7.0 hr and 73% of the dose was excreted into the 0-24 hr urine.[85] Clinical efficacy of oral OFL (100 to 600 mg/day) has been described for the treatment of respiratory and genitourinary infections.[86] SAR studies have not been reported; the synthesis of OFL has been described.[87] OFL is under development in Japan, Europe, and the US.



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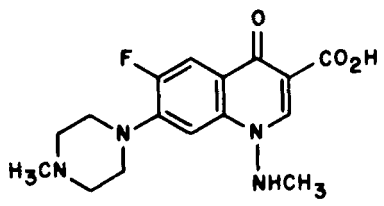
Amifloxacin (WIN 49,375) - AMI (9) has a 1-methylamino function reported to be an ethyl equivalent.[88] Against Gram-negative bacteria amifloxacin shows in vitro activity comparable to NOR but is less potent than CIP.[89] AMI has low activity against B. fragilis,[90] but is more active than aztreonam or cefotaxime against aminoglycoside-resistant P. aeroginosa.[91] It is more active than moxalactam or ceftazidime and equally active as imipenem against other P. aeruginosa,[92] and is more active than tobramycin or cefotaxime against methicillin-resistant S. aureus.[93] AMI shows comparable oral and parenteral efficacy in mouse protection tests against Gram-negative bacteria including P. aeruginosa.[94] Excretion of urinary antibacterial activity was greater than NOR or PEF with orally dosed rats,[95] and AMI showed complete bioavailability by the oral route.[96] The major urinary metabolite of AMI in monkeys is the piperazinyl N-desmethyl derivative which shows similar in vitro activity to AMI, NOR and PEF.[88,96] Correlation between the steric bulk of 1-alkylamino substituents and antibacterial activity in vitro and in vivo was in general agreement with studies involving 1-alkyl and 1-alkoxyquinolones.[88] The 1-methylamino analogue of OXO showed comparable in vitro and in vivo activity vs. E. coli.[97] The ROS analogue retained in vitro activity only, while for this substitution in NAL, significantly less activity was found.[97] AMI is in US clinical trials.



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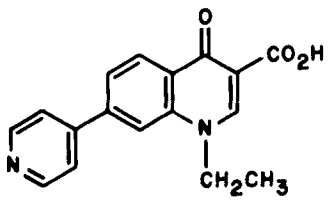
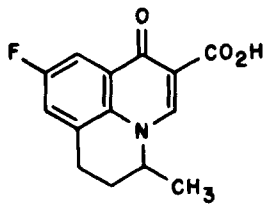
Rosoxacin (WIN 35,213) - ROS (10) has good in vitro activity against common

urinary pathogens with less activity against gram-positive cocci.[98,99] The activity of ROS vs. penicillinase-producing N. gonorrhoeae (PPNG) was greater than spectinomycin, comparable to NOR and OFL but less than CIP.[100] ROS was more active vs. U. urealyticum than PEF, OXO, FLU, NOR, NAL and pipemidic acid.[28] ROS was efficacious against systemic infections in mice with little difference noted between the po and sc route of medication.[99] A single 300 mg oral dose in man gave a mean peak serum concentration of 4.9 µg/ml 2.7 hr post-medication with an elimination half-life of ca. 3.4 hr.[101] The urinary metabolites of ROS in primates are its glucuronide conjugate and N-oxide.[98] Human prostatic tissue concentrations were about one-half those of serum concentrations in man.[102] ROS has been evaluated for the treatment of uncomplicated gonococcal infections in men and women.[98,103,104] The synthesis of ROS and derivatives with differing substitutions in the pyridine ring and at position-1 has been reported.[105] The 2,6-dimethylpyridinyl analogue was more effective than ROS vs. S. aureus[106] and comparable to NOR vs. S. pneumoniae.[107] ROS is currently marketed outside the US.



10

Flumequine (R-802) - FLU (11) was generally more potent in comparative in vitro studies than NAL,[108] comparable to OXO,[109] 2- to 8-fold less potent than PEF[110] and 8- to >64-fold less potent than NOR[108] against most Enterobacteriaceae. The efficacy of FLU against experimental urinary tract infections in rats was greater than NAL but less than OXO with equal weight oral doses.[109] The major metabolite of FLU in humans, the 7-hydroxy derivative, was 1/8 as potent as FLU.[111] This metabolite was not detected in human plasma where levels of FLU reached 40 µg/ml following an 800 mg oral dose. The 7-hydroxy metabolite was present in human urine at 2- to 5-fold greater levels than the parent drug which represented <5% of the oral dose.[111] FLU has been used to treat uncomplicated gonorrhea[112] and UTI.[113] The 8-(4-methyl-1-piperazinyl)- and 8-(1-piperazinyl)-analogues of FLU inhibit bacterial DNA gyrase.[114] FLU is available for human and veterinary use in Europe.



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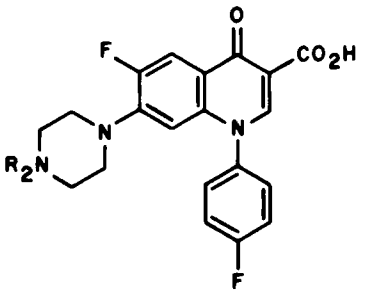
Miloxacin (AB-206) - MIL (12) was 4- to 8-fold more potent in vitro than NAL and comparable to OXO except against P. aeruginosa and staphylococci, where OXO was more potent.[115] Against systemic Gram-negative bacterial infections in mice, oral MIL was 2- to 4-fold more efficacious than NAL and comparable to OXO. MIL was inactive against infections due to S. pyogenes. In human volunteers receiving 500 mg oral MIL, a Cmax of 7.7 µg/ml was observed at 1-2 hr after dosing.[116] Of the drug appearing in the 0-8 hr urine, 5.4% was MIL and 87% was in the biologically inactive glucuronide form.[116] MIL is marketed in Japan.

A-56619, A-56620 - The in vitro activities of A-56619 (13) and A-56620 (14) were similar to NOR but showed greater activity against B. fragilis.[117] Against systemic infections in immunosuppressed mice, both were more efficacious than NOR (po and sc).[118] Peak serum levels of A-56620 and A-56619 in mice following a 100 mg oral dose were 10- and 40-times greater, respectively, than an equal dose of NOR. The serum half-life in

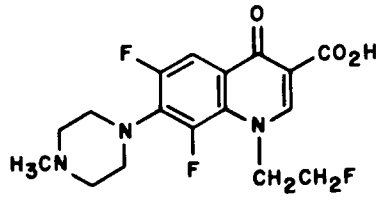


13, R_2 = CH_3
14, R_2 = H

dogs (10 mg/kg, po) for A-56619 was 8.2 hr with the drug showing enterohepatic circulation.[119] Similar *in vitro* potencies were generally noted in each pair of 7-(1-piperazinyl) and 7-(4-methyl-1-piperazinyl) analogues in which the substitution on the 1-phenyl ring was varied.[120] The 4-hydroxy and 2,4-difluorophenyl analogues were the only mono- and disubstituted derivatives having *in vitro* activity similar to A-56619. Replacement of the 1-phenyl with 3- or 4-pyridinyl diminished activity. A-56620 and its 1,8-naphthyridine analogue had similar antibacterial activities.[121] Both A-56619 and A-56620 are in the preclinical phase of testing in the US.

AM-833 - AM-833 (15), has *in vitro* activity similar to NOR except it is more potent vs. *S. aureus*.[122] AM-833 was ca. 5- to 14-fold more potent than NOR *in vivo* (po) vs. *S. aureus*, *E. coli* and *P. aeruginosa*. Against most Gram-positive and Gram-negative strains, AM-833 was slightly less active *in vitro* than CIP and showed comparable or slightly greater activity than NOR, ENO or OFL.[123] Peak serum levels and the biological half-life of AM-833 in dogs were ca. 3-fold higher and 2-fold longer than NOR.[124] No clinical studies have been reported.

CI-934 - CI-934 (16) was generally more potent *in vitro* than AMI or OFL vs. Gram-positive cocci.[125] It was less active *in vitro* against the *Enterobacteriacae* than NOR or CIP and showed little activity against *P. aeruginosa* (median MIC = 12.5 μg/ml) or *B. fragilis* (MIC_{90} = 25 μg/ml). Against staphylococcal and streptococcal infections in mice oral CI-934 was more efficacious than any other quinolone tested (CIP, PEF, OFL, ENO) and was comparable to, or somewhat less efficacious than, cephalexin.[126] Against Gram-negative infections in mice CI-934 was 3- to 20-fold less efficacious orally than ENO or CIP. The 7-[3-[(amino)methyl]-1-pyrrolidinyl] moiety of CI-934 has been postulated as a piperazine equivalent and the improved Gram-positive activity of CI-934 *in vitro* and *in vivo* was attributed to this substitution. Comparison of CI-934 and its 8-desfluoro derivative indicated similar gyrase inhibition and *in vitro* antibacterial activities, however, CI-934 showed about 10-fold greater *in vivo* activity by both the oral and subcutaneous routes.[127] CI-934 is in the preclinical phase of testing in the US.



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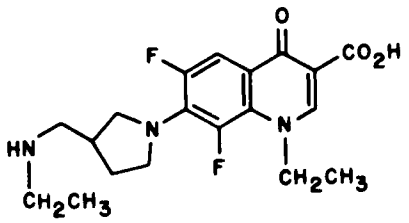

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Chapter 16. Nonclassical Targets for Antibacterial Agents

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Introduction - The antibacterial activity of an antibiotic is the result of several biological properties of the drug. Namely, the antibiotic must be able to (1) penetrate the cell envelope of a microorganism, (2) bind to or act at a target site to inhibit a metabolic step critical for cell growth, and (3) avoid or circumvent any resistance mechanisms used by the microorganism for protection. When these conditions are met the antibiotic will inhibit growth. The antibiotic may kill the organism or inhibit growth without killing depending on the target site and the mechanism of interference.

Although ability to penetrate the cell envelope and to avoid resistance mechanisms are critical to the action of a drug, the specific target site of an antibiotic can determine the practical significance of these two properties. For example, if the target has a membrane location, it may be relatively easy to reach and the antibiotic need not completely penetrate the cytoplasmic membrane. On the other hand, a cytoplasmic location, such as a ribosome, requires complete penetration. The target can sometimes influence the liklihood of resistance development. If all mutations leading to alteration of the target are themselves lethal to the microorganism, resistance development will likely involve another mechanism such as the production of an inactivating enzyme.

An important property of antibacterial agents is whether they have selective toxicity. This can be dictated by the target. If the target is unique to procaryotic organisms, such as peptidoglycan biosynthesis, the antibiotic might be expected to show low toxicity towards the mammalian host - unless, of course, it has secondary effects on cellular functions not unique to the procaryote. The spectrum of activity of an antibiotic can also be influenced by the target since an antibiotic can act on very specialized targets found only in certain microorganisms.

A wide variety of chemical structures are encountered amongst antimicrobial agents yet the majority of the drugs that have been studied can be subdivided into the following categories based on general target sites: (1) inhibitors of nucleic acid biosynthesis; (2) inhibitors of peptidoglycan biosynthesis; (3) inhibitors of lipid biosynthesis: (4) inhibitors of ribosome function; (5) inhibitors of membrane function; (6) inhibitors of folic acid metabolism. Details of molecular modes of action including target sites have been worked out for only a limited number of antibiotics. Those drugs used clinically have been the most extensively studied. There are many more drugs that remain uninvestigated.

The six categories listed above are considered classical targets. A review of the literature quickly shows that the large majority of

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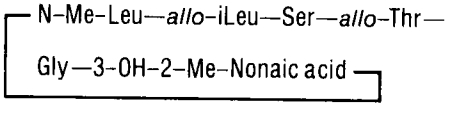
antibiotics that have been studied fall into one of these categories. Perhaps this is related to the empirical approaches so often used to search for and evaluate new antimicrobial agents. Nevertheless, antibacterial agents have been described that inhibit nonclassical targets. These drugs have come from both fermentation screening programs and synthetic efforts. This article will review some examples of antibacterial agents that act on nonclassical targets.

Inhibition of Lipoprotein Biosynthesis - The murein lipoprotein is a major protein in the outer membrane of gram-negative bacteria.[1] This molecule is synthesized as a prolipoprotein in the cytoplasm and is subsequently translocated across the cytoplasmic membrane by a signal peptide. The complete biosynthetic sequence includes modification and processing of the prolipoprotein to a mature form of the lipoprotein. The outer membrane is unique to gram-negative bacteria, and, in spite of its function as a barrier to drugs and other harmful substances, it can provide a selective target for inhibition by antibiotics.

Globomycin (1) is a cyclic peptide antibiotic active against gram-negative bacteria.[2] This narrow spectrum antibiotic is not a general inhibitor of macromolecular biosyntheses.[3] Rather, treatment of E. coli with globomycin causes an accumulation of the modified prolipoprotein in the cytoplasmic membrane.[4] Globomycin appears to inhibit proteolytic cleavage of the modified prolipoprotein by the prolipoprotein signal peptidase. The inhibition of processing leads to spheroplast formation and is followed by cell lysis. Since mutants have been described that lack lipoprotein, the lethal effects of globomycin are most likely due to the accumulation of unprocessed prolipoprotein in the cytoplasmic membrane.[4] Globomycin shares structural similarities with the prolipoprotein,[2] and the inhibition of signal peptidase could result from this relatedness.[5]



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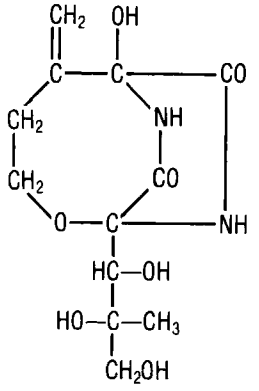

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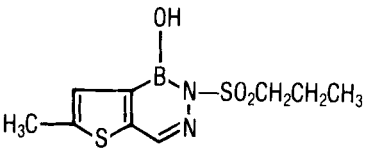
Bicyclomycin (2) is another antibiotic selective against gram-negative bacteria. Inhibition of E. coli by bicyclomycin reportedly inhibits the biosynthesis of the bound form of the lipoprotein.[6] However, since bicyclomycin is active against a mutant of E. coli lacking a murein lipoprotein,[8] the effects on lipoprotein are thought to be a secondary action.[7]

Inhibition of Lipopolysaccharide Biosynthesis - The lipopolysaccharide (LPS) of gram-negative bacteria consists of polysaccharide units linked to a lipid structure (Lipid A) thru 2-keto-3-deoxyoctanoic acid (KDO).[8] LPS is a component of the outer membrane and has a pronounced effect on the virulence and toxicity of many gram-negative pathogens.[9] Changes in LPS structure account for the smooth to rough phase shifts in colony morphology which can affect phagocytosis by polymorphs and macrophages. As a part of the outer membrane of gram-negative bacteria, inhibitors of LPS biosynthesis might be expected to inhibit cell growth. Diazaborines are synthetic antimicrobial agents active against gram-negative bacteria. Hŏgenauer and Woisetshlager have shown that a model diazaborine (3) inhibited galactose incorporation into LPS by a gal-epimerase-negative mutant of E. coli.[10] Since rough mutants having an LPS lacking sugars attached to the KDO moiety are viable, inhibition by the diazaborine must not be directed at the elongation of the sugar units. Rather, the diazaborine must inhibit the incorporation of D-arabinose-5-phosphate, a precursor of KDO, into LPS. It is thought that the drug interferes with the transfer of KDO to the lipid A precursor molecule needed for formation of the LPS. Several diazaborine analogs have been synthesized and compared with respect to their antibacterial activity.[11] All have a gram-negative spectrum but are inactive against Pseudomonas.



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Arabinose 5-phosphate isomerase is required for the biosynthesis of KDO. Bigham, et al. have shown that analogs of the substrates and intermediates can inhibit this enzyme.[12] None of the inhibitors, however, had antimicrobial activity in vitro.

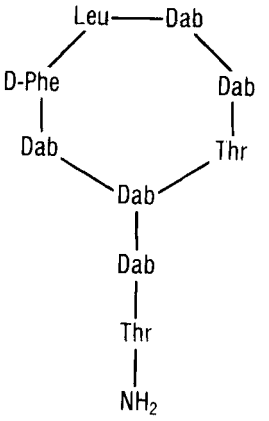
Inhibition of metabolite transport - Makover and Telep have proposed that inhibitors of the phosphoenolpyruvate:sugar phosphotransferase system might be chemotherapeutically useful in treating certain infections.[13] They suggested, based on their studies comparing the virulence of transport mutants and a normal, parental strain, that infections confined to anaerobic sites might be susceptible to treatment with this kind of drug. No known inhibitors were described in this report.

Cladosporin is an isocoumarin derivative that inhibits uptake of uracil and leucine in Bacillus brevis .[14] Inhibition of uptake correlates with inhibition of growth and does not appear to be competitive.

Combinations Acting on Nonclassical Targets - Antibacterial agents are sometimes used in combination when the combined activity is greater than that predicted based on using each drug alone. These synergistic combinations apparently act by mechanisms somewhat different than those used by the individual drugs. The majority of combinations act on classical targets; however, there are several examples of combinations

where one of the drugs has a unique mode of action that can be considered as nonclassical.

Many antibiotics are unable to inhibit selected microbes because they are not able to penetrate the outer layers of the bacterial cell. By virtue of their structure and chemical composition, these layers serve as a barrier and exclude certain antibiotics and other toxic agents. This is particularly apparent in the case of gram-negative bacteria containing a complex outer membrane. Although the outer membrane can serve as a target of antibacterial action it also confers intrinsic resistance towards many antibiotics.[15] Vaara and Vaara have reported that a derivative of polymyxin B [polymyxin B nonapeptide, PMBN (4)] lacking the fatty acyl group and the terminal diaminobutyric acid residue sensitizes the gram-negative cell to several hydrophobic antibiotics normally excluded by the outer membrane.[16]



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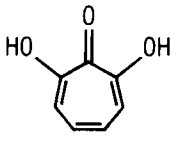
PMBN showed no bactericidal activity when used alone but potentiated the activity of the other drugs. The mechanism is apparently due to the action of PMBN to permeabilize the outer membrane to hydrophobic drugs. Using this technique, these investigators demonstrated that pretreatment of serum-resistant bacteria with PMBN resulted in sensitization to the lytic action of serum complement.[17] In another study, Alatossava, et al. showed that PMBN could be used to influence intracellular potassium and magnesium concentrations in E.coli by potentiating the effects of ionophore antibiotics.[18] PMBN likely interacts with the lipopolysaccharide of the outer membrane causing disorganization and exposing a normally hidden hydrophobic surface.[19] This exposed surface might then serve as a receptor for the lytic component of complement as well as a mechanism of entry for numerous hydrophobic antibiotics.[16] Similar results have been reported for colistin nonapeptide.[20] Chapman and Russell suggested that treatment with colistin itself could sensitize Proteus to TRIS by interacting with phospholipids and/or the LPS layer.[21]

In addition to the nonapeptides, other polycationic agents such as protamine salmine and a polylysine polymer sensitize gram-negative bacteria to hydrophobic antibiotics.[22,23] Sensitization occurs at sub-bactericidal concentrations of the polycation. Interestingly, treatment with polycations make these strains as sensitive as deep

rough strains which lack much of the LPS structure in the outer membrane. The polycations probably bind to the acidic core and lipid A portions of the LPS thereby disorganizing the outer membrane.[24] Protamine and polylysine do not sensitize to the same extent as PMBN. Whereas these polycations seem to cause release of part of the LPS, PMBN does not, indicating that the mechanisms of membrane disorganization might differ for these drugs.

Combinations that Overcome Resistance - Based on drug screening programs, we know that it is possible to find compounds that can interfere with or inhibit antibiotic resistance mechanisms in bacteria. A combination of an inhibitor plus the antibiotic to which the organism was originally resistant can often overcome the resistance and confer susceptibility. Most reports have dealt with inhibitors of drug-inactivating enzymes. Although the antibiotic activity is usually directed against a classical target, inhibition of a drug-inactivating enzyme can be considered nonclassical.

Several drugs are known that facilitate the activity of β-lactam antibiotics against β-lactam-resistant bacteria by inhibiting β-lactamases and sparing the β-lactam antibiotic from enzymatic hydrolysis. The naturally occurring β-lactamase inhibitors include clavulanic acid,[25] olivanic acids,[26] izumenolide[27] and panosialin.[28] The penicillanic acid sulphones[29] and halo-penicillanic acids [30] are semi-synthetic β-lactamase inhibitors. Clavulanic acid has been the most extensively studied inhibitor. It potentiates the activity of penicillins and cephalosporins against β-lactamase-producing strains *in vitro* [31,32] and is effective when combined with amoxicillin in treating amoxicillin-resistant infections.[33] Inhibition of an aminoglycoside-modifying enzyme can potentiate the activity of certain aminoglycosides against strains producing the enzyme. 7-Hydroxytropolone (5) is a



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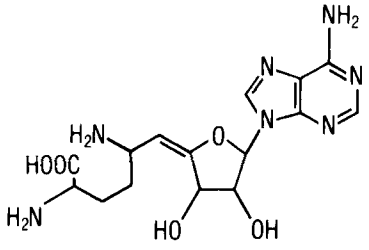

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specific inhibitor of 2"-O-adenylyltransferase.[34,35] This inhibitor facilitates activity against the enzyme-containing strains when combined with aminoglycosides that serve as substrates for the enzyme. Williams and Northrup have described a very potent inhibitor of a gentamicin acetyltransferase.[36] Unfortunately, this inhibitor does not act synergistically when combined with gentamicin presumably because it is excluded from the cell. Miyamura, et al. have described an inhibitor of

chloramphenicol acetyltransferase isolated from a streptomycete.[37] Increased inhibitory activity was detected against several chloramphenicol-resistant bacteria when the inhibitor was combined with chloramphenicol.

In rare cases, combinations of antibiotics can inhibit strains which are resistant or nonsusceptible to the individual drugs. A combination of penicillin and erythromycin inhibits strains of S. aureus that are inducibly resistant to both erythromycin and penicillin. The effectiveness of the combination is due to the inhibition of the induced synthesis of β-lactamase by erythromycin in these strains.[38] An analog of S-adenosylhomocysteine (6), which is a methyltransferase inhibitor,[39] inhibits the development of macrolide resistance in inducibly resistant S. aureus.[40] Presumably, (6) inhibits the methylation of ribosomal RNA and prevents the development of macrolide resistance in these strains.

Inhibition of Virulence Targets - Antibiotics treat infectious disease, because they kill or inhibit the growth of pathogenic microorganisms. While acknowledging the success of antibiotics, it is important to recognize that antibiotic treatment alone is sometimes insufficient without assistance from host defense mechanisms. This is evident from the fact that bacteriostatic drugs are effective in treatment, and from the difficulties in treating infections in the immunocompromised patient. As an adjunct to classical antibiotic therapy, other methods can be used to modulate the progress of an infection. There is considerable interest now in manipulating the immune response by using immunotherapeutic drugs to prevent and treat infectious disease. This area has been reviewed recently by Ades, et al.[41] Another approach to treatment is the use of drugs that interfere with the pathogenesis of the disease. Compounds with this kind of activity would not necessarily have classical antibiotic activity but could be useful for treatment by interferring with a nonclassical, virulence target.

One of the first steps in pathogenesis is adherence of the pathogen to a host surface.[42] This step preceeds the more destructive colonization and invasive stages of a disease. Although adherence per se has little immediate effect, the consequences of this are enormous because without adherence, onset of disease would be less likely. Bacterial adherence results from multiple interactions between ligands on the surface of the bacterial cell (adhesins) and receptor molecules in host cells. Adhesins can be frimbriae or other surface components. Chemical interference or inhibition of this process should interfere with establishment of a disease and could assist in treatment. Studies have shown that purified adhesins, receptor materials or chemical analogs will competitively inhibit adherence.[43,44] Moreover, low concentrations of antibiotics can inhibit synthesis and/or function of adhesins.[43] Whether or not drugs that specifically affect adherence could be used to treat infectious disease will be decided when and if these kinds of compounds are found. An alternative approach would be to look for compounds that would stimulate bacterial adherence to phagocytic cells since phagocytosis is dependent on recognition of the bacterial surface by phagocytic cells.[45]

Streptococcus mutans and other streptococci found in the mouth produce a dextran polymer that coats tooth surfaces giving rise to plaque and leading to caries development. The bacteria elaborate glucosyltransferase which uses sucrose as a substrate to form this dextran polymer. Mutastein is a large molecular weight protein that is an inhibitor of glucosyltransferase.[46,47] It inhibits the formation of

water-insoluble glucan which probably accounts for its inhibition of sucrose-dependent adherence of S. mutans to glass surfaces. Ribocitrin is another inhibitor of glucosyltransferase which also inhibits adherence of S. mutans to glass surfaces without inhibiting growth.[48-51] The drugs could be useful for control of dental plaque formation and subsequent caries development. There are reports[52,53] that cerulenin has an effect on the production of glucosyltransferase but this could be an indirect effect resulting from inhibition of lipid biosynthesis and/or inhibition of the secretion of the enzyme.[54] Other reports describe inhibitory or modulating effects of sodium and potassium ions,[55] pyridine analogs,[56] or flavinoids[57] on glucosyltransferase activity.

Microorganisms require iron for metabolism and growth. Since pathogenic microorganisms must obtain iron from the host, they must scavenge iron from iron-binding proteins such as transferrin. To carry out this task, pathogens have evolved their own iron-binding molecules (siderophores) so as to facilitate growth under conditions where iron is limiting. Enterochelin is an E. coli siderophore and its synthesis has been studied extensively.[58] Following its biosynthesis, enterochelin is transported out of the cell where it binds ferric iron and is taken back up by the cell via a receptor in the outermembrane that recognizes ferric enterochelin. The iron is released intracellularly where it can be used for cell metabolism. Compounds have been described that act as iron-binding agents and lower available iron or by blocking siderophore receptor sites.[59] One can also envisage compounds that interfere with the biosynthesis of a bacterial siderophore or interfere with the receptor site surface so as to prevent import of the iron-carrying siderophore. Drugs having this activity would most likely be bacteristatic rather than bactericidal. Another approach that takes advantage of the specificity of transport systems in E. coli is to use drugs that gain entrance via the iron-siderophore recognition pathway. For example, albomycin is transported into E coli via the same uptake system as that used by the ferrichrome complex[60] due to structural similarities between ferrichrome and albomycin. One disadvantage is that mutations in the transport system can occur and make the organism resistant to the antibiotic. Antibiotics have been described which act as iron chelating or complexing agents.[61,62] It is suggested that this activity could account for the antibiotic properties of these molecules. Deferoxamine is an iron chelator with antibacterial activity which is lost if the deferoxamine is saturated with iron.[63]

Considering the large numbers of new antimicrobial agents discovered each year, it is somewht surprising that so few are reported to inhibit unique or nonclassical targets. Perhaps this is because the specific target sites are known for a relatively small number of these compounds. However it is also possible that the same kinds or types of inhibitors are found because the same screening and discovery methods are used. Several of the agents described in this review were discovered as a result of using unique and nonclassical screening methodologies. A logical conclusion to this observation is that the future discovery of unique drugs to treat infectious diseases requires the use of novel screening strategies.

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Chapter 17. Antineoplastic Agents

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General _ In 1984 numerous advances in our understanding of the biology of cancer came forth. Intensive efforts to unravel the role of oncogenes [1,2] in carcinogenesis[3] and the etiology of specific cancer types, are ongoing. [4,5] The role of growth factors in oncogene expression and control was reviewed.[6-8] A review of the evidence for gene amplification as a mechanism of drug resistance appeared.[9] A more detailed account of antitumor drug resistance was also published.[10]

Several reviews dealing with clinically active drugs or classes of drugs have appeared. Comprehensive reviews of the structure activity relationships of dihydrofolate reductase (DHFR) inhibitors[11] as well as the biological effects of folic acid antagonists were published.[12] Recent progress in the chemistry and biology of anthracyclines was reported.[13] A detailed comparison of the biology and pharmacology of several anthracyclines currently undergoing clinical trial has been published[14] as has a review of anthracycline glycoside-membrane interactions.[15] The potential of taxol as an antitumor agent with a new mechanism of action was presented. [16] The chemistry, biology, mechanism of action, and clinical activity of etoposide (VP-16) have been published.[17] In 1984 a number of papers have appeared implicating topoisomerase II in the mechanism of action of etoposide,[18-20] doxorubicin,[21] 4'-(9-acridinylamino)-methanesulfon-m-anisidide (mAMSA),[22] ellipticine and 9-hydroxy-N2-methylellipticinium acetate (NMHE).[23]

Additional reports on the use of DNA footprinting techniques using DNase I[24] or methidiumpropyl-EDTA Fe(II)[MPE Fe(II)][25] as the DNA cleaving agent have appeared. It has also been shown that common binding locations and site sizes are involved whether one attaches the cleaving agent directly to the DNA binding drug (distamycin) or uses exogenous MPE Fe(II) as the cleaving agent.[26] By attaching the cleaving agent at different positions on the DNA binding agent (distamycin), information concerning sequence specificity of the drug-DNA interaction and drug orientation was obtained.[27] The footprinting technique has been applied to the study of echinomycin interaction with pBR322 DNA.[28] Taken together with a report on the molecular structure of a DNA-triostin complex as determined by x-ray spectroscopy,[29] valuable insights have been obtained on the interaction of bis-intercalators with DNA. Use of ^{1}H NMR to study drug-DNA interactions for seventy agents has given information on the kinetics of drug binding and the specificity of drug for AT or GC base pairs.[30]

Alkylating Agents - Further insights into the mechanism of activation for cyclophosphamide were provided by an NMR study of 4-hydroperoxy-cyclophosphamide decomposition under reducing conditions. The

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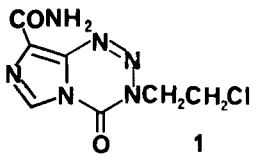
intermediacy of 4-hydroxycyclophosphamides and aldophosphamide were clearly demonstrated as was decomposition of the latter compound to acrolein and a phosphoramide mustard.[31] Several papers describing preclinical studies on 4-(2-sulfonatoethylthio)-cyclophosphamide cyclohexylamine salt (ASTA Z 7557, mafosfamide) have appeared.[32,33]

The synthesis and biology of 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]1,2,3,4-tetrazin-4(3H)-one (1) was reported.[34,35] Compound 1 is felt to be a prodrug modification of the acyclic triazene, 5-[3-(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide. The synthesis of a cis-4-[[[(2-chloroethyl)nitrosoamino]carbonyl]methylamino] cyclohexanecarboxylic acid (N-Me-cis-CCCNU) was reported.[36] This compound was curative in both early and staged Lewis lung carcinoma. Phase I clinical trial reports on teroxirone (α-triglycidyl-triazinetrione)[37] and anaxirone (α-/β-triglycidyl-urazol)[38] have appeared. Severe phlebitis was noted with teroxirone while anaxirone appeared to be superior in this regard. Responses were observed with anaxirone in two adenocarcinomas of unknown origin and in two patients with lung cancer.



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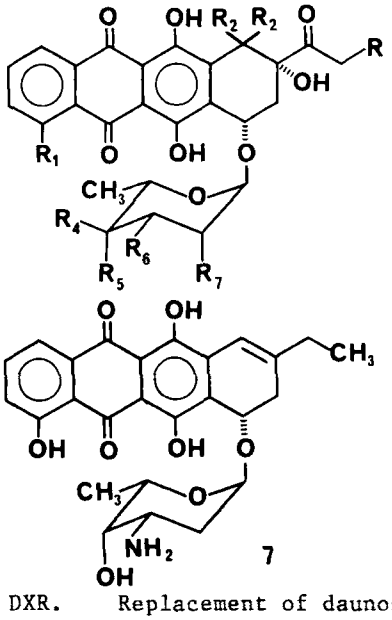
Antimetabolites - Reviews on the folate antagonists have been published in two-volumes covering from the biochemistry up to clinical trials.[39] The interaction of methotrexate (MTX) and 5-fluorouracil was summarized.[40] MTX and aminopterin (AMT) analogues in which L-glutamic acid was replaced by L-homocysteic acid or L-cysteic acid showed in vivo L1210 leukemia activity comparable to the parent compounds although they were 10-30 times less potent.[41] Increased alkyl chain length of mono-MTX esters resulted in a decrease of dihydrofolate reductase affinity and an increase of cytotoxicity in CEM cells.[42] A new synthesis of 5,8-dideazaisofolic acid (IAHQ) and its analogues modified at C5 and C9 was described.[43]

The chemistry and biochemistry of purine and pyrimidine nucleosides were surveyed.[44] Purine nucleoside phosphorylase was examined in a review as a possible target for chemotherapy.[45] Pyrrolo[2,3-d]pyrimidine nucleosides including tubercidin, toyocamycin, and sangivamycin were also the subjects of a review.[46] The adenosine deaminase-resistant 2-chloro-2'-deoxyadenosine lowered blast count by at least 50% in a phase I trial in patients with advanced leukemia.[47] Against murine tumors a high schedule dependancy of 2-bromo-2'-deoxyadenosine was observed.[48] Pyrazolo[3,4-d]pyrimidine-4(5-H)-selenone nucleosides were prepared and found to be active against P388 and L1210 leukemia cells in vitro.[49] Carbocyclic xylofuranosyl 8-azaadenines, showed significant in vivo activity against P388 leukemia.[50] A potential use of p-nitrobenzyl-6-thioinosine to protect normal cells from 9-deazaadenosine was indicated in immunosuppressed mice bearing human pancreatic carcinoma.[51]

Tiazofurin, 2-β-D-ribofuranosylthiazole-4-carboxamide, continues to be a compound of interest.[52] Its congeners, 2-β-D-ara- and 2-β-D-xylofuranosylthiazole-4-carboxamides, however, showed much reduced in vitro cytotoxicity against P388 leukemia cells.[53] The 3-(3-oxoprop-1-enyl) derivatives of thymidine, 2'-deoxyuridine, and 5-iodo-2'-deoxyuridine, were shown to strongly inhibit DNA synthesis in HeLa cells.[54] An inhibitor of ornithine decarboxylase, (2R,5R)-6-heptyne-2,5-diamine (MDL 92175) was reported to cause 80% reduction in growth of EMT 6 sarcoma in mice and HTC hepatoma in rats.[55]

Anthracyclines - Three analogues of doxorubicin (DXR) are currently undergoing clinical trial, 4'-epi-doxorubicin (2), 4'-deoxydoxorubicin (3), and 4-demethoxydaunorubicin (4). Phase II evaluation of 2 has indicated activity in rectal, breast, lung, gastric, ovarian, and head and neck cancers.[56,57] Phase I studies on 3 have been reported. Partial responses in DXR sensitive tumors were noted.[58,59] Compound 4 has been shown to be orally absorbed and largely metabolized to its 13-dihydro derivative which has a prolonged elimination half-life.[60,61] Several additional examples of 4-demethoxy- doxo- and dauno-rubicin analogues have been synthesized.[62] These new analogs are reported to be orally absorbed, more potent, and as active as daunorubicin or doxorubicin. Further details of the synthesis and biological evaluation of 3'-deamino-3'-(3-cyano-4-morpholinyl)doxorubicin (5) have been published.[63] The compound has been shown to be highly potent and active in P388, P388/DXR, L1210, and Gross leukemias. There is little evidence of cardiotoxicity.[64]

Synthesis of compound 6 having two methyl groups substituted at C10 of the chromophore was reported.[65] The compound proved to be inactive, reinforcing the known SAR at this position. A surprising result was the reported activity of akrobomycin 7 in P388 leukemia.[66] The presence of a 9-hydroxy function has long been considered a prerequisite for activity. Syntheses and biological activities of several halosugar analogues were reported.[67] Compounds 8 and 9 have been shown to be orally active, more potent and less cardiotoxic than DXR. The activity of these analogues in P388, Gross leukemias, C3H mammary tumors, and Lewis lung carcinoma were comparable to or better than DXR. Replacement of daunosamine by an iodo sugar as in 10 was also shown to result in a highly active, potent analogue.[68] One of the more intriguing reports in 1984 described the reversal of DXR resistance in ovarian cancer cell lines using the calcium channel blocker verapamil.[69] Verapamil completely reversed doxorubicin resistance in moderately resistant cell lines and partially reversed resistance in highly resistant cells.



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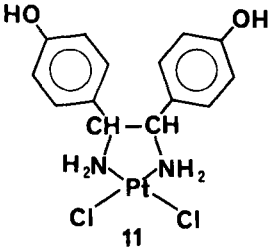
	R_1	R_2	R_3	R_4	R_5	R_6	R_7
2	CH_3O	H	OH	OH	H	NH_2	H
3	CH_3O	H	OH	H	H	NH_2	H
4	H	H	H	H	OH	NH_2	H
5	CH_3O	H	OH	H	OH		H
6	H	CH_3	H	H	OH	NH_2	H
8	OCH_3	H	OH	H	I	NH_2	H
9	OCH_3	H	OH	H	Br	NH_2	H
10	H	H	OH	OH	H	OH	I

Metal Complexes - It has been known for some time that cis-dichloro-diammine platinum (II) (cis-DDP) produces intrastrand cross links between adjacent guanine bases in DNA. The effect of such crosslinks on adjacent base pairs in the DNA helix of a decanucleotide has been studied using ^1H NMR spectroscopy. It was shown that while the GC base pairs at the lesion can be maintained in the duplex, vertical stacking

interactions between adjacent base pairs are distorted.[70] It has also been shown that R,R-1,2-cyclohexanediamine platinum (II) complexes also produce GC intrastrand crosslinks.[71] The synthesis of a number of platinum (IV) analogues of cis-DDP and a study of their interactions with DNA were reported.[72,73] It was concluded that platinum (IV) analogues do not directly interact with DNA and that the likely mechanism of action involves prior in vivo reduction to platinum (II) species.



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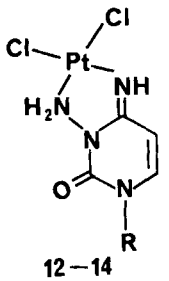

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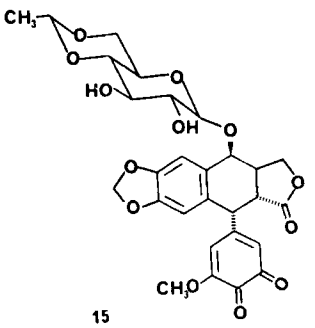
In an effort to provide for specific delivery of a platinum complex to a tumor, a series of complexes which would be expected to exhibit preferential activity in estrogen receptor positive tumors were prepared.[74] The RR and SS isomers of 11 exhibited markedly greater activity in [^{3}H]-thymidine incorporation into MDA-MB 231 breast cancer cell lines and ADJ/PC6 plasmacytoma cells than the meso isomer. The most active compound was the S,S isomer. Syntheses of a series of N-aminated nucleoside complexes of cis-dichloroplatinum (II) were reported (e.g. compounds 12-14).[75] The compounds were much less potent than cis-DDP in L1210 leukemia. In vitro, compound 12 (R=β-D-ribofuranosyl) inhibited RNA synthesis while 13 (R=2-deoxy-β-D-ribofuranosyl) and 14 (R=β-D-arabinofuranosyl) inhibited DNA synthesis.

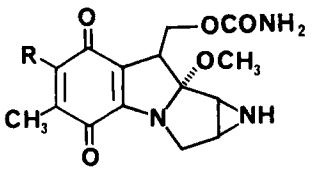
In a direct comparison of [cis-$RuCl_2(DMSO)_4$] with cis-DDP, the ruthenium (II) complex was shown to be as active, if not more active, than cis-DDP in several tumor models.[76] A study of chiral ruthenium complexes found enantiomeric selectivity in binding to a right handed DNA duplex.[77] A paper appeared reporting the use of germanium containing porphyrins as potential antitumor agents.[78] Dimethyl 5,10,15,20-tetrakis[3',5'-di-tertbutylphenyl]porphynato germanium (IV) is active in Walker 256 rat carcinoma and also shows borderline activity in IMC carcinoma and B16 melanoma.

Natural Products - Natural products from microbial sources and higher organisms continue to provide novel chemotherapeutic agents along with new perspectives in their mechanisms of action. Structure-activity relationships of podophyllotoxin analogues including VP-16 and 4'-demethylepipodophyllotoxin thienylidine glucoside (VM-26) in cytotoxicity and DNA breakage activity were published.[79] It was proposed that epipodophyllotoxins interfered with the breakage-reunion reaction of topoisomerase II by stabilizing the cleavable complex.[20] It has also been reported, however, that VP-16 binds irreversibly to DNA and microsomal proteins, with orthoquinone 15 as a proposed reactive intermediate.[80]



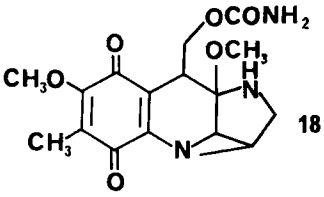
15

IR and NMR characterization studies indicate that, contrary to the previous supposition, leucomitomycin B and C have a finite stability.[81] When activated by NADP-cytochrome P-450 reductase or xanthine oxidase, mitomycin C radical anion was implicated as the reactive species which was postulated to undergo alkylation at C1[82] or to generate hydroxy radicals.[83] Circular dichroism spectroscopy was used to investigate mitomycin C-DNA adducts.[84] BMY-25282, 7-N-(dimethylaminomethylene)mitomycin C (16), was disclosed to have superior P388 and B16 in vivo activities compared to mitomycin C.[85,86] A cure rate of 60-70% in B16 bearing mice by an ip-ip route was reported. Superior activities were also found with RR-150, 7-cysteaminemitosane (17).[87] Syntheses of mitomycin C analogues with aryl substituents on the 7-amino group were published along with their biological activities.[88] A mitomycin A congener (18) with a novel ring system was isolated from Streptomyces caespitosus.[89]



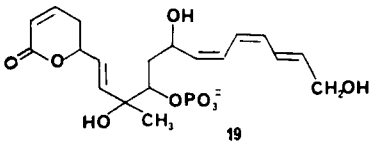
16 R=$(CH_3)_2NCH=N-$

17 R= $HSCH_2CH_2NH-$

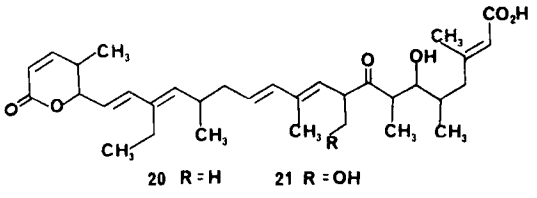


18

In the reaction of CC-1065 with DNA, the adduct was shown to be a cyclopropane ring opened product linked to N3 of adenine.[90] Several synthetic efforts aimed at the preparation of CC-1065 were reported.[91-94] Two novel antitumor antibiotics, PD 114,759 and PD 115,028, of unknown structure, which are three times more potent in vivo and six times more potent in vitro than CC-1065, have been isolated from an Actinomadura species.[95] Structurally novel lactone phosphate esters, C1-920 (19), PD 113,270, and PD 113, 271 were reported.[96] Against L1210 leukemia C1-920 is curative in ca. 10% of mice and gives T/C's higher than 250.[97] Although quite dissimilar in structure, there is some evidence suggesting C1-920 is transported by the same system for reduced folate in L1210 leukemia cells.[98] New polyene lactones, PD 114,720 (20) and PD 114,721 (21) were also reported.[99] The former appears to be identical to leptomycin B.[100]



19

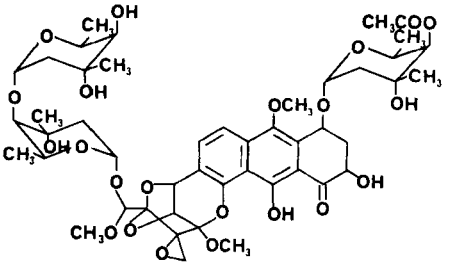


20 R=H 21 R=OH

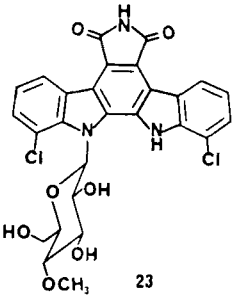
New structure-activity relationships in the saframycin series support the proposed major mechanism of action of saframycin A.[101] Quinocarcin was found to primarily inhibit DNA synthesis in B. subtilis and was involved in generation of free radicals.[102]

New antitumor antibiotics LL-D49194 α_1 (22), β_1,β_2 which are related to trioxacarcins [103] were disclosed.[104] Details of the isolation of phyllanthoside and glycosides of the phyllanthostatins were published.[105] Although rapimycin was only marginally active against murine leukemias,

it was found to be quite active against the colon 38 tumor.[106] The structure of cleocidin, a P388 active fungal metabolite, was determined to be a dimeric diterpene of the clerodane type.[107] Rebeccamycin (23) represented a new chromophore among the antitumor antibiotics.[108] The major components of largomycin FII chromophore were determined to be pluramycin and deacetyl-pluramycin.[109]



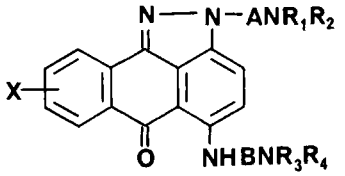
22



23

The marine environment continues to provide structurally interesting compounds. Didemnin B, one of the depsipeptides isolated from Caribbean tunicates, was found to inhibit synthesis of protein much more than syntheses of DNA or RNA.[110] In addition to previously reported bryostatins, a new P388 active bryostatin-4 was isolated.[111] A macrocyclic lactone possessing an epoxide in the side chain was isolated as a potent cytotoxic agent from Tedania ignis.[112]

Synthetic Agents - Phase II trials of mitoxantrone in breast cancer patients revealed 33-35% overall response rates.[113,114] In these studies the dose limiting toxicity was myelosuppression. In comparative studies of mitoxantrone and doxorubicin against breast cancer, there was little difference between the two drugs in terms of the total response rate and the duration of responses.[115,116] The nucleoid sedimentation technique detected intercalation of bisantrene but not of mitoxantrone in L1210 cells.[117] By using electron microscopy, however, both drugs were found to intercalate and the apparent difference between the two drugs was explained by mitoxantrone's offsetting compacting effect on pBR 322 DNA.[118] Related pyrazoles 24 were synthesized, many of which were found to give T/C's higher than 200 in in vivo P388 experiments.[119] Continued syntheses of mAMSA analogues were reported. These include orally active 4-methyl-5-methylcarbamoyl-mAMSA,[120] 3'-alkylaminoderivatives of AMSA,[121] and dibasic 9-aminoacridin-4-carboxamide.[122] Evidence was presented to indicate the mAMSA-induced DNA cleavage is mediated by topoisomerase II.[123] A possible role of DNA mAMSA Cu(II) ternary complex in the generation of superoxide radicals was also cited.[124]



24

A phase II trial of 9-hydroxy-2N-methylellipticinium acetate (NMHE) was carried out in patients with advanced solid tumors, giving an overall response rate of 19%.[125] To overcome the membrane transport problem amino acid adducts of NMHE were synthesized.[126] The resulting four compounds, however, exhibited a decreased efficacy against L1210 leukemia. The dimeric intercalators of 7H-pyridocarbazole were shown to

have different properties from the monomers in terms of the delayed toxicity and the cell-cycle specificity.[127]

The syntheses and *in vivo* P388 activities of bis[(carbamoyloxy)-methyl] derivatives of pyrrolo[2,1-*a*]isoquinoline were reported.[128] One of them was also found to be active against B16 melanoma, $CD8F_1$ mammary tumor, L1210 leukemia, colon 38 tumor, and MX-1 mammary xenograft. A synthetic inhibitor of microtubles, tubulazole, showed good *in vivo* activities against MO_4 sarcoma, L1210 leukemia, and TA_3 carcinoma.[129] Having a unique mode of action, mopidamol was shown to inhibit membrane transport of thymidine and 2-deoxyglucose in L1210 cells.[130] A brief report on the status of vitamin A in cancer chemotherapy appeared.[131] Syntheses of some 13-*cis* and all-*trans*-retinamides were reported.[132] In the study of conformationally restricted retinoids, chroman and thiochroman analogues were found to be less toxic than retinoic acid, yet they had antipapiloma activity similar to that of retinoic acid.[133]

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Section IV-Metabolic Diseases and Endocrine Function

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Chapter 18. Interleukin 1

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INTRODUCTION

The physiological role of Interleukin 1 (IL-1), a protein produced by cells of the macrophage/monocyte lineage, is just beginning to be elucidated but a number of studies suggest that IL-1 is important in certain disease states. Interleukin-1 has been shown to be present in joint fluids obtained from patients with any of several different arthritides including rheumatoid arthritis, psoriatic arthritis, Reiter's syndrome, osteoarthritis, gout, traumatic arthritis, rubella arthritis and acute synovitis.[1-3] It is not known whether increased levels of IL-1 are an underlying cause of the disease or if the disease induces a change in IL-1 production. However, in light of the effects of IL-1 on bone, cartilage, synovial cells and fibroblasts (see below), it may contribute to either the initiation or exacerbation of the tissue destruction observed in an arthritic joint.

Additional studies demonstrate that altered IL-1 production is associated with certain other diseases. Decreased in vitro production of IL-1 by cells obtained from patients with diseases such as systemic lupus erythematosus (SLE),[4,5] systemic sclerosis,[6] sarcoid,[7] lepromatus leprosy[8] and some but not all types of cancer has been reported.[9-12] Although the clinical significance of these changes in IL-1 production is not known, several of those diseases are associated with suppressed immune responsiveness, a possible result of decreased IL-1 production. Along these lines, IL-1, together with Interleukin-2 (IL-2), has been shown to enhance significantly in vitro pokeweed mitogen (PWM)-induced immunoglobulin secretion by peripheral blood lymphocytes obtained from patients with SLE.[13] In contrast to those reports of decreased production, a higher level of IL-1 production by alveolar macrophages was found in patients with high-intensity alveolitis as compared with that from control patients.[14]

Interleukin 1 may also play a role in other host responses associated with either acute or chronic infections, inflammatory responses or traumatic injury.[15] The numerous non-immune effects of IL-1 cited below support this. The importance of IL-1 in mediating these effects in the various disease states, relative to other endogenous or exogenous stimuli, has yet to be elucidated.

BIOCHEMICAL CHARACTERIZATION AND GENE CLONING OF IL-1

Background. Lymphocyte Activating Factor (LAF), a substance produced by human peripheral blood leukocytes, which potentiates the proliferative effect of certain lectins on mouse thymus cells or peripheral blood T-cells, was first described by Gery and co-workers,[16-18] and was later found to be produced by spleen, thymus or bone marrow cells from several species including rabbit, rat, mouse and human.[19]

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Further characterization of these cells demonstrated that adherent cells, or macrophages, are responsible for production of LAF.[20]

Subsequent to these initial studies, other investigators described additional macrophage-derived factors which were named on the basis of their biological activity. These include mitogenic protein,[21] helper peak-1,[22] B-cell differentiation factor (BCDF),[23] and B-cell activating factor.[24-26] In most cases, these factors were not purified to homogeneity but preliminary biochemical characterization suggested that they could be identical to LAF. As a result of the biochemical similarities of the proteins responsible for this range of biological activities, in 1979, the Second International Lymphokine Workshop recommended that LAF and certain other specific macrophage-derived factors be considered identical biochemical entities and that they be designated IL-1.[27] Proof that each of these factors is indeed identical with LAF and that IL-1 possesses a broad spectrum of biological activities will require confirmation of earlier findings with purified proteins. In light of the recent progress in obtaining relatively large amounts of murine IL-1 by recombinant DNA technology, these studies are now possible and should lead to final resolution of some of these questions. For the purposes of this review, IL-1 is considered to be synonymous with LAF and tentatively identical to the other factors discussed below with reservations as noted pending final experimental confirmations. The best characterization of IL-1 has been achieved for mouse and human species.

Murine IL-1. Biochemical studies reveal that IL-1 produced by stimulated murine macrophages and macrophage tumor cell lines is a single polypeptide chain with a molecular weight (MW) ranging between 12,000 and 19,000 daltons.[28] Extensive biochemical characterization of this material has not been possible until recently, because it has been difficult to prepare sufficient amounts of IL-1 for structural and other studies.

Recently recombinant DNA technology has been applied to the study of IL-1 derived from the P388D1 cell line. Lachman and co-workers were the first to show that P388D1 cells produce IL-1.[29] Subsequent purification of this material to apparent homogeneity by Mizel and co-workers[30] revealed its size and charge heterogeneity. The activity of this IL-1 is resistant to treatment with urea, SDS, reducing and alkylating reagents, trypsin, chymotrypsin or papain under non-denaturing conditions.[31] Activity is abolished, however, by treatment with proteinase K or with papain in the presence of 8M urea or upon reaction with phenylglyoxal.[31,32] Mizel *et al.* purified the major species of P388D1-derived IL-1 and determined its amino acid composition.[30] Goat anti-IL-1 antibodies which inhibit both normal and cell line-derived IL-1 activity in the thymocyte proliferation assay have been generated.[33] Interleukin-1 purified with this antibody exhibits the same size and charge heterogeneity as that purified in a conventional manner. These results suggest that differential processing of a single primary protein may explain the IL-1 polypeptide variability.

The availability of the anti-IL-1 antibody and the application of recombinant DNA technology have permitted a detailed analysis of the biosynthesis and structural characteristics of mouse IL-1. Cell-free synthesis experiments using mRNA prepared from superinduced P388D1 cells have demonstrated that a 33,000 MW polypeptide reacts specifically with the anti-IL-1 antibody and, hence, represents the primary translation product of IL-1 mRNA.[34] Pulse labeling experiments indicated that the 33,000 MW polypeptide is synthesized by stimulated normal and cell-line macrophages, and is the precursor to the heterogeneous collection of low molecular weight IL-1 polypeptides found in the culture fluid of stimulated cells.[35] The mRNA coding for the IL-1 precursor has been cloned.[34] The nucleotide sequence of this gene predicts a protein of 270 amino acids with a calculated MW of 31,026. Partial amino acid sequence studies on immunoaffinity-purified P388D1-derived IL-1 suggested that IL-1 activity is derived from the

carboxy-terminal region of the 270 amino acid precursor. As a result, the 156 carboxy-terminal amino acids were expressed in E. coli and yielded protein with the expected size (17,400 MW). This recombinant protein is immunoprecipitated by the anti-IL-1 antibody and is active in the murine thymocyte proliferation assay with a specific activity identical to that of purified natural murine IL-1.[34]

In order to explain the size and charge heterogeneity characteristic of IL-1 preparations, a model has been proposed based on the murine gene cloning and expression results.[34] The model predicts that the 270 amino acid IL-1 precursor is synthesized and secreted from stimulated macrophages, and is enzymatically converted to a lower molecular weight form by one or more proteases released by macrophages (Fig. 1). The primary processing site may be the tetrabasic Lys^{86}-Lys^{87}-Arg^{88}-Arg^{89} sequence resulting in the release of the carboxy-terminal region consisting of amino acids 89-270. Subsequent proteolytic attack at basic amino acids present in the newly exposed amino-terminus would generate a heterogeneous population of molecules. Future work will be necessary to substantiate this model and to show whether recombinant IL-1 derived from a single gene possesses all the activities that have been ascribed to IL-1.

Human IL-1. Human IL-1 has been shown to be produced by stimulated normal human peripheral blood leukocytes or monocytes,[19] leukemic cells obtained from acute monocytic and myelomonocytic leukemia patients,[36-38] and a number of human tumor cell lines.[39-43]

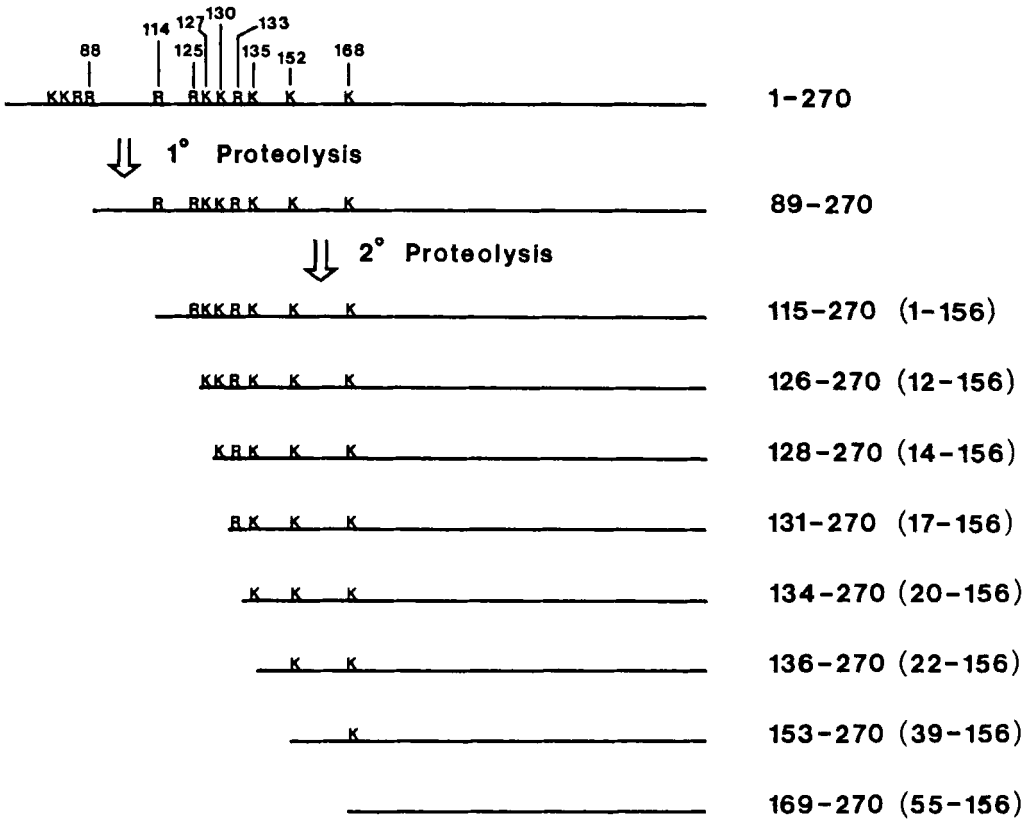
Human IL-1 is sensitive to elevated temperatures[20] and is resistant to a number of proteolytic enzymes.[44] A single isoelectric point of approximately 7.1 has been reported for IL-1 derived from acute monocytic leukemia cells.[45] In contrast, IL-1 derived from normal human peripheral blood monocytes is heterogeneous with respect to charge and major isoelectric points of approximately 5.1 and 6.8 for the 15,000 MW species have been found.[41] In addition, human IL-1 may differ from mouse IL-1 with respect to its amino acid sequence or post-translational modification since mouse and human IL-1 differ in their reactivity to anti-mouse IL-1 antibody.[33]

Recent reports describe the purification of human IL-1 to apparent homogeneity by high performance liquid chromatography (HPLC).[46,47] In addition, a gene which is distinct from the previously described mouse gene and which may code for human IL-1 has been cloned.[48] Expression of this gene and characterization of its product may clarify the relationship between these different proteins.

Other IL-1-like Factors. Endogenous Pyrogen (EP) was first described by Beeson[49] and is thought to be responsible for the febrile responses associated with most, if not all, pyrogenic materials.[15] Recent evidence suggests that EP is secreted by monocytes and macrophages. Rabbit EP has been purified to apparent homogeneity[50] and appears to exist in two forms with molecular weights and isoelectric points similar to rabbit IL-1.[50,51] These results suggest that IL-1 and EP may be identical molecules. Additional evidence for the identity or close similarity of IL-1 and EP comes from studies examining their biological activities.[52,53] Based on their results Damais and co-workers, however, question the identity of EP and IL-1 and suggest that the activities between the two monokines can be dissociated.[54]

Murine and human keratinocytes have been shown to produce an IL-1-like activity designated epidermal T-cell activating factor.[55,56] This factor exhibits some of the activities of IL-1 including the ability to increase the production of serum amyloid A protein (SAA), to promote muscle cell proteolysis, to induce fever, and to act as a mitogen for fibroblasts.

Figure 1. Model for the Processing of Mouse IL-1 Precursor Protein[a,b]



a. ref. 34

b. The presence and location of basic amino acids (R = arginine, K = lysine) surrounding the putative amino-terminus of processed "mature" IL-1 are indicated along the 270 amino acid IL-1 precursor. A primary processing event at the tetrabasic KKRR releases the carboxy-terminal protein numbered 89-270. Subsequent secondary proteolysis releases a collection of molecules with different amino termini (numbered with respect to the original positions in the 270 amino acid precursor; in parenthesis, with respect to the 156 amino acid protein expressed in E. coli).

Other cell types have also been reported to produce IL-1-like activities. Mouse glial cell cultures and rat C_6 glioma cells, upon stimulation with lipopolysaccharide, produce a 13,000-16,000 MW protein which enhances mitogen-induced thymocyte and fibroblast proliferation and increases production of SAA.[57,58] Human glioblastoma cells have also been reported to release an IL-1-like factor.[59] In addition, two other factors may resemble IL-1: one which possesses LAF activity and is produced by an Epstein-Barr virus transformed B-cell line,[60] and catabolin, which is produced by synovial fibroblasts and which has cartilage-resorbing activity.[61]

Further purification and chemical characterization of these various factors are needed to establish their structural relationship to IL-1.

EFFECTS OF IL-1 ON LYMPHOID CELLS

Effect on T-Lymphocytes. Interleukin 1 was first recognized for its ability to induce thymocyte proliferation in the presence or absence of lectin.[17,20] Subsequent to these initial observations, a number of investigators have attempted to delineate the role of IL-1 in T-cell-mediated responses and the mechanism by which IL-1 regulates T-lymphocyte function.

The activation of T-lymphocytes is thought to require two separate accessory cell (macrophage) signals.[62] The first signal is the presentation of antigen to T-lymphocytes in the context of an antigen : Ia complex. The second signal is thought to be provided by accessory cell release of IL-1. A number of studies support this two-signal model for T-cell activation. For example, antigen-pulsed UV-irradiated Ia+ accessory cells, which are deficient in IL-1 production, were unable to present antigen in a manner which induces T-lymphocyte proliferation.[62,63] The addition of IL-1 to antigen-pulsed UV-irradiated accessory cells restored their ability to induce the proliferation of T-cells.[62,63] In other studies, depletion of adherent accessory cells from lymphoid cell preparations prevented antigen-induced activation of T-lymphocytes.[64,65] The addition of IL-1 to accessory cell-depleted cultures did not restore responsiveness suggesting, in these systems, that IL-1 alone does not totally replace accessory cell function.

The mechanism by which IL-1 enhances the proliferation of T-cells has not been completely defined. Although receptors have been described for other cytokines, detailed IL-1 receptor-binding studies have not yet been reported. However, in one report a T-cell tumor line was shown to absorb IL-1 activity,[66] suggesting the presence of IL-1 receptors on these cells. Interaction of IL-1 with the cell apparently leads to at least two processes which contribute to the ability of IL-1 to augment T-lymphocyte proliferation. The first of these is the secretion of lymphokines,[67] including IL-2 from helper T-cells. This is supported by observations that IL-1 enhances lectin- or antigen-induced IL-2 production from helper T-cell clones,[68] thymocytes,[69,70] a T-cell line,[66] a T-cell hybridoma[71] and splenic T-lymphocytes.[72,73]

The second process by which IL-1 may contribute to T-lymphocyte activation is through the induction of IL-2 receptor expression. Kaye et al.[74] have shown that a cloned helper T-cell line releases IL-2, but does not proliferate, following antigen receptor cross-linking. The addition of IL-1 induces cell proliferation. The ability of IL-1 to augment cell proliferation is accompanied by an increase in IL-2 receptors on the T-cell surface. Thus, in this model system, the primary role of IL-1 in inducing T-cell proliferation appears to be the induction of IL-2 receptors rather than the induction of IL-2 release.

In summary, IL-1 appears to play a key role in the activation of T-lymphocytes. The processes triggered by IL-1 may include both the secretion of IL-2 and induction of receptors for this growth factor.

Effect on B-Lymphocytes. B-lymphocytes are the cells of the immune system which produce antibodies or immunoglobulins. Antigenic stimulation of B-cells results in their proliferation and differentiation leading to the secretion of antibodies to the antigen. These events involve complex cellular interactions, several of which are mediated by soluble lymphoid cell-derived immunoregulatory cytokines.

Early studies suggested that IL-1 restores the ability of B-cell-enriched cultures to generate antibody secreting cells.[22,24-26] These reports show that IL-1 synergizes with T-cell-derived lymphokines to induce antibody production and to make B-cells responsive to subsequent T-cell signals.[75,76] Although these early

studies suggested that IL-1 had a role in the induction of antibody secretion by B-cells, the presence of residual T-lymphocytes in the B-cell preparations did not rule out the possibility that the IL-1 effects on B-cell function resulted from an indirect induction of T-helper cell function.

Recent studies in both human and murine systems have utilized more highly enriched B-cell preparations. Thus, the effects of IL-1 observed in these studies may result from its direct action on B-cells.[75-79] The most extensive studies in the murine system, were performed by Howard and co-workers, who utilized anti-IgM to mimic antigen activation of B-cells, and showed that IL-1 synergizes with B-cell growth factor (BCGF) to induce B-cell proliferation.[75] The synergy was greater for low density cultures of B-cells compared with high density cultures of B-cells presumably due to the presence of contaminating IL-1-producing accessory cells. Since IL-1 is effective as late as 14 hrs after the addition of BCGF, it apparently acts at an early stage of B-cell activation.[78] Booth et al.[80,81] confirmed these findings, reporting that IL-1 facilitates anti-IgM-induced B-cell proliferation, and in the absence of anti-IgM, induces B-cells to secrete immunoglobulin.[80] Interleukin 1 also stimulated the synthesis of immunoglobulin by a pre-B-cell line.[82]

Similar studies to those described above have been performed with human B-lymphocyte preparations. Lipsky et al.[83] have shown that an anti-IL-1 antibody inhibits PWM-induced B-cell immunoglobulin secretion. Falkoff et al.[84] have demonstrated that IL-1 synergizes with suboptimal concentrations of anti-IgM antibody in inducing B-cell proliferation but does not enhance BCGF-induced B-cell proliferation. In addition, IL-1, although not active alone, synergized with BCDF in inducing immunoglobulin secretion by S. aureus Cowan-activated B-cells.[85]

In summary, these data suggest that IL-1 can modulate B-cell function through a direct interaction with B-cells. However, the magnitude and nature (i. e., proliferative or differentiative) of the effect can vary depending on the presence of other lymphocyte-derived signals and the type of B-cell stimulus.

Effect on Natural Killer Cells. Natural killer (NK) cells are a population of immune cells which demonstrate cytotoxic activity against selected tumor cells without in vitro or in vivo priming. These cells are thought to be involved in the host's natural surveillance against tumor cells. The activity of these cells can be enhanced in vitro by treatment with interferon[86] or IL-2.[87] Although not active alone, IL-1 was found to synergize with both leukocyte interferon and IL-2 to enhance human NK cell activity.[88] The mechanism of this enhancement has not been determined.

NON-IMMUNE EFFECTS OF IL-1

Effect on Liver Cells. The acute phase response includes the increased hepatic production of a number of plasma proteins such as SAA, serum amyloid P protein (SAP), C-reactive protein, fibrinogen and haptoglobin and is generally observed during periods of infection and/or inflammation.[15]

Sipe and co-workers identified a mediator from endotoxin-stimulated macrophages, which induced an increase in SAA production in mice in vivo[89] and was later found to be closely related or identical to IL-1[90]. In the latter study, highly purified mouse IL-1 induced SAA synthesis; phenylglyoxal treatment of IL-1 abolished both its thymocyte proliferating and SAA-inducing activities.[90] Human EP has also been shown to induce SAA synthesis by mouse hepatocytes in vitro.[91]

The effect of IL-1 on SAP production is more controversial. In one study, partially purified mouse and rabbit IL-1 produced a minimal elevation of SAP above normal values in the mouse.[92] In another report, partially purified IL-1 induced

SAP synthesis in vivo and in vitro; that activity was unaffected by phenylglyoxal treatment although the thymocyte proliferating activity was abolished.[93]

An effect of IL-1 on fibrinogen and haptoglobin synthesis has also been reported but it is not clear if this is a direct effect of IL-1 or if it is mediated by other substances, such as prostaglandins (PGs) and free fatty acids, which are induced by IL-1.[15]

Effect on Plasma Divalent Cations. IL-1 may play a role in regulating changes in plasma divalent cation (i. e., zinc, iron, copper) levels associated with chronic inflammation or infectious diseases.[15] Evidence for the potential involvement of IL-1 in this process is based on the assumption that EP and IL-1 are the same molecules. In studies with crude or partially purified preparations of rabbit[94] or human[95] EP, fractions containing pyrogenic activity also possessed the ability to regulate plasma zinc and iron levels. In addition, the fraction released from rabbit peritoneal macrophages, which produced fever in rabbits and reduced zinc plasma levels, also contained LAF activity.[96]

Effect on Cartilage. A 12,000-17,000 MW protein from cultured human monocytes has been shown to stimulate synthesis of collagenase and neutral proteoglycans by human articular chondrocytes and to promote release of glycosaminoglycans from intact cartilage.[97] This protein co-purified with IL-1 as assessed by its LAF activity.[97,98] Both the LAF and chondrocyte-stimulating activities are destroyed by phenylglyoxal.[97,98] However, Pradke and co-workers[99] found that murine P388D1-derived IL-1, as well as IL-1 from other species, was unable to stimulate rabbit chondrocyte protease secretion. McGuire-Goldring and co-workers[100] speculate that these different findings may be due to a cross-species barrier.

Production of PGE and plasminogen activator by human articular chondrocytes is also stimulated by an IL-1-like factor, as assessed by its LAF activity.[100]

Effect on Bone. Cultured human monocytes produce a factor which stimulates bone resorption in vitro.[101] Further purification of this factor and comparison with other reported activities of IL-1 suggest that it is similar to IL-1 and may induce Ca^{++} release from bone in a manner which is independent of PG synthesis.[102] The stimulation of bone resorption by IL-1 is accompanied by an inhibition of ^{3}H-proline incorporation into collagen.[103]

Effect on Synovial Membrane. A human macrophage-derived factor, which may be similar or identical to IL-1, has been shown to stimulate collagenase and PG release by rheumatoid synovial cells.[104,105] This activity co-purified with mouse P388D1 derived IL-1.[106] Furthermore, a recent study showed that a single preparation of partially purified human IL-1 had lymphocyte activating, chondrocyte-stimulating, and cartilage-resorbing activities as well as the ability to enhance synovial cell proliferation.[98]

Effect on Fibroblasts. Interleukin 1 may also be a modulator of fibroblast proliferation. Partially purified mouse and human IL-1 stimulated the proliferation of several human dermal fibroblast cell lines.[107] The effect of human IL-1 on fibroblast proliferation cannot be separated from its thymocyte proliferating activity by gel filtration and HPLC.[108] Interleukin 1 also stimulated production of collagenase by cultured fibroblasts and elicited the release of PGs.[109]

Effect on Muscle Cells. Treatment of muscle tissue with human EP results in net protein degradation and marked synthesis of PGE_2; both effects are blocked by inhibitors of the cyclo-oxygenase pathway.[110] These studies suggest that IL-1 may be a mediator of muscle cell proteolysis.

MODULATION OF IL-1 ACTIVITY

As described above, IL-1 is thought to be an important mediator of immune cell interactions and to contribute to a number of disease manifestations and physiological processes. Thus, agents that modulate either IL-1 production or IL-1 activity on target tissues may be therapeutically beneficial and have been the focus of a number of investigations.

Modulation of IL-1 Production. A number of stimuli have been shown to induce the release of IL-1 in vitro and in vivo.[15,111] These include particulate agents such as latex particles[112] and bacteria,[15] adjuvants such as lipopolysaccharide,[16,20] lymphokines,[113,114] and a variety of organic compounds including phorbol myristate acetate,[115] 1,25-$(OH)_2$ D_3[116] and steroids.[117] In addition, in some instances UV irradiation can induce the release of IL-1 in vivo and in vitro.[118,119]

In contrast to the large number of reports identifying agents which induce IL-1 production, relatively little information is available concerning agents that modulate the production of IL-1. Dinarello et al.[120] examined the effect of inhibitors of arachidonic acid metabolism on IL-1 production and reported that 5, 8, 11, 14-eicosatetraynoic acid (ETYA) and 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline (BW755C) inhibit the production of IL-1 by human monocytes stimulated with staphylococci, but the cyclo-oxygenase inhibitor ibuprofen was not active. These results suggest that a product of arachidonate lipoxygenase is involved in the induction of IL-1 secretion by human monocytes. The effect of corticosteroids on IL-1 production has also been examined.[121-124] In one of these studies, hydrocortisone (10^{-6} to 10^{-4} M) was shown to block carrageenan-induced IL-1 secretion.[124] Thus, inhibition of IL-1 synthesis may be a contributing factor to the immunosuppressive properties of corticosteroids.

Modulation of IL-1 Effector Function. The intracellular events induced by IL-1 which trigger functional changes in target tissues are not known; however, a number of selected inhibitors of intracellular metabolic pathways seem to modulate IL-1 activity.

Interleukin 1 induces the secretion of PGs from a variety of sources including hypothalamic tissue,[125] skeletal muscle cells,[126] synovial cells[1] and monocytes.[127] Thus, arachidonate products may mediate certain biological effects of IL-1. Dinarello et al.[127] demonstrated that indomethacin inhibits IL-1-induced PGE secretion and skeletal muscle proteolysis. Ibuprofen prevented IL-1-induced fever in rabbits and PGE production by human monocytes but not IL-1 induced thymocyte proliferation.[127] In contrast, the lipoxygenase inhibitors ETYA and BW755C inhibited IL-1-induced thymocyte proliferation.[127] These data suggest that IL-1 possesses the capacity to activate the arachidonic acid pathway in target tissues and that products or inhibitors of this pathway may mediate selected effector functions of IL-1.

Cyclosporine A (CSA) also modulates several activities of IL-1 including inhibition of IL-1-induced bone resorption, PG synthesis by chondrocytes and production of osteocalcin by osteoblasts.[128] In contrast, proteinase secretion by chondrocytes is not affected. The mechanism by which CSA inhibited these responses is not known. CSA also inhibited the proliferative response of thymocytes to IL-1 and PHA.[129] This latter activity is presumably based on the ability of CSA to inhibit the transcription of IL-2 mRNA.[130]

Recently, a 20,000 to 40,000 MW glycoprotein, which inhibited IL-1-induced thymocyte proliferation, was isolated from the urine of febrile patients .[131] This protein had no effect on IL-2-induced thymocyte proliferation and may be a specific inhibitor of IL-1 although its mechanism of action is not known.

CONCLUSIONS

In summary, IL-1 is a molecule which possesses a wide variety of biological activities. The recent cloning and expression of the IL-1 gene should provide the single gene product needed to gain more definitive information concerning its array of activities. This information and additional developments in IL-1 research, should lead to an understanding of the mechanism by which IL-1 induces each of its effector activities, of the role of IL-1 in various disease states and of the means by which specific activities of IL-1 can be modulated.

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Chapter 19. Growth Hormone Releasing Factors (Somatocrinins)

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Introduction - Following the proposal by Reichlin[1] of the existence of a growth hormone releasing factor and the demonstration by Deuben and Meites[2] of its presence in hypothalamic extracts, numerous investigators had sought to characterize this elusive hormone. In spite of the success in identifying the other hypothalamic releasing factors, thyrotropin releasing hormone (TRH)[3,4], luteinizing hormone-releasing hormone (LHRH)[5,6] and corticotropin releasing hormone (CRH)[7], the early efforts led only to the isolation and characterization of a potent inhibitor of growth hormone secretion: somatotropin release inhibiting factor, somatostatin (SRIF).[8,9]

Late in 1982, peptides with growth hormone releasing activity were isolated from human pancreatic islet tumors, obtained from patients with acromegaly, and were characterized independently by Vale et al[10,11] and Guillemin et al.[12,13] Three growth hormone releasing factor (GRF) peptides, which are homologous from the amino-terminus, were characterized: GRF(1-37)-OH, GRF(1-40)-OH and GRF(1-44)-NH_2. Although GRF(1-40)-OH was the predominant peptide obtained from the tumors, GRF(1-44)-NH_2 (Figure 1)* has been confirmed as the primary structure on the basis of molecular cloning and DNA sequence analysis.[14,15] The trivial name somatocrinin has been designated for GRF.[16] The 44-amino acid peptide isolated from human pancreatic tumors (hpGRF) and possessing an amidated carboxy-terminus has been shown to be identical to GRF found in human hypothalamus (hGRF).[17-19] Accumulation of GRF in pancreatic islets is considered to be ectopically produced[12] and there is no evidence which

FIGURE 1

PRIMARY SEQUENCE OF HUMAN GROWTH HORMONE RELEASING FACTOR (SOMATOCRININ)

H-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-
Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-
Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-
Ala-Arg-Ala-Arg-Leu-NH_2

*Abbreviations used in this paper follow the rules of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN): Eur. J. Biochem., 138, 9 (1984).

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supports the production of hGRF outside of the hypothalamus. Measurement of growth hormone-like immunoreactivity (GRH-LI) in human tissue extracts confirmed that hGRF is biosynthesized in the hypothalamus, transported to and stored in the stalk median eminence for release into the portal vessels.[20,21]

Significant regions of sequence homology have been observed between hGRF and several intestinal peptides of the secretin-glucagon family.[22] The most extensive structural similarity exists with a peptide isolated from porcine gut[23], PHI-27, in which there is homology to hGRF in 12 of the 27 amino acid residues. Nevertheless, hGRF has been reported to stimulate the secretion of growth hormone (GH) specifically, both in vitro and in vivo, without any effect on the secretion of other pituitary hormones.[12,24] The specificity of each of the four hypothalamic releasing factors (TRH, LHRH, CRH and GRF) on their respective target cells has been confirmed and there are no significant interactions among the releasing factors on anterior pituitary hormone secretions.[25] Somatostatin has been shown to inhibit (non-competitively) hGRF-induced growth hormone secretion both in vitro and in vivo.[26]

Extremely large doses of hGRF(intravenously) have been reported to produce a mild tranquilization effect, a minimal blood pressure lowering of short duration, transient facial flushing, and a rise in pulse rate.[27] Intraventricular administration of hGRF gives an overall tranquilizing effect, as determined by EEG monitoring during slow wave sleep.[28] Marked behavioral and motor effects have been reported in rats within 5 minutes of intracerebroventricular administration of hGRF.[29] These included sniffing, grooming and exploring as well as behavior characterized as a fear response 2 hours after injection.

Structural Differences Among Species - Hypothalamic growth hormone releasing peptides have been isolated from porcine,[30] bovine,[31] caprine,[19] ovine[19] and rat[32] sources and their structures elucidated (Figure 2). Porcine GRF (pGRF) is identical to hGRF with the exception of three substitutions (Arg^{34}, Gln^{38}, Val^{42}). Bovine GRF (bGRF) and caprine GRF (cGRF) are identical to each other and have five

FIGURE 2

SEQUENCES OF GRF PEPTIDES FROM VARIOUS SPECIES

hGRF	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL-NH_2
pGRF	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGARVRL-NH_2
bGRF	YADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGAKVRL-NH_2
cGRF	YADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGAKVRL-NH_2
oGRF	YADAIFTNSYRKILGQLSARKLLQDIMNRQQGERNQEQGAKVRL-NH_2
rGRF	HADAIFTSSYRRILGQLYARKLLHEIMNRQQGEENQEQRSRFN-OH

substitutions from the human sequence (Asn^{28}, Arg^{34}, Gln^{38}, Lys^{41}, Val^{42}). Ovine GRF (oGRF) is related to bGRF and cGRF with one additional substitution, Ile^{13} in place of Val^{13}. Rat hypothalamic GRF (rGRF), a 43-amino acid peptide with a carboxyl terminal-COOH function, only has 67% homology with hGRF, with substitutions in fourteen positions. In spite of the structural differences of GRF among species, it has been shown that hGRF not only stimulates growth hormone release in humans,[33] but also in a number of other species. However, rGRF is a more potent secretagogue than hGRF in the rat.[34] It has been shown that hGRF-induced GH secretion in rats is not age-related.[35] Evidence has been presented which demonstrates that hGRF secretion in rats varies according to the hour of administration.[34] To explain this observation, it has been hypothesized that superimposed on the steady state release of somatostatin and GRF is an additional rhythmic surge of these peptides with a periodicity of 3-4 h , about 180° out of phase.[34]

Synthesis and Structure-Activity Relationships - Solid phase peptide synthesis[36] has been successfully applied to the preparation of hGRF[11,12,19,32] and enabled its rapid synthesis for structural confirmation and biological evaluation. Solution syntheses have also been reported for hGRF(1-44)-NH_2,[37-39] hGRF(1-40)-OH[40] and hGRF(1-37)-NH_2.[41] The observation that GRF(1-29)-NH_2 possesses the full intrinsic activity [where full intrinsic activity is defined as the maximum GH-secretion obtained by hGRF(1-44)-NH_2] in the *in vitro* assay[11] established the importance of the amino-terminus domain of the molecule and led to the synthesis of a series of carboxy-terminus deletion analogues.[42-49] A substantial loss (to 12%) in biological potency [where potency is defined as the relative dose or concentration of an analogue required to produce the equivalent effect with hGRF(1-44)-NH_2] was observed with hGRF(1-27)-NH_2; hGRF(1-21)-NH_2 was the shortest fragment with full intrinsic activity despite very low potency.[49] The GRF analogues having a free carboxy-terminus possess approximately one-half the potency of the corresponding carboxy-terminal amidated fragments. N^{α}-Biotinyl substitution also resulted in diminished biological potency.[41] The omission analogues GRF(1-15)-(20-44)-NH_2 and GRF(1-15)-(18-44)-NH_2 were inactive.[39]

Methionine at position 27 is not required for biological activity and has been replaced by Ile or Val with partial loss of biological potency.[43,46] Replacement of Met^{27} by Nle gave analogues with increased potency by a factor of three.[43] Replacement of Tyr^{1} by other aromatic residues (with the exception of histidine, which is the amino-terminus residue in rGRF) resulted in diminished bioactivity.[43,48] D-Amino acid substitutions generally produced analogues with diminished biological potencies.[43,48] [D-Ala^{2}]-GRF(1-29)-NH_2 was reported to be 50 times more potent than GRF(1-29)-NH_2 in an *in vivo* bioassay both in pigs and rats;[47] however, in limited human clinical trials (GH release), it was only equipotent with GRF(1-29)-NH_2 and GRF(1-40)-OH.[50]

A series of peptides structurally unrelated to the somatocrinins have also been reported to elicit growth hormone secretion specifically. These peptides do not act at the same pituitary receptor site and may be involved in a different mechanism of GH release.[51] The initial peptide in this series, Tyr-D-Trp-Gly-Phe-Met-NH_2, is structurally related to [Met]-enkephalin, and has been termed growth hormone-releasing peptide (GHRP).[52] This observation led to the design of a series of GHRP

analogues which were based on conformational energy calculations.[53-56] The most potent GHRP analogue,[57] [His^1,Lys^6]-GHRP, was shown to stimulate GH release specifically both *in vitro* and *in vivo*.[51] Although an *in vivo* comparison with GRF by s.c. injection was not possible due to kinetic variations (e.g. distribution, metabolism) of the GH response, [His^1,Lys^6]-GHRP, like GRF, released GH in a number of species.[51]

Biological Activity - The isolation and purification of hGRF and the determination of its intrinsic activity and potency was facilitated by the development of a sensitive quantitative bioassay utilizing a dispersed rat pituitary primary cell culture and a specific rat GH radioimmunoassay.[26] The same assay system was used to confirm the specificity of hGRF-induced GH release[12] and its non-competitive inhibition by somatostatin.[58] GH stimulates release of insulin-like growth factors,[59] IGF I (somatomedin C) and IGF II, which in turn mediate many of the anabolic effects of GH.[60] IGF I and IGF II also inhibit hGRF-induced GH release but epidermal growth factor and fibroblast growth factor do not effect GH secretion.[61]

In addition, hGRF-stimulated GH release is correlated with the stimulation of adenylate cyclase and cAMP formation.[62-64] The kinetics of hGRF regulation of transcription of GH gene expression suggests that cAMP is not a required intermediate.[65,66] Calcium ion is also required for GH release, and somatostatin can block both cAMP-induced and Ca^{++}-mediated release.[67] Prostaglandin E_2 stimulated hGRF-induced GH release in an additive fashion.[67] Phosphatidyl inositol labelling in cell culture was also stimulated by hGRF in a dose- dependent manner, an effect which could be blocked by somatostatin.[68] The pulsatile release of GH in response to hGRF in a pituitary cell perfusion system mimicks the pulsatile endogenous GH secretion in intact rats.[69] The necessity for hGRF in somatic growth was demonstrated with the reduction of the rate of growth in rats resulting from administration of antibodies to hGRF.[70]

Human GRF has been administered to a variety of animals including: rats[11,42], rabbits[71], goldfish[72], fowl[73-76], dogs[12], goats[77], cattle[78-80], sheep[81] and monkeys[82]. Although a significant dose dependent GH response was seen in all species, hGRF is substantially less potent in monkeys. Shorter substitution analogs have been reported to be more potent in rats and pigs.[83]

Interactions between hGRF and other hormones have been studied *in vitro* and *in vivo*. Corticosterone is required for hGRF-stimulated GH secretion *in vitro* during the four-day pre-incubation period of the rat pituitary cell bioassay procedure.[26] Although estradiol also increases cell content of GH *in vitro*, it exhibited no direct effects on GH secretion or content in the presence of corticosterone.[84] Glucocorticoids enhanced the pituitary response to hGRF *in vivo*.[85]

Clinical Studies - The considerable amount of information that has been accumulated *in vitro* and *in vivo* in animals over a short period of time has facilitated the use of hGRF in human subjects. Hundreds of human volunteers and patients have received acute i.v. bolus injections of hGRF and some have been infused for several hours. Both hGRF-(1-44)-NH_2 and hGRF(1-40)-OH are highly potent in causing the specific release in humans of rapid but variable peaks of GH. Responses to hGRF can be seen within 5 min of injection with a peak response after 30 min and lasting for 1-

2 h.[33,86,87] Dose response studies in humans, in general, suggest that 0.5-1.0 μg/kg i.v. will produce a maximum first peak response.[88] Higher doses will prolong the peak or produce a second peak, and cause transient facial flushing. Similar first peak responses are achieved with insulin-induced GH secretion. Infusion of hGRF after a preliminary bolus injection elicits a GH response between 20 and 45 min which cannot be maintained by the infusion. In addition, a second bolus of hGRF after infusion does not induce a significant increase in GH.[89] There are no definitive reports of the effects of low level continuous hGRF infusion over a prolonged period of time in humans. Such a study should determine whether the pituitary synthesis and secretion of GH can be elevated chronically. If young men are given 4 consecutive 90-min infusions of hGRF(1-40)-OH at doses of 1, 3.3, 10 and 33 ng/kg/min preceded and followed by 90-min saline infusions, GH levels can be significantly elevated by each GRF infusion.[90] Dose responses can be observed as well.

Reduction in response to hGRF occurs with aging in normal men. Significant decreases in acute responses occur when 1.5-1.7 μg/kg of hGRF-(1-44)-NH_2 is administered by i.v. bolus to men 40-80 years of age.[91] Mean GH baseline levels do not differ with age nor does the pituitary content of GH.[92] Normal adult women have been given 0.3-3.0 μg/kg of hGRF(1-40)-OH in early follicular, late follicular and mid-luteal phases of their menstrual cycles.[93,94] No significant differences exist between the maximal peak GH responses to hGRF between men and women or at different times in the menstrual cycle of the women.

The primary use of hGRF has been as a diagnostic tool in patients with growth deficiencies or in acromegalics. The etiology of growth hormone deficiency (GHD) is heterogenous. In adults, hGRF elicits a low GH response in idiopathic GHD.[95,96] When injections are given every 3 h for 5 days, the second challenge stimulates some of the patients who do not respond to the first injection. Somatomedin C levels are increased in some patients 24 h after initiation of treatment and to higher levels after 5 days of hGRF administration.[97]

Children with GHD states such as constitutional short stature, intrauterine growth retardation, or organic hypopituitarism showed variable but significantly better GH responses to hGRF than to arginine,[98] L-Dopa,[98] insulin[99] or glucagon-propranolol[99] provocation tests. The localization of GH deficiency to a hypothalamic defect has been demonstrated in many GHD children.[100-102]

Administration of hGRF to acromegalics has elicited a variety of responses from high to moderate to delayed or even no GH responses.[103,104] Peak responses, above the already elevated GH levels, are higher than usually seen in normal men and may result in part from adenomatous tissue as a source of GH. Administration of hGRF-(1-29)-NH_2 at equivalent doses to hGRF(1-44)-NH_2 produces similar GH releasing activity in normal males and females or patients with prolactinomas.[105]

Prolonged administration of hGRF(1-44)-NH_2 (1 μg/kg every 3 h over 10-12 days) increased mean peak GH response and somatomedin C levels and accelerated the velocity of lower leg growth in two GHD children.[106] In separate studies in which two children were pulsed every 3 h with hGRF(1-40)-OH *via* an infusion pump for 4 months, significant acceleration of growth response was observed.[107]

Many more chronic studies with hGRF(1-44)-NH_2, hGRF(1-29)-NH_2 and analogues will be required before one can draw conclusions concerning appropriate dose levels, routes and frequencies of administration and suitable galenic forms for the various types of GH deficiencies. Expansion of these studies to other applications such as burn and wound healing, obesity, bone repair, cachexia and growth retardation secondary to other pathologies will be necessary before the full potential of the somatocrinins can be realized.

Conclusions - In only 2.5 years since the long sought hypothalamic releasing factor peptide, somatocrinin, was isolated and sequenced, it has been synthesized and evaluated in vitro and in vivo in animals and humans and has been used as a diagnostic tool in various growth deficient patients. Its potential in growth promotion and improved milk production, feed utilization and nutrient partitioning between fat and muscle in animals is currently being explored. Somatocrinin analogues with enhanced biological potency have been prepared and recombinant DNA methods of production of GRF are eagerly awaited so that larger quantities may become available more economically. These achievements are expected to facilitate more extensive testing in other human pathologies.

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Chapter 20. Platelet-Activating Factor: Multifaceted Biochemical and Physiological Mediator

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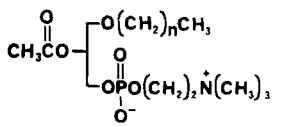
Introduction - In the relatively few years since the discovery of platelet-activating factor as the first bioactive phospholipid, an increasingly significant role in biochemical and physiological events has been ascribed to this novel mediator. Platelet-activating factor (PAF) was first detected as a soluble lytic component capable of rabbit platelet stimulation, and later was isolated from antigen-stimulated IgE-sensitized rabbit basophils. Independently, a similar substance was isolated from the renal medulla which demonstrated significant hypotensive properties, and was termed antihypertensive polar renomedullary lipid (APRL). Chemical characterization and semi-synthesis from bovine heart plasmalogen, followed by total synthesis, established the structure as 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, predominantly composed of the hexadecyl and octadecyl homologs 1a and 1b. Based on this determination, the structurally descriptive abbreviations PAF-acether, AGEPC and AAGPC have also come into use. Since the first overview of PAF chemistry and biology here in 1982,[1] over 500 research papers, symposium proceedings[2] and a number of updated and specialized summaries have appeared,[3-7] attesting to the rapid growth of PAF research. This chapter will summarize some of these recent developments.



	n
1a	15
b	17

Synthesis of PAF - Procedures for both semi-synthesis and total synthesis of PAF on a sufficiently large scale for biochemical investigations have been refined and improved. A facile semi-synthetic procedure utilizes 1-O-alkyl-2-fatty acyl-sn-glyceryl-3-phosphorylethanolamines isolated from bovine erythrocytes. Methanolysis of the acyl residue, followed by mild acetylation and quaternization, provides PAF with either 16:0, 18:0 or 18:1 ether substituents.[8] The total synthesis of PAF used to confirm the proposed structure involved steps severely limited by the potential for acetyl migration. These problems have been addressed by the original workers, who have provided suitable modifications for tritylation of batyl alcohol and, more importantly, for detritylation under conditions mild enough to avoid the possible acetate isomerization.[9] Various other total syntheses of PAF, enantio-PAF and rac-PAF from differentially protected glycerol derivatives have been described.[10-14] The stereochemistry of D-mannitol, from which most chiral glycerol synthons are prepared, dictates that these routes most readily provide enantio-PAF, necessitating inversion of the pro-2-sn-glycerol position for the preparation of PAF. This has been accomplished by extensive protective group manipulation and/or some type of nucleophilic displacement. Alternatively, routes to the required glycerol derivative from either L-serine or L-ascorbic acid can be used. The potential for acid-catalyzed racemization of isopropylidene glycerols prompted the use of either D- or L-tartaric acid as the chiral source, providing PAF or enantio-PAF, respectively, by a very direct route

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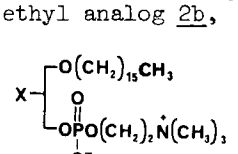
in good overall yield.[15] Notable in most all reported sequences is that acetylation of lyso-PAF (2a) is the final step, tacit recognition that the 2-O-acetyl group is by far the most labile functionality. The single exception to this utilizes a very mild phosphorylation step which avoids the problem of acetyl migration.[11] Finally, PAF labeled with tritium in the alkyl chain was obtained by reduction of the 9,10-octadecenyl analog with tritium gas over palladium oxide.[16]

Physico-Chemical Studies - The profound influence of solvent on both the proton and carbon NMR spectra was examined, showing that PAF exists as aggregates in aqueous media and as a monomeric species in methanol.[17] A later study of the critical micellar concentration of PAF revealed that, at the molar concentrations employed in biological studies (10^{-7} to 10^{-11}), PAF will exist as a monomeric species, suggesting that aggregates do not play a role in its effects.[18] A method for the quantitative analysis of PAF in amounts as low as 10 ng using fast atom bombardment mass spectrometry (FAB-MS) has also been developed.[19]

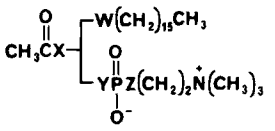
PAF Analogs and Structure-Activity Parameters - As with most biologically active molecules, the synthesis of analogs of PAF serves three purposes: first, to probe the structural requirements for activity, here commonly measured as stimulation of platelet aggregation; second, to enhance the desirable biological actions (e.g., antihypertensive activity) while diminishing or eliminating untoward effects (e.g., anaphylaxis); and third, to discover antagonists of PAF. To these ends, PAF analogs in a number of classes have been prepared. The most numerous and straightforward of these are variations of the 1-O-alkyl and 2-O-acetyl groups, prepared by substitution of the appropriate analog synthon in a PAF synthesis. As observed earlier and now confirmed, both the long alkyl chain (saturated and unsaturated C_{16-18}) and short acyl residue are almost always essential for both proaggregatory and hypotensive activity.[20-24] Some analogs incorporating major changes at position 2, including the ethyl ether 2b, methyl carbamate 2c and nitrate 2d,[22] were surprisingly well tolerated, whereas some others, such as the trifluoro- and trichloroacetyl derivatives 2e and 2f, were not.[25] Replacement of the 2-acyl moiety with fluorine or chlorine (2g and 2h) also reduced proaggregatory activity significantly.[26] Uniformly, the enantiomers and positional isomers of PAF and analogs have been found to be essentially inactive.[20,27] Compounds lacking the oxygen at position 2 were prepared by standard malonic ester alkylation, followed by reduction to afford 2-alkyl-1,3-propanediols. Mono-O-alkylation and phosphorylation gave racemic 2-desacetyloxy-2-alkyl PAF analogs. Of these, 2-desacetyloxy-2-n-propyl PAF (2i) exhibited activity comparable to the above cited 2-O-ethyl analog 2b, while the 2-isopropyl and 2-isobutyl analogs 2j and 2k were significantly less active. Notably, analog 2i was not hypotensive, indicating the possibility of separation of biological effects.[28]



	X				
2a	HO	e	CF_3CO_2	i	n-Pr
b	C_2H_5O	f	CCl_3CO_2	j	i-Pr
c	CH_3NHCO_2	g	F	k	i-Bu
d	NO_3	h	Cl		

Isosteric analogs of PAF have been a prime target as possible agonist/antagonist probes. Replacement of oxygen by either methylene, sulfur or nitrogen represents minimal structural disruption and a possible method to block the action of catabolic enzymes. Systematic replacement of a single oxygen at each of the four possible positions to give the carba analogs 3a-d has been accomplished.[29-33] Of these, only the sn-3 phosphonate analog 3c has exhibited activities comparable to PAF, suggesting that this position is the least important of the four to biological activity. The trimethyl-

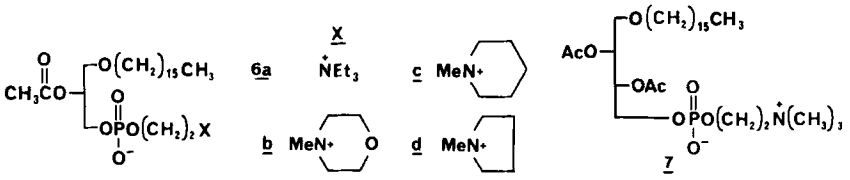
ammoniumethyl phosphonate analog 3e, one methylene group shorter than phosphonate 3d, was also less active.[34] Two isosteres (4a, 4b) substituting sulfur for oxygen at the sn-1 or 2 positions have been prepared. The sn-1 thioether 4a was significantly less active as a proaggregatory stimulant,[36] while the activity of 4b remains unreported. The 2-acetamido analog of PAF (5a), prepared from L-serine,[37] was marginally active as both a hypotensive and platelet stimulant. Other homologous amides and the 2-amino lyso-analog were essentially inactive. The 1-thioether-2-acetamido analog 5b, a combination of two of these isosteric features, has been prepared, but no biological data has been reported.[38]



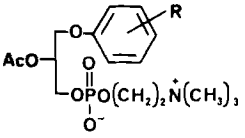
W,X,Y,Z = O except

3a	W=CH$_2$	4a	W=S
b	X=CH$_2$	b	X=S
c	Y=CH$_2$		
d	Z=CH$_2$	5a	X=NH
e	Z= –	b	X=NH, W=S

A series of compounds incorporating modification of the polar head group was prepared from a common bromoethyl synthetic intermediate, replacing the trimethylammonium portion of choline with other nitrogen-containing moieties [triethylammonium (6a), N-methylmorpholinium (6b), N-methylpiperidinium (6c) and N-methylpyrrolidinium (6d)].[39] Each member of this series was a potent hypotensive, 6a and 6b being essentially equipotent to PAF, with 6c and 6d being 3 and 10 times more potent than PAF, respectively. These are the first examples of analogs significantly more potent than PAF in any biological screen.

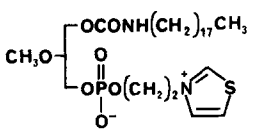


A novel congener 7 of PAF, incorporating an additional acetoxymethylene unit into the glycerol backbone prepared from diacetone glucose, was shown to be a very weak stimulant of aggregation.[40] In another attempt to change the structure markedly, a series of 1-O-alkylphenyl PAF analogs 8a-c was prepared.[31] Comparison of the three showed that the meta analog 8b exhibited activity in the range of PAF, with the para analog 8c less active and the ortho analog 8a completely inactive in both platelet aggregation and antihypertensive studies.

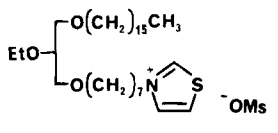


	R
8a	o-$C_{16}H_{33}$
b	m-$C_{14}H_{29}$
c	p-$C_{13}H_{27}$

Receptor-Specific Antagonists - Reported successes in the search for specific receptor antagonists of PAF action can be divided into two categories. The first group is comprised of lipids possessing a thiazolium polar head group. CV-3988 (9), which incorporates an octadecyl carbamate in position 1, methyl ether in position 2, and thiazolium ethyl phosphate in position 3, was discovered as part of a program to investigate carbamate analogs of lysophospholipid.[41] This compound inhibits PAF-induced aggregation, while having no effect on aggregation induced by arachidonic acid, ADP or the calcium ionophore A-23187, and also dose-dependently inhibits PAF-induced hypotension in anesthetized rats.[42] Variations of the structure of 9 which replace

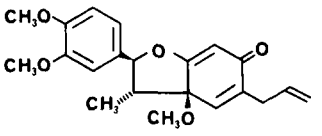


9

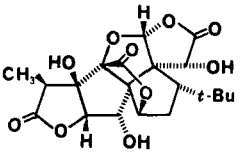


10

the thiazoliumethyl phosphate with a thiazoliumheptyl moiety at position 3 and also incorporate other PAF features, gave a series of compounds an order of magnitude more potent than 9 as PAF antagonists, the most potent of which is the thiazolium mesylate 10 (ONO-6248).[43] A second group is comprised of two natural products structurally unrelated to PAF. Kadsurenone (11), a pseudolignan isolated from the Chinese medicinal herb haifenteng, is a potent PAF antagonist active both *in vitro* and *in vivo*.[44] Ginkgolide B (BN 52021, 12) has also been shown to be a potent and specific PAF antagonist, and has been compared to 11 in a number of assays.[45] Both of these compounds, along with the more lipid-like analogs, will be of great use in the study of the receptor-mediated effects of PAF.



11



12

Biosynthesis and Metabolism - Detailed examination of the release of PAF from a variety of cell types [e.g., platelets, macrophages, polymorphonuclear cells (PMN)] utilizing a number of stimuli (e.g., calcium ionophore, opsonized zymosan) has provided new information regarding the two biosynthetic pathways for production of PAF. The de novo pathway involves transfer of a phosphocholine group to 1-O-alkyl-2-acetyl-sn-glycerol from CDP-choline catalyzed by a choline transferase, and postulates dihydroxyacetone and fatty alcohol as the required biochemical precursors of PAF. The second route, referred to as the deacylation-reacylation mechanism, is a two-step process involving phospholipase A_2 (PLA_2) cleavage of endogenous 1-O-alkyl-2-acyl glycerophosphocholine (GPC) to lyso-PAF, followed by acetylation catalyzed by a specific calcium-dependent acetyltransferase.[1,46] The demonstration of the necessary role of phospholipase A_2 was accomplished by the use of PLA_2 inhibitors in both rat alveolar macrophages[47] and rabbit platelets.[48] Evidence for enhanced levels of acetyltransferase activity and its dependence on extracellular calcium ions and other stimuli in murine macrophages[49,50] and peritoneal cells,[51] rat peritoneal[52] and alveolar[47] macrophages, rat kidney cells,[53] and human eosinophils[54] and PMN[55,56] has also been presented. Regulation of the rate-limiting acetylation step by calmodulin was demonstrated in stimulated PMN by the use of two calmodulin antagonists,[57] and may occur by an activation of the enzyme requiring phosphorylation.[58] The presence of sufficient alkyl acyl GPC precursor for PAF biosynthesis has been demonstrated in a variety of PAF sources.[59,60] Although labeled hexadecanol was not incorporated into PAF by rabbit PMN under conditions which stimulate PAF production,[61] evidence that the proposed last step of the de novo pathway is operative has been demonstrated. The neutral lipid 1-O-alkyl-2-acetylglycerol has been found to be hypotensive in rats,[62] and to be metabolized to PAF in rabbit platelets,[63,64] both presumably occurring after the action of a choline-phosphotransferase to produce PAF.

The most recent development in PAF biosynthesis has provided a surprising link between PAF itself and the arachidonate metabolites. In studies conducted in rabbit platelets[65] and neutrophils,[66] human platelets[67] and PMN,[68] and rat alveolar macrophages,[69] 1-O-alkyl-2-arachidonoyl-GPC has been found to be both a precursor to and product of the lyso-PAF deacylation-reacylation cycle. On one hand, it becomes a common chemical precursor for both PAF and arachidonate under conditions which stimulate platelet aggregation. Alternatively, it acts as a trap for lyso-PAF generated by PAF hydrolysis in target cells. This coupling of two potent endogenous agents of platelet aggregation will no doubt

provide key information in the study of platelet-mediated inflammatory processes.

Conflicting studies report the chemical composition of PAF synthesized by stimulated human PMN, as determined by FAB-MS after HPLC purification and bioassay. In one case, while added radiotracer PAF containing a mixture of 14:0, 16:0 and 18:0 alkyl chains could be separated into the three components, PAF biological activity eluted as a single component characterized as the 16:0 homolog.[70] In the other, five components which exhibited activity were detected: one was identified as the 16:0 homolog, while two others were tentatively found to be the 15:0 and 18:0 homologs; two other components remain unidentified.[71] This apparent homogeneity, or lack thereof, in the production of PAF by PMN may be due to the difference in stimuli used (opsonized zymosan or calcium ionophore[70] vs FMLP[71]), but remains unexplained.

Interactions with Target Cells - The structural requirements for, and the existence of antagonists of, PAF activity both directly point to the presence of specific PAF cell membrane receptors in platelets. Saturable binding of PAF to human platelet receptors has been conclusively demonstrated by two groups using different PAF sources and experimental conditions. The earlier study presented evidence for two distinct binding sites differing about 37-fold in affinity for PAF.[72] The first was a saturable, high affinity site, while the second demonstrated nearly infinite binding capacity. The later experiments confirmed the existence of the high affinity site, albeit possessing a different K_a, and attributed the second type of PAF binding previously observed to unsaturable, nonspecific uptake and metabolism.[73] In addition, platelets desensitized to PAF by prior incubation failed to bind a second dose of labeled PAF, demonstrating the loss of available sites. Later studies provided confirming results for rabbit platelets[74,75] and guinea pig smooth muscle[76] and demonstrated the lack of specific PAF receptors in rat platelets, consistent with species specificity studies.[77,78] Sepharose-bound PAF affinity chromatography of isolated human platelet plasma membranes and subsequent gel electrophoresis revealed the existence of a single protein of molecular weight 180,000, a possible constituent of the PAF receptor.[79]

The role of calcium ions in the biosynthesis and metabolism of PAF mentioned above is also manifested at the cellular level, and is intimately associated with the first steps in the mechanism of platelet activation. In studies with human,[80,81] rabbit[82-84] and horse[85] platelets, PAF rapidly induced a transient loss of phosphatidylinositol-4,5-bisphosphate by activation of phospholipase C, producing 1,2-diacylglycerol, and phosphatidic acid, a calcium ionophore. The initial reduction of polyphosphoinositides is directly linked to mobilization of membrane-bound calcium ions by the phosphatidic acid without the need for extracellular calcium. The calcium ions thus provide the required stimulation for protein kinase C which phosphorylates a 40,000-dalton protein, ultimately releasing serotonin from the activated platelet.[86,87] The associated preliminary shape change, the first measurable physiological platelet response, has been demonstrated to be independent of both extracellular divalent cations and arachidonic acid metabolism.[88] PAF itself does not release the necessary microsomal calcium by direct ionophoric activity,[89] but synthetic PAF phosphatidate, the product of possible phospholipase C and diacylglycerol kinase action, has been shown to be a potent ionophore in the red blood cell.[90] However, the presence of PAF phosphatidate as a transient metabolite of PAF in platelets has never been demonstrated.

Since PAF had been found to stimulate rabbit platelet aggregation with both cyclo-oxygenase inhibitors and ADP scavengers present, and was released upon platelet stimulation with thrombin, collagen and calcium

ionophore, it was proposed as the endogenous mediator of the so-called third pathway of aggregation.[1] However, in later studies using human platelets from normal[91,92] and other control groups,[93,94] PAF-induced aggregation was found to be mediated through both cyclo-oxygenase and ADP-dependent pathways. Both aggregation and serotonin release were inhibited by 2-deoxyglucose, antimycin A, mepacrine, PGI_2, indomethacin and aspirin. The ADP scavengers creatine phosphate and creatine phosphokinase inhibited the second wave of aggregation but not secretion.[91] In addition, PAF-induced aggregation was found to depend on formation of prostaglandin endoperoxides and TXA_2, and, at low concentrations, on ADP secretion.[92,93] Finally, PAF itself was discovered to be a weak agonist of human platelet thromboxane production,[94] and an inhibitor of rabbit platelet adenylate cyclase.[95] A later study in human platelets seems to contradict some of these findings;[96] this discrepancy may be attributed to differences in methods of platelet preparation. The cyclo-oxygenase and ADP-independent pathway, termed as either membrane modulation or stimulus-response, was shown to be operative only under conditions where threshold amounts of PAF and epinephrine were allowed to act synergistically, an effect inhibited by the $\alpha 2$-blocker yohimbine.[97,98] However, since both PAF and epinephrine are released in blood, platelet activation by PAF under these limited conditions may be relevant in some physiological responses. A report on one such apparent difference, comparing PAF-induced platelet aggregation and reptilase clot retraction, may be an example.[99]

The recruitment of human neutrophils to sites of local inflammation may be activated by many stimuli, including PAF.[100,101] The original studies of the mechanism of the migration-aggregation response linked the production of leukotriene B_4 (LTB_4) to the PAF stimulus directly in both rabbit[102] and human PMN[103] but this interrelation has been modified of late. A feedback mechanism for both PAF and LTB_4 levels regulated by cyclic AMP has been suggested.[104,105] Inhibition of PAF, but not LTB_4, neutrophil response by a 5-lipoxygenase inhibitor suggests that an intact 5-lipoxygenase is necessary for the response to PAF, but that LTB_4-mediated chemotaxis is independent of 5-lipoxygenase activity.[106] Further separation of activities for both mediators has been noted by utilizing combinations of arachidonate metabolites and PAF,[107] and by desensitization studies and observation of the additive effects of PAF and LTB_4,[108] strongly suggesting the existence of two distinct receptors.[109,110] Thus, it is likely that some combination of the products of PAF biosynthesis, i.e. metabolizable arachidonate and PAF itself, and not a single mediator, cooperate to produce the observed response in neutrophils, and, quite likely, in all target cells.

In guinea pig peritoneal macrophages, PAF triggered the oxidative burst as measured by both luminol-dependent chemiluminescence and hydrogen peroxide release, an effect which could be inhibited by superoxide dismutase and catalase.[111] Further study in mouse peritoneal macrophages suggests that, while PAF may be an important initiator of the oxidative burst, arachidonic acid modulates the response, with lipoxygenase products being stimulatory and cyclo-oxygenase products being inhibitory.[112] Cells liberating PAF in close contact with macrophage populations thus may cause the release of oxygen species known to be highly toxic.

PAF and Anaphylaxis - Although PAF is a potent platelet-aggregating agent, detailed investigations of the pathophysiological manifestations of the effects of PAF have focused attention on its role as a key anaphylactomimetic mediator,[113-115] operating, in some cases, by mechanisms independent of platelet activation. The hypotensive activity of PAF, one of the first noted and the most pronounced pharmacological property exhibited on either i.v. or oral administration, is attributable to a direct relaxation of vascular smooth muscle, observable in the rat whose platelets are

refractory to PAF.[113] Early evidence proposed that the acute vascular response was mediated by α-adrenergic antagonism. However, exogenous noradrenaline and angiotensin II showed pressor activity after PAF.[116] Pharmacological blockade with specific cholinergic, histaminergic or β-adrenergic receptor antagonists, did not block the dose-dependent hypotensive action of PAF in the normotensive rat.[117] Other studies in guinea pigs, rabbits, dogs and rats confirm the absence of action of PAF on central or autonomic receptors, suggesting that the hypotensive effect of PAF is mainly due to an endothelium-dependent vasodilation.[118-120] Blood flow distribution studies also indicated a decrease in vascular resistance in specific organs.[121,122] The antagonist 9 significantly inhibited PAF-induced hypotension, while not affecting that induced by PGI_2, PGE_2, bradykinin, histamine or acetylcholine.[123] Thyrotropin-releasing hormone and an analog (MK 771) both reversed PAF-induced hypotension in guinea pigs, possibly accounting for the beneficial cardiovascular effect of this peptide in anaphylactic shock.[124] Gross cardiovascular effects of PAF in isolated guinea pig heart preparations[125,126] and anesthetized open-chested dogs[127,128] include a potent, dose-dependent reduction in coronary blood flow and left ventricular contractile force which are unaffected by cyclo-oxygenase or lipoxygenase inhibitors. These observations suggest that PAF may contribute to contractile failure, reduced coronary flow and conduction arrhythmias of cardiac anaphylaxis.

As an indication of the potency of PAF as a mediator of inflammation, intradermal injection of as little as 0.1 pmole of PAF induced an acute increase in vascular permeability, as measured by the skin-blueing model in guinea pigs, confirming the high degree of activity of PAF relative to histamine ($x10^{3-4}$), bradykinin and serotonin ($x10^{2-3}$), leukotrienes D and E ($x10^2$) and C5a (equal).[129] The course of inflammation in guinea pig skin has been charted by radioisotope[130] and histopathological[131] studies. In man, intradermal PAF injection produces an early weal and flare response (1-2 h), succeeded by an area of late-onset erythema (3-6 h), a biphasic response characteristic of the dual response to allergen.[132,133] The acute bronchoconstriction, pulmonary hypertension and edema observed on PAF administration has been demonstrated to be a platelet-independent response mediated by the formation of leukotrienes C_4 and D_4 and TXA_2 in studies carried out on isolated rat, rabbit, cat and guinea pig lung tissue[134-139] and in guinea pigs,[140] rabbits,[141] baboons,[142] and rhesus monkeys.[143-144] While one mechanistic inhibitor study concluded that the action of PAF on the contractile cells was a direct one, independent of endogenous histamine, arachidonic acid metabolites or trapped platelets,[145] a later study showed that PAF interacts with pulmonary sites distinct from those for bradykinin, LTC_4 or arachidonic acid to stimulate TXA_2 production.[146] This release of spasmogens by activated platelets, alveolar macrophages and neutrophils has been proposed as the key step in the development of the acute lung injury associated with adult respiratory distress syndrome (ARDS),[137,139] and in the late-stage allergic reaction in asthma.[142,147,148] Since PAF is formed within the lung during initiation of an allergic reaction, it could account for the appearance of the late-onset reactions and airway hyperreactivity of asthma or the more serious pulmonary hypertension and hydrostatic edema of ARDS. Of particular note is the finding that the therapeutically effective disodium chromoglycate, whose action against asthma has been attributed to mast cell stabilization, has now been shown to be an effective inhibitor of PAF-induced cutaneous inflammatory responses, which are independent of mast cell activation and which resemble the biphasic response to allergen.[149] The implications are consistent with PAF being a key mediator of the pathogenesis of lung injury in a variety of conditions.

Finally, the diverse range of biological activities exhibited by PAF

have yielded two tools for pharmacological research. Specific antibodies against PAF were raised in rabbits. The anti-PAF IgG significantly agglutinated PAF antigen and inhibited PAF-induced platelet aggregation.[150] Prophylactic glucocorticoid pretreatment of mice exerted a highly protective effect against dose-dependent PAF-induced mortality in mice, an event uninhibited by indomethacin, verapamil or nifedipine. This observation suggests that such a model,[151] and the results of a similar study in rabbits,[152] may provide a useful screen for agents protective against anaphylactic reactions mediated by PAF.

Conclusion - The still expanding range of biological activities exhibited by PAF coupled with the variety of cell types which produce PAF firmly establish it as a key mediator of inflammatory and anaphylactic events. While it is unlikely that PAF itself is solely responsible for the pathogenesis of acute and allergic inflammation, it is quite apparent that PAF must be included among the important mediators of normal and abnormal acute allergic and inflammatory reactions.[114]

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Chapter 21. Luteinizing Hormone Releasing Hormone (LHRH) Analogues

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INTRODUCTION

Luteinizing hormone releasing hormone (LHRH; 1) agonists are agents which induce gonadotropin release from the pituitary gland when given acutely; LHRH antagonists counteract the effect of LHRH agonists at the LHRH receptor and so inhibit gonadotropin release.

$$\text{pGlu}^{1}\text{-His}^{2}\text{-Trp}^{3}\text{-Ser}^{4}\text{-Tyr}^{5}\text{-Gly}^{6}\text{-Leu}^{7}\text{-Arg}^{8}\text{-Pro}^{9}\text{-Gly}^{10}\text{-NH}_2 \qquad 1$$

Brief reports on LHRH agonists and antagonists have been included in chapters in recent volumes of this series.[1,2] In addition, excellent reviews on the pharmacology of LHRH agonists and antagonists and their clinical utility have recently appeared.[3-9]

This chapter will review recent data on structure-activity relationships for LHRH agonists and antagonists and their pharmacological activities and clinical applications.

CHEMISTRY

Agonists - Potent LHRH agonists have earlier been synthesized by replacing the Gly residue in position 6 with D-amino acid residues and the Gly-amide moiety in position 10 with various alkyl amide residues or azaglycine (Azgly) amide.[10-12] New bulky hydrophobic D-amino acid residues in position 6 either alone or together with some of the C-terminal changes mentioned above have now given more potent analogues. Incorporation of the D-isomers of 3-(3,4,5-trimethoxyphenyl)alanine [D-Tmo], 3-(2-naphthyl)alanine [D-Nal(2)], 3-[p-(trifluoromethyl)phenyl]-alanine [D-Ptf], 3-(2,4,6-trimethylphenyl)alanine [D-Tmp] and 3-(p-biphenyl)alanine [D-Bpa] in position 6 of LHRH leads to very potent agonists.[13] The potency of these compounds at suppressing estrus in adult female rats is 1.4 to 2 times that of [D-Trp^6, Pro-NHEt^9]-LHRH or 140 to 200 times that of LHRH; [D-Tmp^6]- and [D-Nal(2)^6]-LHRH are the most potent compounds in this series.

One of the objectives of this synthetic program was to study the relationship between hydrophobicity and biological activity. No clear relationship between these parameters was found. [D-Nal(2)^6]-LHRH and [D-Nal(1)^6]-LHRH are essentially identical in hydrophobicity but the former compound is about 4 times more potent than the latter. [D-Tmo^6]-LHRH, which is much less hydrophobic, is as potent as the more hydrophobic [D-Ptf^6]-LHRH and [D-Bpa^6]-LHRH. Pro-NHEt^9 substitution in these compounds gives variable results. [D-Nal(2)^6]-LHRH is twice as potent as [D-Nal(2)^6, Pro-NHEt^9]-LHRH, but equipotent to [D-Nal(2)^6, MeLeu^7]-LHRH and [D-Nal(2)^6, MeLeu^7, Pro-NHEt^9]-LHRH.[13]

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Replacement of the Gly^6 residue of LHRH with bulky heterocyclic amino acid residues, e.g., 3-(2-benzoxazoly)alanine [D-Boa] and 3-(5,6-dimethylbenzimidazo-2-yl)alanine [D-Dmb], also gives compounds more potent than [$D\text{-}Trp^6$, $Pro\text{-}NHEt^9$]-LHRH in the estrous suppression assay.[14] These analogues are less hydrophobic than [$D\text{-}Trp^6$, $Pro\text{-}NHEt^9$]-LHRH. In this series of compounds, Pro-NHEt substitution in position 9 leads to an improvement in potency which is not found in the more hydrophobic series. [$D\text{-}Bia^6$, $Pro\text{-}NHEt^9$]-LHRH and [$D\text{-}Tba^6$, $Pro\text{-}NHEt^9$]-LHRH are 2.5 to 3 times more potent than [$D\text{-}Bia^6$]- and [$D\text{-}Tba^6$]-LHRH [Bia=3-(2-benzimidazolyl)-alanine; Tba=3-(4,5,6,7-tetrahydrobenzimidazol-2-yl)alanine].

With Azgly substitution in position 10 of LHRH along with some of the above hydrophobic amino acid residues in position 6, the resulting compounds are even more potent.[15] Thus, [$D\text{-}Dmb^6$, $Azgly^{10}$]-LHRH and 2 (Table 1) are 1.9 and 2.3 times respectively more potent than [$D\text{-}Trp^6$, $Pro\text{-}NHEt^9$]-LHRH. The most potent compound (230 times LHRH) to originate from these studies is 2.

Replacement of Gly^6 in LHRH with t-leucine (Tle) and with Pro-NHEt at the C-terminus gives [Tle^6, $Pro\text{-}NHEt^9$]-LHRH, a potent analogue superior to [$D\text{-}Ala^6$, $Pro\text{-}NHEt^9$]-LHRH at inducing follicle stimulating hormone (FSH) and LH release in ovariectomized heifers.[16] The duration of effect was also significantly greater than that of [$D\text{-}Ala^6$, $Pro\text{-}NHEt^9$]-LHRH.

A number of LHRH agonists have entered clinical trial, including [$D\text{-}Leu^6$, $Pro\text{-}NHEt^9$]-LHRH (leuprolide, Abbott); [$D\text{-}Ser(tBu)^6$, $Pro\text{-}NHEt^9$]-LHRH (buserelin, Hoechst); [$D\text{-}Nal(2)^6$]-LHRH (nafarelin acetate, Syntex); [$D\text{-}Trp^6$, $MeLeu^7$, $Pro\text{-}NHEt^9$]-LHRH (lutrelin acetate, Wyeth) and [$D\text{-}Ser(tBu)^6$, $Azgly^{10}$]-LHRH [Zoladex^R, Imperial Chemical Industries (ICI)].

Antagonists - Potent antagonists of LHRH which show good in vivo activity were obtained earlier by various modifications in positions 1, 2, 3, 6 and 10 of LHRH.[4,6,17,18] In the hope of improving the potency and duration of effect, some of these have now been further modified (Table 1).

In the 3 series of analogues, replacement of the $D\text{-}Phe(p\text{-}Cl)^2$ residue with other substituted D-Phe residues (m-Cl, o-Cl, p-Me, o-Me, p-OH, p-NH_2, 3,4-di-Cl) results in less potent compounds, but the $D\text{-}Phe^1$ residue can be replaced with substituted D-Phe (p-Cl, m-Cl, p-F) or D-Trp residues to give an improvement in potency.[19] Compound 4, the most potent compound from this series, blocked ovulation completely at a dose of 10 µg/rat.[20] Replacement of the $D\text{-}Ala^{10}$ residue in 4 with D-Ser caused some loss of potency but the $D\text{-}Leu^{10}$ analogue was markedly inferior. Further improvement in potency is obtained when $D\text{-}Phe^6$ in 4 is replaced with D-Lys, D-Arg or D-(p-aminomethyl)phenylalanine residues and $D\text{-}Phe(p\text{-}Cl)^1$ is replaced with D-Nal(2).[21-23] Thus, 5 and 6 blocked ovulation at a dose of 3 µg/rat; 6 even had some activity at 1 µg/rat. A similar analogue, [Ac-$D\text{-}Trp^{1,3}$, $D\text{-}Phe(p\text{-}Cl)^2$, $D\text{-}Lys^6$, $D\text{-}Ala^{10}$]-LHRH, inhibited ovulation completely at a dose of 1.5 µg/rat and when administered daily (5 µg/rat s.c.), suppressed ovulation for 3-4 estrous cycles.[24]

Substitution of D-Arg for Gly^6 does not lead to an increase in potency in every case. In another series of compounds, [$Ac\text{-}Pro^1$, $D\text{-}Phe(p\text{-}F)^2$, $D\text{-}Nal(2)^3$, $D\text{-}Arg^6$]-LHRH was about 7-fold less effective than [$Ac\text{-}Pro^1$, $D\text{-}Phe(p\text{-}F)^2$, $D\text{-}Nal(2)^{3,6}$]-LHRH in an anti-ovulatory assay.[25]

Similarly, [Ac- Δ^3-Pro^1, D-Phe(p-F)^2, D-Trp^3, D-Arg^6, Azgly^{10}]-LHRH is much less potent than [Ac- Δ^3-Pro^1, D-Phe(p-F)^2, D-Trp^{3,6}, AzGly^{10}]-LHRH, which completely blocked ovulation at a dose of 6 μg/rat.[26]

Table 1. LHRH (1) Agonist and Antagonist Analogues.

No.	Residues substituted in positions 1	2	3	6	10
1	pGlu	His	Trp	Gly	Gly
2	pGlu	His	Trp	D-Nal(2)	Azgly
3	Ac-D-Phe	D-Phe(p-Cl)	D-Trp	D-Trp	Gly
4	Ac-D-Phe(p-Cl)	D-Phe(p-Cl)	D-Trp	D-Phe	D-Ala
5	Ac-D-Phe(p-Cl)	D-Phe(p-Cl)	D-Trp	D-Arg	D-Ala
6	Ac-D-Nal(2)	D-Phe(p-Cl)	D-Trp	D-Arg	D-Ala
7	Ac-D-Nal(2)	D-Phe(p-Cl)	D-Trp	D-hArg(Et)$_2$	D-Ala
8	Ac-D-Phe(p-Cl)	D-Phe(p-Cl)	D-3-Pal	D-3-Pal	D-Ala
9	Ac-D-Nal(2)	D-Phe(p-Cl)	D-3-Pal	D-Arg	D-Ala
10	Ac-D-Phe(3,4-di-Cl)	D-Phe(p-Cl)	D-3-Pal	D-Arg	D-Ala
11	Ac-Δ^3-Pro	D-Phe(p-F)	D-Trp	D-Trp	Gly
12	Ac-Δ^3-Pro	D-Phe(p-F)	D-Nal(2)	D-Nal(2)	Gly
13	Ac-D-Nal(2)	D-Phe(p-F)	D-Trp	D-Arg	Gly
14	Z-Gln	des-His	Trp	D-Pse*	-NHEt
15	Z-Gln	des-His	Trp	D-Phe	-NHEt
16	BOC-Ser(Bzl)	des-His	Trp	D-Trp	Gly
17	Ac-D-Trp	D-Phe(p-Cl)	D-Trp	D-Phe	D-Ala

* D-Pse = threo-D-phenylserine

In 5, D-Arg^6 can also be replaced with D-homoarginine or N^G, $N^{G'}$-dialkyl D-homoarginine [D-hArg(Me$_2$), D-hArg(Et$_2$)] residues with retention of full biological activity;[27] the D-hArg(Pr$_2$)^6 analogue is less effective. LHRH analogue 7 is twice as potent as 5 and is also effective when injected 24 hours earlier. In the LH suppression assay in castrated adult male rats, 7 reduced the serum LH concentration to 20% of control values for 24 hours at a dose of 50 μg/kg; the LH concentration returned to control values between 24 and 48 hours.

Replacement of D-Trp^3 in 4 and 5 with D-(2-, 3- or 4-pyridyl)-alanines (Pal) also gives potent antagonists.[28] Compounds 7, 8 and 9 are the most potent of the series; 9 blocked ovulation completely at 0.5 μg/rat. When Leu^7 in compound 9 is replaced by L-Trp, the resulting analogue is somewhat more potent than 9. Other replacements in position 7 [e.g., Nal(2), Phe(p-Cl), Phe, Ile] give much less effective compounds.[29]

LHRH antagonists with restricted conformation have been synthesized in order to obtain more information about the overall conformation of the molecule.[30] Two such compounds containing an α-methyl amino acid residue in position 2, [Ac-Ala^1, α-Me-D-Phe^2, D-Trp^{3,6}]-LHRH and [Ac-Ala^1, α-Me-D-Phe(p-Cl)^2, D-Trp^{3,6}]-LHRH, are as potent as the parent peptides but [Ac-Ala^1, D-Phe^2, α-Me-D-Trp^3, D-Trp^6]-LHRH is inferior.

To restrict the conformation even further, compounds with a disulfide bridge between residues 1 and 4 were prepared. The most potent, [Ac-D-Cys^1, D-Phe(p-Cl)^2, D-Trp^{3,6}, Cys^4]-LHRH, showed only 22% inhibition of ovulation at 200 μg/rat.

In an attempt to increase stability to enzymatic degradation, compounds with up to six D-amino acid residues were prepared.[31] [Ac-Thr^1, D-Phe^2, D-Trp^{3,6}, D-Ser^4, D-Tyr^5, D-Arg^8]-LHRH blocked ovulation (50-70%) at a dose of 25 μg/rat. Compounds prepared with 4 or 5 D-amino acid residues were either inactive or much less effective.

A few LHRH antagonists have recently been reported to be active by the oral route.[24,31,32] For example, 5 inhibited ovulation completely at a dose of 2 mg/rat; compounds 9 and 10 at a dose of 0.5 mg/rat inhibited ovulation to the extent of 56 and 63%, respectively. Other potent antagonists of LHRH, 11, 12 and 13, also blocked ovulation in regularly cycling female rats when given by the i.g. route.[33] The most potent of these, 13, completely blocked ovulation at a dose of 12.5 mg/kg and was also more potent than 11 and 12 in another *in vivo* assay (inhibition of LH secretion in ovariectomized rats) in which it caused an 81% fall in LH secretion, which lasted for more than 12 hours. The higher potency of 13 was not seen in other test systems. In a receptor binding assay using [^{125}I-D-Ala^6, MeLeu^7, Pro-NHEt^9]-LHRH and rat anterior pituitary homogenates, 12 and 13 were much less potent than 11. In an *in vitro* assay (suppression of LHRH-mediated LH secretion by cultured pituitary cells), 11, 12 and 13 were equipotent.[33]

Recently, vascular and behavioral toxic effects have been reported with the potent LHRH antagonist 13.[34] When given s.c. to rats at a dose of 1.25 mg/kg, transient edema of the face and extremities occurred; intravenous injection caused rapid and pronounced peripheral vasodilation followed by respiratory depression, cyanosis and death. Lower doses produced qualitatively similar physiological effects but most rats survived treatment. These effects were not observed in mice and rabbits. In rhesus monkeys, i.v. administration of 100 μg/kg caused an immediate flushing of the face and a depression of spontaneous activity. Higher doses produced more marked effects of longer duration. The partial reversibility of these effects with Benadryl^R suggests that histamine release may account for some of these toxic manifestations. The analogues 11 and 12 do not appear to have this property at the doses tested which encourages the belief that activity and toxicity can be readily separated.[34]

There is also evidence that some LHRH antagonists can elevate blood pressure in conscious normotensive and hypertensive rats.[35] Intraperitoneal injection of either 14, 15 or 16 caused acute increases in blood pressure in normotensive male Sprague-Dawley rats in a dose-related manner. Compound 15 also raised blood pressure in spontaneously hypertensive rats. LHRH itself at doses up to 50 mg/kg had no effect on blood pressure in either normotensive or hypertensive animals.[35]

FORMULATIONS

Because LHRH analogues are poorly absorbed by the oral route, considerable effort has been expended to devise suitable formulations for clinical trials. Although daily s.c. injection has been used, efforts have concentrated on two alternative delivery systems, namely nasal sprays and long-acting depot formulations.

Hoechst was the first to develop and apply a nasal spray formulation of their LHRH agonist, buserelin, in clinical trial;[5] nasal spray formulations of nafarelin acetate[36] and lutrelin acetate are also known to be available. These formulations, although more acceptable than daily injection, suffer from low, and to some extent, variable absorption

and may still give rise to compliance problems. As a consequence, administration of these drugs by the nasal route requires application several times daily.

A once-monthly, biodegradable depot formulation of Zoladex^{R} (ICI) has been developed for s.c. delivery.[37-39] This formulation consists of a homogeneous dispersion of the drug in a rod of d,l-lactide-glycolide copolymer which releases the drug for at least 28 days.[40] Recent clinical trials in patients with prostate cancer show that serum testosterone concentrations are reduced to values observed after castration and maintained with a total dose of 3.6 mg given every 28 days.[41-43] This is accompanied by clinical improvement; there are no lesions at the injection site, compliance is good and the only side effects observed are those related to the LHRH analogue itself.

Similar long-acting formulations of LHRH agonists have been made by microencapsulation of nafarelin[44] and [D-Trp^{6}]-LHRH[45] to give injectable microspheres. These formulations are also highly effective in animal models but at present have only a relatively low drug incorporation (1-2%). A sustained-release delivery system for leuprolide, made by radiation-induced polymerization of an unspecified monomer, is also claimed to be clinically effective for several months.[46]

CLINICAL APPLICATIONS

LHRH agonists can stimulate gonadal function if given at low dose as a series of pulses. If given at higher doses at least once daily, they produce a response equivalent to castration, which is frequently, but inappropriately, referred to as their paradoxical effect. This latter effect is, in fact, predictable and represents the well recognized process variously known as desensitization, tachyphylaxis or receptor down-regulation. The induction of a castration state with LHRH agonists probably represents the first example of successful therapeutic application of the phenomenon of tachyphylaxis. Clinical application of LHRH antagonists has not yet been reported.

Cancer - Extensive clinical trials have been conducted with a number of LHRH agonists in the treatment of prostate cancer;[41-43,47-59] leuprolide and buserelin are now marketed in some European countries for this indication. All of the analogues studied have induced an initial stimulation of testis function for about 7 days followed by a rapid decrease in serum testosterone concentrations to within the castrate range in 3 to 6 weeks; good clinical responses are also usually found. Buserelin has been given both subcutaneously and intranasally, although there is a tendency in the latest trials to use a high parenteral dose to induce the response and then to give intranasal maintenance therapy. [D-Trp^{6}]-LHRH and leuprolide given s.c. have also been studied clinically. Zoladex^{R} has been largely given either s.c. daily or monthly in the depot formulation. The combination of LHRH agonists with pure anti-androgens has also been studied in prostate cancer and preliminary results suggest that survival may be improved.[60]

A few efficacy studies[58,61-63] of LHRH agonists in the treatment of premenopausal women with breast cancer have been undertaken. Leuprolide, Zoladex^{R} and buserelin, under varying dose regimens, caused a fall in circulating estrogen concentrations and a blockade of ovulation, accompanied by clinical responses. After treatment with LHRH analogues, responses have also occurred in post-menopausal women with breast cancer;[62,64] this may suggest that these drugs do not simply act by

inducing a chemical oophorectomy. There is preliminary evidence that buserelin has a direct antitumor effect on human mammary cancer (MCF-7) cells in culture, although the magnitude of the effect is small.[65,66]

The clinical utility of LHRH analogues in other forms of cancer remains to be determined, but there is evidence that they induce remission of animal tumors of the pituitary gland[67,68] and pancreas[69] as well as a transplantable chondrosarcoma[70] and osteosarcoma.[64]

It has also been suggested that temporary suppression of gonadal function with LHRH agonists may protect fertility in young patients being treated with cytotoxic agents for leukemias and lymphomas. However, the experimental evidence provided to support this hypothesis[71] was obtained in mice which have been shown to be resistant to gonadal suppression with LHRH agonists[72] so these findings remain perplexing.

Non-Malignant Disease - Clinical use has been made of both the stimulatory and inhibitory effects of LHRH agonists on gonadal function. Regression of uterine fibroids[73-75] and marked improvements in endometriosis[76-80] have been achieved with several compounds at doses which cause a reduction in circulating estrogen. The castration effect produced is reversible and since serum concentrations of sex hormone binding globulin are not influenced by such treatment, these analogues may have advantages over danazol therapy which markedly reduces the concentrations of this protein and hence increases the proportion of free (hormonally-effective) estrogen.[78] LHRH agonists may also be effective in the treatment of polycystic ovarian disease.[81]

The gonadal suppressing action of LHRH agonists has also been used to treat successfully precocious puberty in both boys and girls; preliminary results suggest that it has advantages over other available therapies.[82-85] [D-Trp^6, Pro-NHEt^9]-LHRH, at a dose which suppressed ovarian function, was able to induce remission in a woman with idiopathic anaphylaxis.[86]

Although the early literature suggested daily injections of LHRH agonists would stimulate gonadal function, it is now clear that small doses given in a regular pulsatile manner are needed to treat male and female infertility. Usually LHRH has been used because the high potency of the analogues appears to offer little advantage in the treatment of this condition. Ovulation can be reliably induced and pregnancies have been achieved in women with anovulatory infertility following pulsatile administration of LHRH from modified infusion pumps.[87-93] Similarly, clinical improvement has been reported in men with hypogonadotropic hypogonadism when they were given pulsatile LHRH treatment.[94-101] Induction of puberty with LHRH analogues has proved to be difficult largely because pituitary desensitization has occurred on repeated treatment with relatively high doses of drug.[102-103] However, when given either on alternative days or at low dose in a pulsatile manner, stimulation of gonadal function, sometimes over many months, can be achieved.[104-107]

Several papers describe the use of LHRH and agonists to induce descent of the testis in patients with cryptorchidism.[108-112] Mixed results were obtained and there were frequent relapses. However, in most studies excessive doses of drug were given which led to inhibition of gonadal function. Studies employing a pulsatile mode of administration have not been reported.

Contraception - There has been widespread interest in the use of LHRH analogues as contraceptive agents and numerous studies have been conducted in primates and man. When the antagonist 17 or the potent agonist [D-Trp^6]-LHRH was administered to normally cycling cynomologous monkeys (Macaca fasicularis) during the first 25 days of the menstrual cycle, ovulation was completely inhibited in all animals.[113] Recovery of ovulatory function generally occurred earlier in the animals treated with the antagonist. Repeated episodes of breakthrough bleeding were seen in some animals treated with the agonist. When the antagonist 17 was administered to regularly cycling rhesus monkeys (Macaca mulatta) from days 10 to 14 of the menstrual cycle, a significant delay in the pre-ovulatory LH peak and in ovulation occurred in 5 of 7 animals.[114]

The post-ovulatory administration of LHRH agonist or antagonist analogues has given less satisfactory results. The agonist [D-Trp^6]-LHRH consistently induced luteolysis in rhesus monkeys when injected on day 3 or 5 post-ovulation but had no effect when given on day 7. The luteolytic effect was prevented by human chorionic gonadotropin (hCG) when given either alone from days 6 to 10 post-ovulation or together with the agonist.[115] Post-ovulatory administration of the antagonist 17, starting from the first day after ovulation until the onset of the next menstrual period, did not have any significant effect on the length of the cycles and luteal phases of rhesus monkeys.[116] Circulating LH and FSH levels were suppressed throughout the treatment but the concentrations of progesterone and estradiol did not differ from normal. These studies suggest that luteal function after ovulation is independent of gonadotropin secretion. A similar conclusion can also be drawn from studies in monkeys hypophysectomized immediately after ovulation.[117] Circulating levels of LH and FSH have also been shown to be decreased in castrate male and female monkeys after the administration of 11 or 17.[118,119]

Although there are no data yet on the antifertility use of LHRH antagonists in women, there have been several studies on the effects of LHRH agonists. Repeated administration of several potent agonists has led to suppression of gonadotropin secretion, a reduction in serum estrogen concentrations and a blockade of ovulation.[120-122] Alterations in bleeding patterns occurred in the majority of patients but there was no evidence of hypo-estrogenism. A rapid return of ovulatory function occurred following cessation of treatment. Concern about the possible adverse effects of unopposed estrogen on the endometrium led to a recommendation that a progestin be co-administered during the second half of a monthly treatment.[123] However, recent data suggest that long-term treatment with LHRH agonists does not cause endometrial abnormalities.[120]

As in monkeys, LHRH agonists will cause luteolysis if given during the early luteal phase in women but they will not terminate luteal function in the face of stimulation by hCG.[124-125] There is evidence that LHRH analogues may influence release of hCG from the placenta. The synthesis and release of placental LHRH, immunologically and biologically indistinguishable from hypothalamic LHRH, has been clearly demonstrated.[126] In the absence of a pure placental LHRH sample, synthetic LHRH was shown to stimulate the release of the α sub-unit of hCG, hCG and progesterone from human placental tissue,[127-129] and this led to a hypothesis that human chorionic LHRH regulated the release of these hormones. In a recent experiment, increased production rates and release of hCG were found in midterm and term placental cultures in response to LHRH.[129] Specific binding of LHRH and the agonists [D-Ala^6, Pro-NHEt^9]-LHRH and buserelin to the receptors present on partially

purified cell membranes from midterm and term placentas was also demonstrated.[129] The antagonist [D-Phe^{2,6}, D-Pro^{3}]-LHRH was inactive in displacing radioactive LHRH from these receptors at concentrations between 10^{-9} and 10^{-4}M, although another antagonist, [Ac-Pro^{1}, D-Phe(p-Cl)^{2}, D-Nal(2)^{3,6}]-LHRH, was able to suppress the release of the α subunit of hCG, hCG and progesterone from midgestation human placentas *in vitro*.[130] There is evidence from studies in baboons that a fall in circulating chorionic gonadotropin induced by LHRH agonists results in pregnancy termination,[131] but studies in women will be required to confirm that this mechanism is operative during human pregnancy.

Chronic administration of LHRH agonists is reported to suppress testis function and fertility in primates[132-135] and man;[136-137] an arrest of spermatogenesis can be produced but this is accompanied by a reduction in serum androgen concentrations and a loss of libido. Androgen replacement therapy sustains libido but also restores spermatogenesis; azoospermia has not yet been induced by such treatment.

Finally, LHRH-like factors have been found in rat testis tissue[138] and human seminal plasma[139] and LHRH analogues have been demonstrated to have direct effects on secretion of testosterone by rat Leydig cells.[140-142] There is no evidence for a direct functional effect in the human testis. No LHRH receptors have been found in human testis tissue[143] and even after chronic treatment, LHRH agonists fail to prevent the rise in testosterone induced by LH or hCG.[50,144-146] Man seems to be like the primate and the mouse, where LHRH analogues do not appear to have a direct action on the testis.[72,135]

Veterinary Applications - Although chronic administration of LHRH analogues does inhibit gonadal function in dogs,[147-149] suppression of estrus in bitches and unwanted social behavior in male dogs would require a depot formulation to be practical. In view of the probable cost of such a formulation, veterinary application of LHRH analogues is more likely to be concentrated on small doses given over a few days to initiate breeding during seasonal anestrus in sheep and postpartum anestrus in cattle. Several studies have already shown that, in contradistinction to primates, continuous low dose therapy with LHRH and analogues will initiate follicular development, ovulation and normal luteal function in ewes[150-152] and lead to fertile estrus.[152]

Conclusions - Since many highly potent and safe LHRH agonists are available, the main improvement which remains to be achieved is that of oral activity. LHRH agonists will find a place in the treatment of hormone-responsive cancers, benign uterine diseases and precocious puberty. Pulsatile administration of small doses of these analogues is becoming established as a treatment for certain types of anovulatory infertility. Use of LHRH agonists as antifertility agents is uncertain and still requires extensive study.

There is room for improvement in the potency of LHRH antagonists and ideally in the identification of orally active agents. There is some concern over the toxicity of certain antagonists but the clear indication that efficacy and toxicity are separable provides a challenge for the medicinal chemist.

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Section V - Topics in Biology

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Chapter 22. Sodium/Calcium Exchange and Calcium Homeostasis in Excitable Tissue

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Introduction - Electrically excitable cells, such as those derived from cardiac, smooth muscle, neural or neuroendocrine tissue, are distinct from inexcitable cells in that they possess unique pathways which determine plasma membrane permeability to ions (eg. Na, K, Ca, Cl). This channel mediated flux allows rapid conduction of ions down their respective electrochemical concentration gradients, which in turn gives rise to action potentials and other electrical phenomena typical of excitable cells. Among the different ionic species that traverse the membrane in this fashion, the flux of Ca^{2+} is of great interest because of the central role which this cation plays in the regulation of excitation-response coupling. Free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is maintained at very low levels in these cells, although significant quantities must routinely be processed. Under resting conditions, the magnitude of the transmembrane Ca^{2+} concentration gradient is ca. 10^4. However, when maximally elevated, $[Ca^{2+}]_i$ may increase from ca. 10^{-7} to 10^{-5}M. Excitable cells, therefore, have evolved a number of mechanisms that function critically in the maintenance of cytoplasmic Ca^{2+} within this range. These processes, acting in dynamic equilibrium, maintain Ca^{2+} homeostasis and prevent Ca^{2+} overload which would result in cytotoxicity. An ensemble of these mechanisms is shown in Figure One.

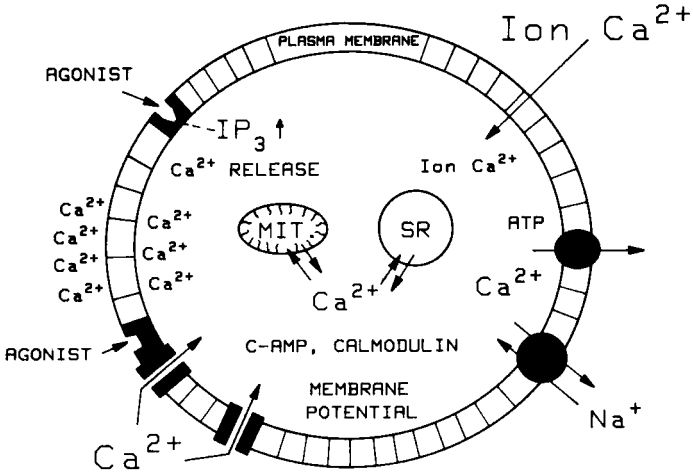

FIGURE 1: Ca^{2+} HOMEOSTASIS

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The influx of Ca^{2+} via voltage-gated channels is directly controlled by the plasma membrane electrical potential ($\Delta\Psi$).[1] Channel activity is also modulated by various hormones which act on intracellular regulatory pathways. In addition, receptor operated Ca^{2+} channels, phosphatidylinositide metabolism, and, to a lesser extent, passive influx of Ca^{2+} provide means of elevating $[Ca^{2+}]_i$ in some cells. To reduce $[Ca^{2+}]_i$, two general mechanisms are employed.[2] One involves sequestering Ca^{2+} by binding to intracellular proteins or by energy-dependent concentration in intracellular organelles (eg. sarcoplasmic reticulum (SR) or mitochondria). The other employs plasma membrane active transport systems to eject Ca^{2+} from the cell. Two means of accomplishing the latter have been identified. The first, a Ca, Mg-ATPase, is an ATP-dependent Ca^{2+} pump of low capacity but high affinity which acts solely in the unidirectional extrusion of Ca^{2+}. The second, a Na-Ca exchange antiporter, is a carrier mechanism which transports Ca^{2+} coupled to Na^+ movement in the opposite direction. Because Na-Ca exchange is completely reversible, transmembrane ion movement can occur in either direction, as dictated by conditions which influence carrier activity (see below). Since these are the only systems which function in net Ca^{2+} removal from excitable tissues, it is important to assess the relative role of each in Ca^{2+} homeostasis. This review will focus on Na-Ca exchange, its characteristics, regulation and possible roles in the physiology and pathology of excitable cells. For more complete treatments of these topics, the reader is directed to several other recent reviews.[2-9]

Background - The concept that Na-Ca exchange is involved in cellular Ca^{2+} flux originated from early studies assessing the effects of Na^+ and Ca^{2+} on myocardial contractility. Either elevating $[Ca^{2+}]_o$ or reducing the extracellular Na^+ concentration ($[Na^+]_o$) increases heart muscle tension. Dependency of contractile force on these ions is a function of $[Na^+]_o^2/[Ca^{2+}]_o$, and the monovalent cation involvement is specific for Na^+ (for review see ref. 3). These results led to the postulate that Na^+ and Ca^{2+} compete for a unique transporter which promotes Ca^{2+} influx in heart. Reuter and Seitz demonstrated Na-Ca exchange in atria by showing that $^{45}Ca^{2+}$ efflux is sensitive to either $[Na^+]_o$ or $[Ca^{2+}]_o$ and that these ions compete 2:1 in promoting efflux by acting at a common membrane site.[10] Moreover, increasing $[Na^+]_i$ stimulates $^{45}Ca^{2+}$ influx in atria, suggesting a reversible carrier mechanism.[11]

Since these original studies, Na-Ca exchange has been identified in several tissues, including both excitable (eg. squid axon,[12] crustacean neuron[13] and muscle,[14] smooth muscle,[15] skeletal muscle,[16] brain synaptosomes,[17] retinal rod cells[18]) and inexcitable (eg. kidney,[19] intestine,[20] bladder,[21] liver,[22] sperm[23]) varieties. However, characterization of this reaction has been hindered by technical difficulties associated with flux measurements in intact preparations, by problems in measuring minute currents associated with transport using conventional electrophysiological techniques, and by uncertainties in assigning cellular responses to action of Na-Ca exchange. To circumvent such problems, Kaback's laboratory was the first to develop and characterize an osmotically intact bacterial cytoplasmic membrane vesicle preparation in which vectorial transport reactions could be studied.[24] This novel approach has been successfully applied to eukaryotic cells for preparing plasma membrane vesicles and has substantially aided kinetic and thermodynamic analysis of a number of transport reactions, including Na-Ca exchange. The first description of exchange in vesicles was made by Reeves and Sutko who showed that crude sarcolemmal membranes derived from rabbit heart would accumulate $^{45}Ca^{2+}$ in response to an outwardly directed Na^+ gradient.[25] Similar experiments with plasma membranes derived from

brain,[26,27] smooth muscle,[28,29] skeletal muscle,[16] and pituitary[30] have convincingly demonstrated the presence of Na-Ca exchange in these excitable cells.

Characteristics - Na-Ca exchange is an active transport reaction which occurs without direct involvement of ATP. The reaction is driven by transmembrane ion gradients and electrical potentials. In this way, energy derived from an ion moving down its electrochemical gradient is coupled to movement of another ion in the opposite direction. Exchange is freely reversible, since imposition of a Na^+ gradient directed outward drives Ca^{2+} uptake in vesicles while a Na^+ gradient directed inward promotes Ca^{2+} efflux.[25,31] Transport activity resides in the plasma membrane because of parallel purification with other plasma membrane marker enzymes[25-30] and because inside-out sarcolemmal vesicles, selectively Na-loaded by action of the plasmalemmal Na, K-ATPase, are competent for Na-Ca exchange.[32,33] Na-Ca exchange is also present in mitochondria derived from heart and brain, but this system displays markedly different properties than those of the plasma membrane carrier.[34] The specificity of the cardiac exchanger indicates an absolute requirement for Na^+ as the monovalent cation substrate, since neither Li^+ nor K^+ are able to promote transport.[25] Ba^{2+} and Sr^{2+} can alternate for Ca^{2+}, albeit less effectively, as the divalent cation transported.[9] A number of divalent and trivalent cations inhibit cardiac exchange by interacting competitively at the Ca^{2+} binding site of the carrier.[35]

Stoichiometry - Elucidation of Na-Ca exchange stoichiometry is important for determining both transport mechanism and role of exchange in cell physiology. It had been suggested that 2 Na^+ exchange per Ca^{2+} because of the $[Na^+]_o^2/[Ca^{2+}]_o$ dependence of Ca^{2+} fluxes in atria.[10,11] However, the transmembrane Na^+ gradient will not provide a large enough driving force to reduce $[Ca^{2+}]_i$ to basal levels with a 2:1 stoichiometry.[36] Also, $\Delta\Psi$ influences Na-dependent Ca^{2+} movements in dialyzed squid axon[37] and altering Na-Ca exchange activity changes $\Delta\Psi$ of cardiac cells.[4] These findings suggest that exchange is electrogenic. In cardiac vesicles, simultaneous initial rate measurements of $^{22}Na^+$ influx and $^{45}Ca^{2+}$ efflux yield a 3:1 ratio.[32] Similar results have been obtained by measuring fluxes in fetal mouse cardiac cells.[38] Moreover, a $\Delta\Psi$ (interior negative) is generated in sarcolemmal vesicles,[39,40] and pituitary vesicles[30] during Na_i-dependent Ca^{2+} uptake. These results suggest that exchange generates a net current during carrier turnover. Consistent with this, artificially imposed $\Delta\Psi$'s alter initial rates of vesicular Ca^{2+} flux in a fashion expected if net charge movement occurs.[30,39-42] Finally, a study with sarcolemmal vesicles has used artifically generated $\Delta\Psi$'s to drive exchange in the absence of ion gradients and has calculated an average stoichiometry of 2.97 ± .03 Na:Ca, thereby clearly establishing that the reaction is electrogenic.[43]

Kinetics - Kinetics of Na-Ca exchange have been studied in both whole cells and vesicles. However, because of technical difficulties associated with whole cell measurements, it has often not been possible to determine kinetic parameters with certainty. While vesicle experiments have technical problems of a different nature,[3,9] they do provide a means to quantitatively assess this reaction. Na_i-dependent Ca^{2+} uptake is a saturable function of $[Ca^{2+}]_o$ with a reported apparent K_m of ca. 10-40 μM in a number of systems including heart.[26,30,33,44] However, a significantly higher affinity (1 μM) has been measured in cardiac vesicles under some experimental conditions.[40,45] Investigation of Ca^{2+} uptake in synaptic membrane vesicles indicates a complex dependency on $[Ca^{2+}]$ with a high affinity component (K_m= 0.5 μM) and a much lower affinity component

which could not be resolved.[46] One explanation for variability in vesicle K_m values, and discrepency between these and those measured in intact cells,[3] is that vesicle populations typically contain membranes of mixed polarities. If Na-Ca exchange is asymmetric, then vesicular unidirectional flux measurements would reflect operation of two different activities acting in combination. Indeed, studies of Na-Ca exchange in dialyzed squid axon indicate a significant difference in apparent K_m for Ca^{2+} at the internal (ca. 10 μM) and external (3 mM) membrane surface.[47] However, when inside-out cardiac vesicles were studied preferentially, the affinity for Ca^{2+} (33 μM) was identical to that measured in the entire vesicle population, indicating a symmetrical transport reaction in heart.[33] This apparent low affinity for Ca^{2+} is of some concern in discerning the physiological role of exchange, given $[Ca^{2+}]_i$ fluctuations, since these data indicate that transport would never be functioning at V_{max}. Whether vesicle experiments accurately reflect properties of the transporter in vivo or if exchange activity is regulated by pathways unique to intact preparations is presently not clear. Nonetheless, kinetic experiments do demonstrate that Na-Ca exchange is a high capacity system for transport of Ca^{2+}. In cardiac vesicles, V_{max} rates of 5-30 nmoles Ca^{2+} transported/sec/mg protein have been reported.[44,45] This translates into a flux of 10 pmoles/sec/cm^2 in intact tissue,[2] consistent with a value of 30 pmoles/sec/cm^2 measured for exchange activity in guinea pig atria.[4] Heart contains one of the most active exchange systems since rates measured in brain,[26,46] pituitary[30] and smooth muscle[29] vesicles are ca. 10-100 fold lower in value.

Ion Binding Sites - Na-dependent Ca^{2+} movement in heart[11,38,48] and squid axon[49] displays a sigmoidal dependency on $[Na^+]$ with a Hill coefficient of 3. Na-dependence has been studied for both Ca^{2+} influx and efflux in vesicles. Na_i-dependent Ca^{2+} uptake in sarcolemmal vesicles displays a $K_{1/2}$ (Na^+) of ca. 20 mM[44,48] and a Hill coefficient of 2.3.[44] Na_o-dependent Ca^{2+} efflux has also been measured in cardiac[31,42] and synaptic[50] vesicles and displays similar $K_{1/2}$ (Na^+). Stopped-flow rapid-quench monitoring of Na_o-dependent Ca^{2+} efflux in cardiac membranes also indicates a sigmoidal dependence on $[Na^+]$ with a $K_{1/2}$ of 30 mM and a Hill coefficient of 2.3-3.2.[42] Preferential loading of inside out vesicles with Ca^{2+} by action of sarcolemmal Ca-ATPase reduced $K_{1/2}$ $(Na^+)_o$ to 20 mM suggesting either an asymmetric carrier mechanism or an effect of ATP on the kinetic properties of the exchanger.[45] Taken together, these results are consistent with participation of multiple Na^+ binding sites in the exchange reaction. Several lines of evidence suggest a common Na^+ and Ca^{2+} binding site on the exchanger. Data obtained with heart[10,11] and squid axon[51] indicate that Na^+ and Ca^{2+} compete in a 2:1 ratio. Kinetic experiments with cardiac vesicles demonstrate that Na_o will inhibit Na_i-dependent Ca^{2+} uptake with a biphasic concentration dependence and limiting Hill coefficients of 1 and 2 at low and high $[Na^+]$, respectively.[44] Inhibition is competitive with a K_i of 16 mM at low $[Na^+]$. These results indicate that either 1 or 2 Na^+ bind at the carrier's Ca^{2+} binding site and that a single Na^+ can prevent binding of Ca^{2+}. Of the other monovalent cations investigated, only H^+ interacts effectively at the Ca^{2+} site.[52] In addition to Na-Ca exchange, the transporter will also catalyze nonproductive Ca-Ca and Na-Na exchange reactions, study of which has been useful in deciphering mechanism. It was originally shown in heart[10] and squid axon[12] that $^{45}Ca^{2+}$ fluxes are stimulated by $^{40}Ca^{2+}$ on the trans side of the membrane. Furthermore, rates of trans-stimulated Ca^{2+} movements in dialyzed squid axon are markedly increased by alkali metal ions.[49] Ca-Ca exchange can also be demonstrated in cardiac,[31,53,54] brain,[26] and pituitary[30] vesicles. Cardiac vesicle studies reveal that alkali metal ions

($K \curvearrowright Rb \curvearrowright Li > Cs >> choline$; including low $[Na^+]$) stimulate Ca-Ca exchange by increasing V_{max}.[54] Stimulatory cations are not transported and the rate determining step appears different from that of Na-Ca exchange because Ca-Ca exchange has a greater V_{max}.[26,30,54] Alkali metal ions are reported to stimulate Na-Ca exchange in brain vesicles[55] but no stimulation is observed in the cardiac system.[54] Na-Na exchange via action of the transporter has also been characterized in cardiac vesicles.[3]

Model of Na-Ca Exchange - A mechanism for Na-Ca exchange has been proposed in heart, based on kinetic properties of the reaction.[3,56] In this model, two ion binding sites exist; a site which binds either Ca^{2+} or 2 Na^+ and a second, distinct site, which binds a third Na^+. Occupation of the common Ca^{2+}-Na^+ site (A-site) by 2 Na^+ places the carrier in a different conformation than does Ca^{2+} and induces a change in protein structure which allows a third Na^+ to bind at a second site (B-site). Occupation of A and B sites by 3 Na^+ is necessary for transport to occur and Na^+ movement is coupled to counter movement of Ca^{2+} bound to an A-site on the trans membrane side. When Ca^{2+} is bound at the A-site, the B-site exhibits broader substrate specificity allowing interaction of alkali metal ions which stimulate Ca-Ca exchange. These ions are not transported by the carrier in this conformation but increase V_{max} of transport. This model is consistent with the characteristics of Na-Ca exchange (electrogenicity, stoichiometry, sigmoidal Na^+ dependence, competition between Na^+ and Ca^{2+} and Ca-Ca exchange stimulation by alkali metal ions). Moreover, stopped flow measurements of Na_i-dependent Ca^{2+} uptake[3] and studies with inhibitors[56] (see below) are consistent with this model. A conceptually similar scheme has been proposed for exchange in squid axon.[49]

Purification and Reconstitution - Purification of the Na-Ca exchanger has recently been attempted. Exchange activity can be solubilized from cardiac membranes by cholate extraction and reconstituted into liposomes with a 5-fold increase in specific activity.[57] Using this protocol, it was observed that if solubilized proteins were first treated with pronase and then reconstituted, exchange activity survived while other proteins were degraded.[58] Furthermore, only five major proteins remained in reconstituted vesicles and exchange activity was enhanced 50-fold. Although the activity increase is partially due to proteolytic activation of the transporter (see below), this protocol does provide one purification scheme. The bovine cardiac Na-Ca exchanger has been identified by this proceedure as an 82 KDa protein, the major membrane protein surviving proteolytic degradation.[59] This moiety is a glycoprotein which may account for its resistance to proteolysis.[60] Exchange activity has also been solubilized, reconstituted[61] and purified[62] from synaptic vesicles. Purification was accomplished by isolating exchanger containing liposomes based on density after selectively loading those vesicles with Ca^{2+} via exchange action. Activity coincided with purification of a 72 KDa protein. Purification of the cardiac transporter has also been achieved using this technique.[63]

Regulation - Several means of Na-Ca exchange regulation have been postulated. One involves interaction of ATP with this system. In squid axon and barnacle muscle, ATP modulates Na-Ca exchange activity.[8,49] While the exact mechanism of action is incompletely understood, it is clear that ATP modifies the kinetic properties of the transporter. Recent work with sarcolemmal vesicles has supplied new information on the way in which ATP may act.[45] Cardiac exchange appears to be regulated by phosphorylation and dephosphorylation reactions mediated by a Ca^{2+}-calmodulin-dependent kinase and phosphatase. These results imply that ATP, μM Ca^{2+} and an endogenous kinase may also alter the kinetics of the squid axon

exchanger. Moreover, they provide a general model wherein Na-Ca exchange can be regulated by $[Ca^{2+}]_i$ through kinase and phosphatase reactions working in an antagonistic fashion to control the phosphorylation state of the carrier. In addition to ATP, several other factors influence transport activity. Na-Ca exchange displays a marked sigmoidal dependency on pH in heart[48,52] and pituitary[30] vesicles with activity increasing as pH is increased from 6 to 9. H^+ exerts its effect, in part, by competing at the Ca^{2+} site of the carrier; therefore, conditions which lower pH_i (eg. ischemia) may diminish activity. Exchange in cardiac vesicles is enhanced by proteases (eg. chymotrypsin stimulates exchange 200%).[64] Whether this is due to direct proteolytic modification of the carrier or removal of an endogenous inhibitory protein is not clear. Phospholipase treatment of cardiac vesicles modifies exchange activity. Treatment with phospholipase C stimulates exchange ca. 100% because of membrane enrichment with negatively charged phospholipids.[65] Phospholipase D increases activity ca. 400% upon production of phosphatidic acid.[66] These findings suggest that Na-Ca exchange is sensitive to membrane environment and that activity can be regulated by negatively charged phospholipids. This correlates with physiological studies which demonstrate that Ca^{2+} binding to sarcolemmal phospholipids influences Na-Ca exchange activity and myocardial function.[67] Data concerning stimulation of Na-Ca exchange by phosphatidic acid is particularly intriguing since hormonal stimulation of some cells results in this agent's production via phosphatidylinositol metabolism which in turn elevates $[Ca^{2+}]_i$ by an undefined mechanism(s).[66] Ca^{2+} chelators (eg. EGTA) also stimulate cardiac exchange by decreasing the K_m for Ca^{2+} to 1 μM.[68] Whether endogenous sarcolemmal Ca^{2+} binding sites act as Ca^{2+} chelators in regulating exchange under physiological conditions is unknown. Finally, reactive oxygen species have been shown to influence Na-Ca exchange in cardiac vesicles.[69] Modification by these agents could be important in regulating transport activity during myocardial ischemia.

Inhibitors - Inhibitors of voltage-dependent Ca^{2+} channels have significantly advanced our understanding of the role which Ca^{2+} plays in excitation-response mechanisms.[1] Identification of selective Na-Ca exchange and Ca-ATPase inhibitors, critically needed for assessing physiological roles of these reactions, has lagged behind. Except for inorganic ions and calmodulin antagonists, few selective inhibitors of Ca-ATPase have been reported. Recently, however, several putative inhibitors of Na-Ca exchange have been identified. La^{3+} inhibits exchange in both vesicles[25,27,30] and intact tissues such as heart[70] and squid axon.[51] Because La^{3+} interferes with most Ca^{2+} transport, it is difficult to selectively inhibit Na-Ca exchange, although some progress has been reported with isolated myocytes.[70] A number of organic agents including dibucaine, tetracaine and ethanol,[71] quinidine,[72] verapamil,[73] chloropromazine[40] and amrinone[74] inhibit Na-Ca exchange in vesicle systems, albeit all function at very high concentrations. The antibiotic polymixin B also inhibits cardiac exchange.[64] Adriamycin, an antitumor agent which is cardiotoxic, has been reported to block transport in sarcolemmal vesicles at low concentrations.[75] However, it was found ineffective by others in cardiac[3] and pituitary[30] vesicles, and its inhibitory activity was only partially confirmed by measuring currents related to Na-Ca exchange in heart cells.[72] Quinacrine inhibits cardiac Na-Ca exchange (K_i= 20 μM),[3] but its use is likely to be quite limited because of the many actions of this compound. Charged unbranched alkyl amphiphilic molecules modulate exchange activity in cardiac vesicles.[76] Anionic molecules (eg. dodecyl sulfate) stimulate exchange while cationic derivatives (eg. dodecylamine) are potent inhibitors (I_{50} ca. 20 μM). Because negatively charged phospholipids affect exchange (see above),

these agents may alter the carrier's membrane environment thereby influencing transport in a secondary fashion. Recent work assessing the effects of these detergents on myocardial contractility and Na-Ca exchange activity,[77] should be viewed with this caveat in mind. The Ca^{2+} entry blocker diltiazem and other benzothiazepines and benzodiazepines block Na-Ca exchange in cardiac mitochondria, but have no effect on the sarcolemmal system.[78]

The diuretic amiloride is known to inhibit a number of Na^+ transporting systems.[79] It inhibits Na-Ca exchange in murine erythroleukemia cells,[80] and membrane vesicles derived from brain,[81] pituitary[30] and heart.[82] In addition, it blocks cardiac mitochondrial Na-Ca exchange.[83] However, lack of potency and proper selectivity make it unattractive as a probe of either mechanism of inhibition or physiological roles of Na-Ca exchange. Thus, recent reports of amiloride's action on myocardial contractility[84] are difficult to assess. Various amiloride analogs are more potent and possess greater selectivity than the parent compound in inhibiting other Na^+ transport systems.[85] Using the strategy that some amiloride derivatives might likewise show increased efficiency in inhibiting Na-Ca exchange, Kaczorowski and coworkers identified certain analogs, substituted on the terminal guanidino nitrogen or 5-pyrazine ring nitrogen, which are ca. 100-fold more potent than amiloride in blocking exchange in pituitary, cardiac and brain vesicles.[82,86] A mechanistic investigation in pituitary reveals that both inhibitor classes function noncompetitively with respect to Ca^{2+} but are competitive with Na^+, apparently interacting at the B-site of the carrier. Data obtained with an amiloride analog which is an irreversible photoaffinity label are consistent with this interpretation.[87] These results have been confirmed and extended by studies using benzamil (amiloride substituted with a benzyl group on the terminal guanidino nitrogen) which inhibits exchange in sarcolemmal vesicles.[3,56] Benzamil blocks cardiac Na-Ca and Ca-Ca exchange noncompetitively with respect to Ca^{2+} and alkali metal ions which stimulate Ca-Ca exchange (see above) reverse inhibition. Benzamil also blocks Na-Na exchange and inhibition is alleviated by elevating $[Na^+]$. These results imply that benzamil interacts at the B-site of the cardiac transporter. Although data consistent with this model have been presented for inhibition of synaptic vesicle Na-Ca exchange,[46] others report that amiloride blocks Ca^{2+} competitively in this system.[81] Cardiac Na-Ca exchange has been probed with potent amiloride analogs *in vivo* in an attempt to assess the role of this transport system in myocardial function[82] (see below).

Physiolgical role of Na-Ca exchange - Although Na-Ca exchange has been described in a number of tissues, its physiological role has really only been extensively investigated in heart. Lack of potent selective inhibitors has imposed limitations on work of this type. For example, in smooth muscle the physiology of Na-Ca exchange is the subject of much controversey.[88-90] When Na-Ca exchange and Ca-ATPase activities are compared in vesicles prepared from either ileum or aorta, Na-dependent Ca^{2+} transport has a lower affinity and V_{max} than ATP-dependent Ca^{2+} transport.[29] In contrast, studies with heart,[91] and pituitary vesicles[92] indicate that exchange V_{max} is significantly greater in these systems. Thus, the prevailing opinion regarding smooth muscle is that exchange plays a relatively minor role in Ca^{2+} homeostasis under normal conditions, although it may be critical in certain pathological states such as essential hypertension (see below). Therefore, this section will focus on studies that have assessed the physiological role of Na-Ca exchange in maintaining cardiac Ca^{2+} homeostasis (see refs. 2-7 and 93 for more complete treatments). The relative contribution of exchange compared to

other Ca^{2+} transport systems, sequestration organelles, and binding proteins in maintaining control is complex and unresolved. To provide a conceptual framework for discussion, Na-Ca exchange action can be analyzed within thermodynamic and kinetic constraints imposed by intracellular conditions. A thermodynamic analysis specifies that transport activity is governed by relative magnitudes of transmembrane Na^+ and Ca^{2+} concentration gradients and $\Delta\Psi$. At equilibrium, the relationship formulated by Blaustein[36] applies:

$$\frac{[Ca]_i}{[Ca]_o} = \frac{[[Na]_i]^n}{[[Na]_o]^n} \exp (n-2)\ \Delta\Psi\ F/RT$$

(where n = number of Na^+ transported, $\Delta\Psi$ = membrane potential, F = Faraday constant, R = gas constant and T = absolute temperature)

Recently, intracellular activities of Na^+ and Ca^{2+} ($a(ion)_i$) have been measured in heart by ion selective microelectrodes.[94] Based on experiments at varying $[Na^+]_o$, $[Ca^{2+}]_o$ and $\Delta\Psi$, an average n of 2.7 has been calculated.[3] The noninterger value suggests that Na-Ca exchange is not at equilibrium but rather at steady state with other processes controlling the intracellular ionic environment. Given data with cardiac vesicles, it appears that n = 3.[43] Therefore, exchange favors Ca^{2+} efflux in resting cells with $\Delta\Psi$ = -80 mV. Moreover, if Na-Ca exchange were in equilibrium under these conditions, $[Ca^{2+}]_i$ would be 10-fold lower than the value actually measured.[3] Since exchange is electrogenic and reversible, there is a $\Delta\Psi$ at which direction of flux is reversed. Reversal potential depends on stoichiometry as well as [ion], but for n = 3 a minimum value of -40 mV has been suggested.[2] Thus, normal membrane depolarization (eg. during action potentials) could result in exchange mediated Ca^{2+} influx while repolarization would cause efflux.[7,95] Data consistent with exchange promoting net Ca^{2+} influx or efflux in different cardiac tissues has been obtained with exchange inhibitors that cause a negative inotropic effect in papillary muscle,[82] but produce a small positive inotropic effect in atrial muscle.[96] The notion of reversibility is of prime importance in determining the contribution of Na-Ca exchange to myocardial contractility. However, because of uncertainties in applying equilibrium thermodynamics to intact cells and in values of $a(ion)_i$ which influence transport, a rigorous model of exchange action remains to be elucidated.

Na-Ca exchange is also subject to kinetic constraints. For example, the lowest Ca^{2+} and Na^+ K_m measured in vesicles are 1 μM and 10 mM, respectively. Given the $[ion]_i$ in resting cells, Na^+ could effectively compete Ca^{2+} binding at the carrier's A-site, thereby reducing Ca^{2+} efflux capacity. Moreover, having Na^+ preferentially bound at the inner membrane surface would poise the exchanger for Ca^{2+} influx if the reversal potential is achieved. Elevation of $[Ca^{2+}]_i > 10^{-6}$M during excitation would saturate the A site with Ca^{2+} and promote Ca^{2+} efflux. The extent to which Na-Ca exchange controls $[Ca^{2+}]_i$ at rest or during excitation is unclear, but because of reversibility and high turnover capacity, it could certainly influence total equilibrium cellular Ca^{2+} stores (eg. those in the SR and mitochondria) which ultimately regulate contractility.

Given these unresolved issues, there are, nonetheless, several examples of Na-Ca exchange functioning in intact mammalian cardiac tissue. Measurements of $a(ion)_i$ reveal that lowering $[Na^+]_o$ produces a rapid marked reduction in $a(Na^+)_i$ due to Na-Ca exchange.[2,97] Only a small increase in $a(Ca^{2+})_i$ and tension occurs, although much Ca^{2+} enters the cell,

indicating effective buffering by organelles. Furthermore, increases in $a(Ca^{2+})_i$ are enhanced by membrane depolarization or pretreatment with cardiac glycosides (elevates cellular Na^+), consistent with the action of an electrogenic exchange mechanism.[97,98] While this illustrates Na-Ca exchange action in intact tissue, perhaps more telling in terms of control of contractility are measurements of tension development. Two cardiac preparations have been extensively investigated.[2,4] Amphibian heart cells are small, possess a minimally developed SR, and have relatively high $[Na^+]_i$, suggesting more direct interaction between exchange and contractility than in mammalian tissue. Consistent with this, $[Na^+]_o$ reduction produces a large increase in resting tension and variations in ion gradients or $\Delta\Psi$ expected to directly affect exchange activity produce predicted changes in resting tension.[99] Moreover, tension in frog heart elicited by membrane depolarization also shows a clear dependence on $\Delta\Psi$, $[Na^+]_o$, and $[Ca^{2+}]_o$ indicating that exchange can directly control $[Ca^{2+}]_i$ in this tissue.[2,3] In view of $a(Na^+)_i$ and $a(Ca^{2+})_i$ measurements in frog, Na-Ca exchange appears to be in equilibrium with the transmembrane Ca^{2+} gradient and thus it can be a primary determinant of $[Ca^{2+}]_i$.

In mammalian cardiac tissue, $[Na^+]_o$ depletion does not produce a large increase in resting tension,[4] although if $[Na^+]_i$ is increased by use of Na, K-ATPase inhibitors or if the membrane is depolarized, the effects are more pronounced.[97,98] This probably reflects dominance of intracellular sequestration mechanisms in controlling $[Ca^{2+}]_i$, since agents that stimulate SR Ca^{2+} uptake abolish any modest changes in resting tension.[98] Tension measurements made using a voltage clamp protocol are consistent with this idea. Mammalian cardiac tissue displays only a phasic or twitch tension (ie. a response predominantly controlled by Ca^{2+} influx through Ca^{2+} channels and subsequent SR Ca^{2+} release) unless cells are Na^+ loaded. Elevating $[Na^+]_i$ causes mammalian tissue to display tension behavior typical of amphibian cardiac cells (ie. tonic tension development).[100] These conditions are precisely those which bring Na-Ca exchange into equilibrium with the Ca^{2+} gradient.[3,97] Nonetheless, $[Na^+]_o$ reduction does produce a positive inotropic response in elicited twitch tension, presumably due to increased SR Ca^{2+} loading via exchange action.[97] Elevating $[Na^+]_i$ by increasing electrical stimulation frequency (Treppe response)[101] or by Na, K-ATPase inhibition with cardiac glycosides[102] yield a similar response. Consistent with this, positive inotropic responses due to increasing $[Na^+]_i$ are blocked by amiloride analog inhibitors of cardiac Na-Ca exchange.[82] Therefore, Na-Ca exchange indirectly affects contractility in mammalian heart by controlling Ca^{2+} available for loading intracellular stores. Whether this is due to Na-Ca exchange functioning in an influx, efflux or both modes during cycles of excitation-contraction coupling must be resolved.

Role of Na-Ca exchange in pathology - Although the normal function of Na-Ca exchange in vascular smooth muscle is controversial, it is believed to play a key role in the development of essential hypertension.[103] An excessive Na^+ load produces a hypertensive response, though mechanism remains obscure. If renal Na^+ excretion is impaired, the tendency towards Na^+ and water retention results in volume expansion which promotes secretion of a natriuretic hormone. This hormone is expected to aid in Na^+ excretion by inhibiting Na, K-ATPase in kidney tubule cells. Involvement of Na-Ca exchange is due to presumed hormonal inhibition of smooth muscle Na-pumps which would elevate $[Na^+]_i$ and increase $[Ca^{2+}]_i$ via Na-Ca exchange, thereby promoting muscle contractility, raising vascular tone and elevating peripheral resistance (ie. blood pressure). While this hypothesis is attractive in that it provides a mechanistic basis for the hypertensive response to Na^+, it awaits verification.

In cardiac cells, conditions that lead to Na^+ loading may also have pathophysiological consequences via exchange action. Baker originally suggested that Na-Ca exchange might be involved in cardiac response to digitalis.[51] Whether this accounts for the therapeutic action of cardiac glycosides is not clear, but extensive investigation has shown that these compounds elevate $[Na^+]_i$ and promote Ca^{2+} loading through exchange in heart cells. Ca^{2+} overloading of SR by this route may be responsible for ouabain induced arrhythmogenicity. Spontaneous fluctuations of $[Ca^{2+}]_i$ occur in heart cells due to asynchronous release of Ca^{2+} from SR.[104] If the SR is overloaded with Ca^{2+}, such release could have important physiological consequences since $[Ca^{2+}]_i$ regulates voltage-dependent Ca^{2+} channels and activity of other channels which carry a transient inward current.[93,105,106] Thus, Ca^{2+} release from overloaded SR could interfere with normal cardiac rhythmicity by acting at the level of these channels. Amiloride protects against digitalis toxicity,[84] but, in addition to inhibiting Na-Ca exchange, it has other effects on cardiac action potentials.

Ischemia also causes an elevation in $[Na^+]_i$ and subsequent Ca^{2+} overloading which leads to cardiac damage.[107] Although Na-Ca exchange is minimally imparied in vesicles derived from ischemic heart tissue, cellular damage which occurs upon tissue reperfusion may result from exchange mediated Ca^{2+} influx. As a model of reperfusion injury, the Ca^{2+} paradox has been studied.[108,109] Heart cells perfused with Ca^{2+}-free buffer and then reexposed to Ca^{2+}, take up large quantities of Ca^{2+}, go into contracture, display significant intracellular damage, and lose excitability. Na-Ca exchange is postulated as the basis for these events.[2] Lowering $[Ca^{2+}]_o$ causes $[Na^+]_i$ to rise via exchange. This diminishes Ca^{2+} stores in both SR and mitochondria (via mitochondrial Na-Ca exchange) and promotes further elevation of $[Na^+]_i$ by sarcolemmal exchange. When Ca^{2+} is readmitted to the tissue, Na_i-dependent Ca^{2+} influx occurs and $[Ca^{2+}]_i$ raises to cytotoxic levels. Since high $[Na^+]_i$ blocks accumulation of Ca^{2+} by mitochondria, this eliminates an intracellular Ca^{2+} buffer. Together, these events cause the Ca^{2+} paradox. The extent to which this is mimicked in vivo during ischemia and reperfusion injury must still be assessed. Finally, there are other pathological situations where inhibition of Na-Ca exchange may yield beneficial effects. For example, if loading of cardiac SR Ca^{2+} stores is dependent on exchange and the predominant mode of exchange is to drive Ca^{2+} efflux, then inhibitors might provide a novel route to achieving a positive inotropic response (ie. be cardiotonic agents).

Summary - A Na-dependent Ca^{2+} transport reaction has been identified and characterized in a number of excitable tissues. By studying fluxes in intact cells and isolated plasma membrane vesicles, much has been learned about kinetics, thermodynamics and regulation of this transport system. However, because of multiple Ca^{2+} processing reactions and other complexities associated with investigating Ca^{2+} homeostasis in cells, it has not been possible to unequivocally assign specific roles for Na-Ca exchange in regulating excitatory responses. The development of specific inhibitors, whether they are small organic molecules, antibody reagents directed against the transport protein or natural product toxins, is critically needed to clarify this situation. In addition to being useful in assessing the physiological role of Na-Ca exchange, specific inhibitors could also provide therapeutically effective agents.

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Chapter 23. Possible Roles of Protein Kinase C in Cell Function

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Introduction -- In the past two years, rapid progress has been made in understanding the molecular actions of hormones that induce inositol lipid breakdown. It is now accepted that hormones, neurotransmitters and other agonists, acting at specific cellular receptors, stimulate phospholipase C (PLC) to cleave phosphatidylinositol 4,5 bisphosphate (Ptd Ins 4,5 P2) into inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG).[1] These two compounds act as second messengers of hormone action in the stimulated cells as illustrated in Figure 1.

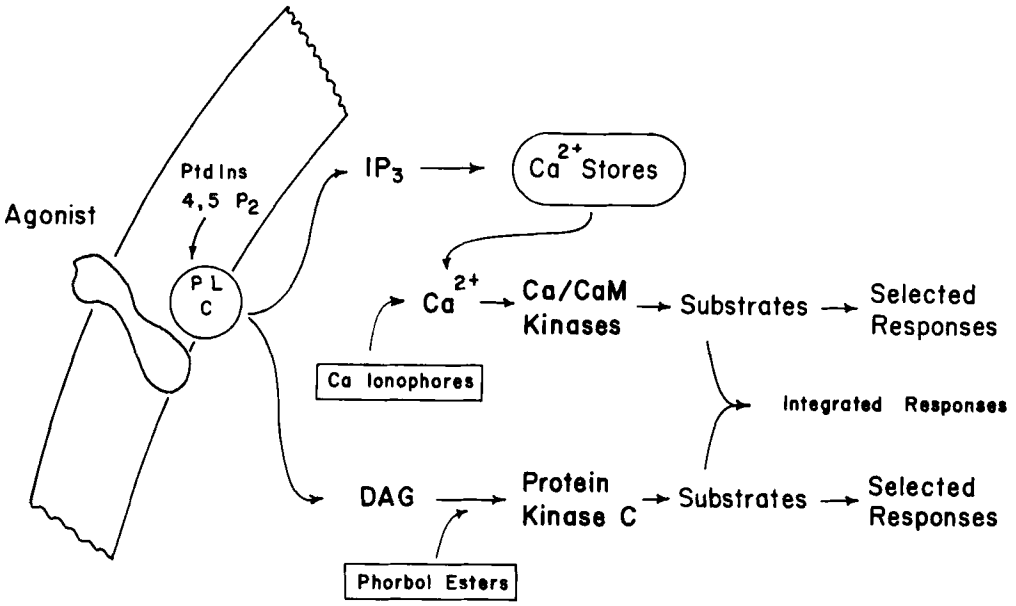

Figure 1. The Roles of DAG and IP_3 as Second Messengers

In a variety of systems, inositol trisphosphate has been demonstrated to initiate a prompt release of Ca^{2+} from intracellular stores (probably microsomal) into the cytoplasm of the cell.[2] This bolus of Ca^{2+} activates

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a number of Ca^{2+} and calmodulin (CaM) dependent enzymes, including calmodulin sensitive protein kinases.[3] Although a variety of enzymes other than protein kinases are regulated by the Ca^{2+}/CaM system,[3] as indicated in Figure 1, a growing body of evidence suggests that protein kinases regulate at least part of the cells' characteristic response to receptor activation.[3-5] The other messenger generated by inositol lipid breakdown, diacylglycerol, activates the Ca^{2+} and phospholipid-dependent protein kinase, protein kinase C. This apparently ubiquitous enzyme regulates another set of cellular responses by phosphorylating a distinct set of substrates.[1,6] In most situations the actions of agonists stimulate both pathways and the cellular responses proceed together. In some cells, the best studied being the platelet, the pathways interact to produce an integrated response such as secretion of ADP and serotonin.[7-9] In other cells, such as the liver, the two pathways appear to act more independently.[10]

Protein kinase C is an enzyme with broad substrate specificity that has been found in most mammalian cells. It has a molecular weight of about 77,000 and exists as a single peptide chain that is thought to be bound to plasma membranes. The enzyme requires Ca^{2+} and a phospholipid such as phosphatidylserine for activity. Unsaturated diacylglycerols such as diolein markedly increase the affinity of the protein for Ca^{2+} ion and activate the enzyme. Membranes usually contain low amounts of diacylglycerols, however, these compounds are produced by receptor stimulated breakdown of inositol lipids giving rise to the hypothesis that protein kinase C plays an important role in the transduction of signals across the plasma membrane.[6]

An important property of the scheme presented in Figure 1 is that each arm of the pathway can be stimulated selectively by certain agents. Ca^{2+} inonophores such as A23187 and ionomycin can raise cytoplasmic Ca^{2+} and activate the Ca^{2+}/CaM pathway.[3,4] Of greater significance is the finding that tumor-promoting phorbol esters can bind to protein kinase C, activating the enzyme and producing the characteristic set of responses for this arm of the pathway.[6] The ramifications of these observations are not fully understood; however, the ability of phorbol esters to regulate cell growth, and the ability of mitogens such as EGF to induce the phosphatidylinositol response, has focused considerable attention on protein kinase C.[6] A number of excellent reviews have appeared summarizing the generation of IP_3 and other phosphorylated inositol molecules,[1] DAGs and the possible role of oncogenes in the phosphatidylinositol pathways,[1] the actions of IP_3[2] and the properties of protein kinase C.[6] This brief review will concentrate on the recognized substrates for protein kinase C and attempt to describe, in as much detail as possible, the relationship between these substrates and the known functions of hormones and phorbol esters on cell function. The subjects covered will include: (a) the effects of phorbol esters and protein kinase C on hormone receptors and membrane function; (b) the possible role of protein kinase C in secretory systems, and (c) a description of the known substrates for protein kinase C as measured *in vitro*. The effects of phorbol esters on protein kinase C and the possible role of this system in regulating cell growth have been covered elsewhere[6] and will not be discussed here.

Effect of Protein Kinase C on Hormone Receptors -- Before phorbol esters such as TPA (4 β-phorbol-12-myristate-13 acetate) were known to activate protein kinase C, it was demonstrated that treatment of 3T3 cells with TPA caused a loss of high affinity EGF binding sites in the cells.[11,12] Recently, a possible explanation for this result was provided by the observation that TPA also induces phosphorylation of serine and threonine residues in the 170,000 dalton EGF receptor in the membrane.[13-16] Increased phosphorylation of the receptor is associated with a decreased ability to act as a tyrosine protein kinase. This result can be demonstrated in both intact A431 cells[13-16] or in membrane preparations incubated with purified protein kinase C.[13]

Other hormone receptors also have their apparent phosphorylation state increased by treatment of ^{32}P-labeled intact cells with TPA (Table 1). In IM-9 lymphocytes, TPA increases the phosphorylation of insulin and somatomedin receptors as determined by immunoprecipitation and gel electrophoresis of solubilized receptors. However, no functional effects of increased receptor phosphorylation were reported in these studies.[17] The phosphorylation state of the Interleukin-2 receptor in cloned T cells also appears to be increased following TPA administration.[19] Similarly, β-adrenergic and possibly α_1-adrenergic receptors are phosphorylated by treating avian erythrocytes and hepatocytes, respectively, with phorbol esters.[20-22] Increased phosphorylation of the β-adrenergic receptor is correlated with a decreased ability of β-agonists (i.e. isoproterenol) to stimulate adenylate cyclase activity in membranes prepared from the cells. However, no measurable decrease in the binding of β-adrenergic ligands occurs.[21] While the phosphorylation state of α_1-adrenergic receptors was not demonstrated to increase in the experiments performed with hepatocytes, the binding of α_1-adrenergic ligands (^{3}H-prazosin) was reduced, as was the ability of norepinephrine to stimulate IP_3 formation.[22]

It appears that a number of hormone receptors in the plasma membrane are substrates for phosphorylation by protein kinase C. This conclusion is based mainly on results obtained following stimulation of cells with TPA, but in two instances (the EGF and β-adrenergic receptors) similar results have been obtained with purified receptors phosphorylated with purified protein kinase C. A unifying theme is that in each case, phosphorylation of the receptor decreases its ability to generate the appropriate intracellular messenger; tyrosine phosphorylation (EGF), cyclic AMP (β-adrenergic), or IP_3 (α_1-adrenergic). Thus, it appears that agents which activate the phosphatidylinositol response enable C-kinase to "feed back" on the receptors for a variety of mitogens and adrenergic ligands. The functional consequences of receptor phosphorylation noted to date appear to be inhibitory. It will be important to determine if C-kinase can phosphorylate a wider variety of hormone receptors and if the functional consequences will always be inhibitory.

Protein Kinase C and Cell Function -- Protein kinase C apparently plays an important role in regulating agonist induced secretion of stored proteins from a variety of secretory cells. Some representative examples are listed in Table 2. In cloned GH_3 cell lines, human platelets, and rabbit neutrophils, it is possible to begin correlating functional studies with protein phosphorylation in intact ^{32}P-labeled cells. An effect common to most cells is that

Table 1. Membrane Receptors that are Substrates for Protein Kinase C

Receptor	Cell Type	Agonist	Functional Change	References
EGF	3T3	TPA[a]	Decrease High Affinity EGF Binding	11-16
	A431	TPA	Decrease High Affinity EGF Binding Decrease Tyrosine Kinase Activity	
Insulin	IM-9 Lymphocytes	TPA	None reported	17
Somatomedin	IM-9 Lymphocytes	TPA	None reported	17
Transferrin	HL-60 Leukemic Cells	PDB	Loss of Receptors	18
Interleukin 2 (TCGF)	T Cells (HUT 102B2)	TPA	None reported	19
β-Adrenergic	Avian Erythrocytes	TPA Purified C Kinase	Decrease in β-Adrenergic Stimulated Adenylate Cyclase	20,21
α_1-Adrenergic [b]	Hepatocytes	TPA	Decrease in α_1 (^{3}H-Prazosin) binding Decrease in Inositol Trisphosphate generation	22

(a) Abbreviations: TPA, 4 β-phorbol-12 myristate - 13 acetate; PDB, 4 β phorbol 12,13 dibutyrate.

(b) Phosphorylation of α_1-adrenergic receptors was not reported in this study, but functional effects were demonstrated.

phorbol esters (usually TPA) stimulate the secretion of stored or newly synthesized cell products. Thus, GH_4C_1 cells are induced to secrete prolactin,[23-25] and platelets to secrete ADP and serotonin.[9,30-32] Similarly adrenal cells are stimulated to produce aldosterone[33] and mouse EL-4 cells to secrete newly synthesized Interleukin-2 (T cell growth factor).[34] Secretion can also be increased by the natural hormone or transmitter (e.g. TRH in GH_3 cells[23] or thrombin in platelets[9]). Usually the increase in secretion caused by the natural agonist is larger and more prompt than that caused by a phorbol ester. This result is explained by the observation that the two pathways shown in Figure 1 act synergistically to enhance secretion. For example, platelets can be treated with low doses of ionophores that elevate cytoplasmic Ca^{2+} to subthreshold levels without causing secretion. When Ca^{2+} levels are elevated, phorbol esters or synthetic diacylglycerols stimulate secretion of ADP or serotonin to a far greater extent than when Ca^{2+} levels are not elevated.[8,9] Similar results can be obtained in other secretory systems.[6] The molecular basis for this synergism is not known, but is presumed to occur via the actions of protein kinase C and Ca CaM dependent kinases.

In non-secretory cells such as the adrenal glomerulosa and the hepatocyte, the Ca^{2+} and DAG signals appear to perform related, but not synergistic, roles. For example, in adrenal cells where the natural hormone angiotensin produces a prompt and sustained rise in aldosterone production, A23187 can mimic the early phase of hormone action and TPA the late phase. Both A23187 and TPA are needed to mimic the entire response.[33] Similarly, in hepatocytes where protein phosphorylation has been measured, neither A23187 or TPA used alone can mimic the actions of a natural hormone such as vasopressin. However, when added together, TPA and A23187 can mimic the effect.[10]

In cell systems where the effects of phorbol esters on protein phosphorylation have been investigated, rather disparate observations have been made. The right half of Table 2 lists the molecular weights and known functions (if any) of the proteins reported to be protein kinase C substrates in intact cells. There are few common substrates among the cell types studied to date, with the possible exception of substrates in the 17-20,000 dalton range. An additional problem is that few of the putative protein kinase C substrates have been identified. However, in the platelet, a phosphoprotein with a molecular weight of 20,000 daltons has been identified as myosin light chain and is thought to function in release of ADP and serotonin via stimulus-secretion coupling.[30-32] This protein is a substrate for both protein kinase C and the Ca^{2+}/CaM dependent myosin light chain kinase (see below). It remains to be determined if the 17-20,000 dalton proteins observed in other cells are myosin light chains. However, in this regard a 17,000 dalton protein has been reported not to be identical with light chains in HL-60 cells.[42] Clearly, the data obtained to date about substrates for protein kinase C in intact cells is fragmentary and a great deal of progress is needed before the roles of these important proteins are understood.

Table 2. Effects of Phorbol Esters on Protein Phosphorylation and Function in Various Cell Types

Cell Type	Functional Effect	Kinase Substrates		References
		MW	Function	
Secretory Cells				
GH_4C_1/GH_3 Cells	Increased Prolactin Secretion	80 K, 66 K		
	Increased Prolactin Synthesis	19 K, 18 K	Unknown	23-25
Islets	Increased Sensitivity to Glucose Increased Insulin Secretion			26,27
Neutrophils	Increased lysosomal enzyme Secretion, aggregation and Superoxide production	98 K, 62 K 20 K, 13 K	 Unknown	28
Parotid	Increased amylase secretion			29
Platelets	Increased serotonin secretion	40-47 K	Unknown	6,9,30-32
	Increased aggregation	20 K	Myosin light chain	
Other Cell Types				
Adrenal Glomerulosa	Increased aldosterone production			33
EL-4 Lymphoma	Increased Interleukin-2 production	92 K, 70 K 54 K, 45 K 32 K, 20 K	Unknown	34,35
Hepatocytes	Unknown	87 K, 70 K	Glycogen Synthase	10,36
		56 K, 35 K	Unknown	
H35 Hepatoma	Unknown	32 K	Ribosomal Protein S6	37
HL-60 Leukemia Cells	Increase H^+/Na^+ Exchange	27 K 17 K	Unknown	38-42

Treatment of both secretory and non-secretory cells with phorbol esters increases the synthesis of some secreted proteins. For example, TPA increases the synthesis of prolactin in GH_3 cells,[25] Interleukin-2 in EL-4 cells,[34] and insulin in islets.[26] Moreover phorbol esters appear to regulate the synthesis of calcitonin in thyroid carcinoma cells[43] and the glycophorin receptor in K562 cells.[44] Clearly, hormones and phorbol esters, apparently acting via protein kinase C, can regulate the synthesis of important stored or secreted cellular proteins. In a number of cells, it has been possible to show that increased protein synthesis is due to increased production of the mRNA for the protein. Thus, TRH or TPA increases the synthesis of prolactin mRNA in GH_3 cells,[25] TPA stimulates Interleukin-2 mRNA production in EL-4 cells,[45] and calcitonin mRNA in thyroid carcinoma cells.[43] Interestingly, TPA and TRH appear to increase the phosphorylation state of a nuclear protein in GH_3 cells prior to production of the new prolactin mRNA. Taken together, these important observations suggest that hormonal signals mediated by DAG and protein kinase C can reach all fractions of the cell, including the nucleus, and exert effects on the production of mRNA for specific proteins.

Substrates for Protein Kinase C -- A number of investigators have studied the actions of purified protein kinase C on purified substrates (Table 3). Since protein kinase C was initially isolated from brain as an enzyme that phosphorylated histone,[6] it might be expected that it has a relatively broad substrate specificity *in vitro*. This has proven to be the case and the kinase has been demonstrated to phosphorylate a large number of substrates including enzymes of glycogen metabolism,[47-48] muscle proteins such as myosin light chain,[51] structural proteins such as MAP-2[6] and ribosomal proteins such as ribosomal protein S6.[53] Consideration of both Tables 2 and 3 shows that few of these substrates match the substrates observed when intact ^{32}P-labeled cells are stimulated with TPA or the cells' natural agonists. Moreover, many of the proteins that are substrates for protein kinase C, e.g. MAP-2 and ribosomal protein S6, are also substrates for a number of other known protein kinases. Thus, these proteins may represent good substrates for any number of protein kinases with broad substrate specificity. Therefore, the results obtained with protein kinase C and purified substrates should be interpreted cautiously.

The above caveats not withstanding, progress is being made with the 20,000 dalton myosin light chain. This protein appears to be phosphorylated in the intact platelet in response to TPA, A23187 or thrombin.[6,9,30-32] Study of the purified protein shows that it is phosphorylated in different sites by protein kinase C and myosin light chain kinase, a Ca^{2+}/CaM sensitive enzyme.[51] Phosphorylation of the light chain by protein kinase C prior to incubation with purified light chain kinase reduces the rate of phosphorylation by myosin light chain kinase. This effect appears to be due to a 9-fold increase in the Km of the light chain for the kinase caused by prior phosphorylation.[51] The maximal velocity of the reaction is not affected. Similarly, prior phosphorylation of the myosin light

Table 3. Substrates for Purified Protein Kinase C

Protein	Demonstrated in Cells (ref)	Functional Change	Reference
Glycogen Metabolism			
Phosphorylase Kinase	--	Increased Activity	47
Glycogen Synthase (87 K)	yes (36)	Decreased Activity	48
Muscle Proteins			
Phospholamban (27 K)	--	Increased Ca^{2+} Uptake sarcolemma membranes	49 50
Other Cardiac Sarcolemma Proteins of Mr = 88,51,42 K	--	Not known	49
Myosin Light Chain (20 K)	yes (30)	Increase Km of MLC Kinase for Myosin Light Chain	51
Structural Proteins			
Vinculin (130 K)	yes (46)	Shape Changes (?)	52
MAP-2 (190 K)	--	--	6
Others			
Ribosomal Protein S6 (32 K)	yes (37)	Not reported	53
Histones	--	Not known	6,54
Retinoic Acid Binding Proteins	--	Not known	55
Guanylate Cyclase	--	Increased Activity	56

chain by the light chain kinase causes a 2-fold increase in the Km of the enzyme for protein kinase C. These data have been interpreted to mean that phosphorylation of the light chain in the intact platelet by protein kinase C will inhibit the effect of the Ca^{2+} stimulus (via myosin light chain kinase).[31,51] Although the exact role of light chains in the platelet release reaction is not known, it will be interesting to attempt to correlate these phosphorylation events with the synergistic effects of DAG and Ca^{2+} on serotonin secretion observed in intact cells.

Summary: Phorbol esters and DAG's activate protein kinase C in intact cells, the immediate consequences of which appear to be the phosphorylation of a host of cellular proteins, many with unknown function. The functional consequences of these phosphorylation events include feedback inhibition of the effects of mitogens and adrenergic agonists at the receptor level, stimulation of the secretion of stored proteins in secretory cells, and stimulation of mRNA accumulation for certain secreted proteins. The link between protein phosphorylation and these latter events is obscure. A major goal of future research should be to identify the functional role of phosphorylation of the proteins that are presently thought to be protein kinase C substrates. It may be possible to approach this goal using a combination of protein chemistry and synthetic drug design aimed at selective stimulation or inhibition of the two pathways presented in Figure 1.

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Chapter 24. Neutrophil Elastase

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INTRODUCTION

Elastases are a family of proteinases that hydrolytically degrade elastin, a structural protein that is insoluble in neutral aqueous media and generally resistant to proteolysis.[1,2,3] In this report, we examine the recent literature pertaining to the biochemical properties, roles in normal physiology, and possible involvement in pathological states of the elastase from human neutrophils.

BIOCHEMISTRY

1) Purification and Physical Properties - Human leukocyte elastase (HLE) is generally isolated from one of four sources[4]: (i) leukocytes obtained by leukapheresis of patients with leukemia[5], (ii) "buffy coat" from normal donors,[6] (iii) purulent sputum,[7,8] or (iv) spleen.[9] While many purification schemes have relied on conventional methods of protein resolution, [4,5,6] several recent approaches have involved adsorption onto Sepharose-bound soluble-elastin,[7] affinity chromatography on Suc-L-Tyr-D-Leu-D-Val-pNA (pNA = para nitroanilide) coupled to Sepharose,[8] and affinity chromatography on Sepharose-bound Ala_3-pNA conducted at -14°C in pH 5 acetate buffer containing 50% ethylene glycol.[9] HLE isolated by these methods is a glycoprotein (MW around 30,000) and exists as at least three iso-forms separable by polyacrylamide-electrophoresis, isoelectric focussing, or cation-exchange chromatography.[4]

2) Catalytic Properties - HLE is a serine proteinase, as evidenced by its inhibition by diisopropyl fluorophosphate, phenylmethyl sulfonyl fluoride, and alpha-1-proteinase inhibitor.[10] It catalyzes the hydrolysis of substrate esters and amides according to a mechanism involving the formation and subsequent hydrolysis of a covalent acyl-enzyme. The intermediacy of such a species is supported by: (i) "burst" kinetics during aza-amino acid ester hydrolysis;[11] (ii) identical k_c values for the hydrolyses of the thiobenzyl ester, p-nitrophenyl ester, ethyl ester, p-nitroanilide, and diolanyl amide of MeOSuc-Ala-Ala-Pro-Val;[12] (iii) nucleophile competition experiments;[12] and (iv) pre-steady-state kinetics.

To define substrate specificity, steady-state kinetics for the HLE-catalyzed hydrolyses of several series of peptide-based p-nitroanilides[13-18] and thiobenzyl esters[19] have been determined. These studies revealed a positive correlation between catalytic efficiency and peptide chain length[16,18] and an almost absolute requirement for Val at P_1.[13,14,15,19,20] However, a recent report indicates that HLE possesses a broad substrate specificity for p-nitrophenyl esters of N-carbobenzoxy-L-amino acids (Z-AA-ONP).[21] Substrates that were

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efficiently hydrolyzed not only included Z-Ala-ONP and Z-Val-ONP, but also Z-Gly-ONP, Z-Phe-ONP and Z-Tyr-ONP. Combined with the studies of peptide-based substrates, these results suggest that substrate chain length regulates P_1 specificity, presumably through subtle enzyme conformation changes that accompany the binding of extended substrates at remote subsites on the enzyme surface.[21]

Remote interactions between HLE and substrate appear to influence not only catalytic efficiency and P_1-specificity, but also which step is rate-determining[12] and operation of the charge-relay system.[22] Specific substrates which offer the enzyme extensive opportunities for remote contacts are hydrolyzed *via* a mechanism that involves charge-relay catalysis, while the hydrolyses of minimal substrates are subject to only simple general catalysis by the imidazole of the active site histidine residue.[22] Subsite interactions influence the rate-limiting step by stabilizing only those transition states leading to the acyl-enzyme; transition states encountered during the hydrolysis of this intermediate appear to be stabilized very little by remote interactions.[12]

3) Specificity Towards Natural Protein Substrates - Human leukocyte elastase is a powerful endopeptidase capable of hydrolyzing amide bonds in a variety of proteins and peptides, including insoluble elastin.[23,24] Such broad specificity is shared by relatively few proteinases and may impart to HLE special roles in pathology and physiology.

Although HLE-catalyzed proteolyses are generally rather poorly characterized, the hydrolysis of elastin by HLE has been studied in more detail, especially with regard to its possible regulation. High ionic strength appears to stimulate elastinolysis by HLE,[25,26] as do cationic lysine rich proteins.[26] The former is thought to involve conformational changes of HLE,[16] while the latter is due to binding of these materials to sites on elastin that otherwise would lead to nonproductive enzyme-substrate interactions. Platelet factor 4 also stimulates elastinolysis by the latter mechanism and, thus, may potentiate the degradation of elastin *in vivo*.[26]

As noted previously, HLE's substrate specificity encompasses many proteins other than elastin and includes the oxidized B chain of insulin,[27] collagen types I,[28] II,[28] III,[29] and IV,[28,30,31] fibrin,[32] fibronectin,[33] fibrinogen[34] proteoglycan,[35] structural proteins of glomerular basement membrane,[36] α_2-plasma inhibitor,[37,38] components of the complement system,[39,40] and immunoglobulins.[41-43]

INHIBITORS

In addition to the inhibitors endogenous to humans, naturally occurring inhibitors of HLE are derived from a variety of sources including plants, microbes, and other animals.

1) Human-Derived Inhibitors - α_1-Proteinase inhibitor (α_1-PI, α_1-antitrypsin) is the most abundant serum proteinase inhibitor (~1.3 gl^{-1}).[44] α_1-PI is a glycosylated protein comprised of 394 amino acid units (54,000 daltons) whose primary structure was recently determined.[45] The oxidative inactivation of α_1-PI has been reported by several investigators using a variety of agents including cigarette smoke condensate, chloramine-T, N-chlorosuccinimide and

ozone.[46,47,48,49] Among the residues oxidized is the critical Met 358. This residue can also be oxidized by phagocyte derived myloperoxidase.[50] However, the physiological relevance of oxidative inactivation is not established. Recently, the expression of a genetically engineered oxidant resistant α_1-PI variant has been reported wherein the susceptible Met 358 is replaced by Val.[51]

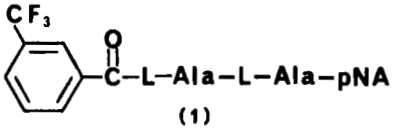
α_2-Macroglobulin inhibits the majority of known proteinases with widely differing specificities from all four catalytic classes including HLE ($K_{ass.} = 3.7 \times 10^7$ $M^{-1}sec^{-1}$).[52] All proteinases tested cleave α_2-macroglobulin within a twenty-seven amino acid peptide sequence occurring between two prolines, and this is believed to represent the outer limits of the "bait region." HLE cleaves at Val-His.[53] HLE, while complexed with α_2-macroglobulin, retains enzymatic activity toward small substrates but retains only limited activity toward tropoelastin and no activity toward insoluble elastin.[54] Detergents such as sodium dodecyl sulfate and long chain fatty acids activate the HLE-α_2-macroglobulin complexes toward degradation of tropoelastin and soluble elastin while inhibiting activity toward small substrate degradation.[55] These investigators suggested that the detergents altered the conformation of the complex, rendering the active site more accessible to soluble elastin. Inhibition of small substrate hydrolysis by these detergents is reported to occur with uncomplexed HLE as well.[55]

Bronchial mucus inhibitor (antileukoproteinase, BSI-TE) is a low molecular weight acid stable inhibitor produced locally by serous cells of submucosal glands in secretory granules of the upper respiratory tract ($K_i = 1.2 \times 10^{-11}$M).[56,57] In the upper respiratory tract, this inhibitor accounts for 80-90% of the elastase inhibitory capacity.[58,59] The complex of this inhibitor with HLE is stable; however, on addition to human serum, HLE dissociates from BSI-TE and forms a complex with α_1-PI.[60] BSI-TE appears identical to HUSI-1 derived from human seminal plasma.[61] An additional bronchial mucus inhibitor was recently reported which has similar molecular mass and amino acid composition to antileukoprotease. However, immunological cross-reactivity between these inhibitor species was not observed.[62] This inhibitor, BSI-E, is present in elevated amounts in obstructive airway disease.[62,63]

2) Non-human Animal, Plant, and Microbial Inhibitors - Arteparon®, a sulfated glycosaminoglycan derived from bovine cartilage tissue, is used outside the United States in the treatment of osteoarthritis and has been reported to be a mixed function inhibitor of HLE,[64] however, the physiological significance of mixed function inhibition is unclear.

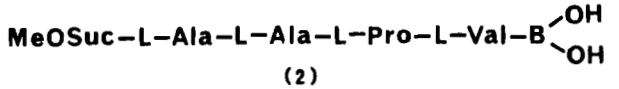
Eglin is a 70 amino acid inhibitor present in the leech Hirudo medicinalis.[65,66] It is a reversible, competitive, slow binding inhibitor of HLE ($K_i = 8 \times 10^{-10}$M).[67]

3) Synthetic Reversible Inhibitors - In recent years a variety of synthetic peptidic compounds have been reported as simple competitive, reversible inhibitors of HLE,[68,69,70,71], the most potent of which is (1).[71]



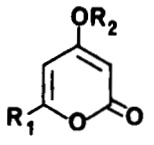
(1)

However, a very recent publication disclosed a boronic acid peptide transition state analog inhibitor of HLE (2) that is extremely potent (K_i = 5.7 x 10^{-10}M).[72]



(2)

Several heterocyclic α-pyrone inhibitors (3) have been reported, which were modeled after elasnin (4), a natural inhibitor.[73,74] A series of potent benzoxazinone (5) inhibitors have also been reported.[75]



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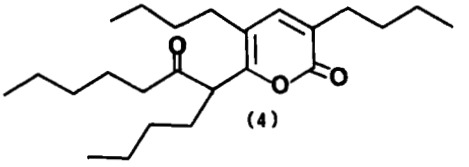

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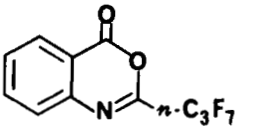
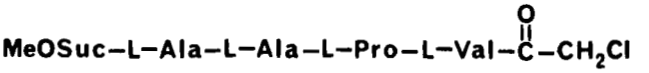


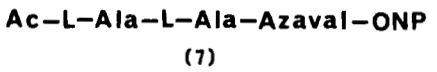
(5)

4) Synthetic Irreversible Inhibitors - A variety of synthetic inhibitors irreversibly inactivate HLE. The most frequently reported class are peptide chloromethyl ketones such as (6) which act by both acylating the active site serine and by alkylating the active site histidine.[76,77,78]



(6)

Other classes of irreversible inhibitors include azapeptides (7),[79] aryl sulfonyl fluorides (8),[80] N-acyl saccharins (9),[81] acyl benzothiazoles (10),[81] 3-chloroisocoumarin (11), and 3,3-dichlorophthalide (12).[82]



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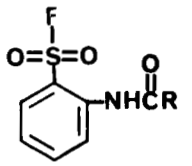

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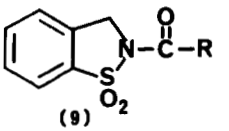

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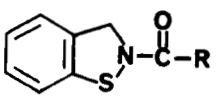
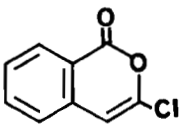
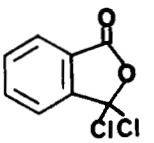


(10)



(11)



(12)

PHYSIOLOGY

1) Location of Elastase in the Cell - Ultrastructural, cytochemical, and immunofluorescence studies of human neutrophils have demonstrated the presence of elastase primarily in the azurophilic granules and, also, in the nuclear envelope, golgi complex, endoplasmic reticulum, and mitochondria.[83,84] Its existence in the mitochondria has been disputed.[85] It is believed that the highly cationic neutral proteinases are neutralized within the liposomal granules by ionic complex formation with anionic proteoglycans which form an acidic granule matrix.

2) Elastase Secretion - Limited secretion of elastase seems to be of physiological importance for modulating inflammatory reactions. Extracellular release of elastase from the neutrophil can occur by either cell death, incomplete closure of the phagosome during phagocytosis or direct active secretion. Secretion can be initiated by soluble stimuli such as C5a, f-Met-Leu-Phe and endotoxin.[86] Some recent evidence suggests that complement receptors on the surface of the neutrophil play a major role in granule enzyme release.[87] Tuftsin, an immunopotentiating tetrapeptide acts as an activator of neutrophil phagocytosis and elastase secretion by binding to specific Fc receptors on the neutrophil cell surface.[88]

Degranulation of azurophils in PMN is believed to require more than one signal. Recent studies have shown that key structures on the surface of the neutrophil can be controlled by extracellular stimuli to initiate translocation of azurophil granules to the cell periphery.[88] Control of elastase secretion from the PMNS can occur as well by agents such as C5a which affect the state of the cytoplasmic microtubules and microfilaments or by agents which influence the level of cAMP or cGMP within the cell.[89]

3) Chemotaxis - In general, proteinases and esterases present in the cytoplasm and membranes of the leukocyte play an important role in the generation of and response to chemotactic stimuli.[90] Cleavage of C5 by elastase releases the chemotactic agent, C5a, which can be further degraded and inactivated by excess elastase. There are conflicting reports regarding the capacity of elastase to efficiently cleave C3 to the chemotactic agent, C3a, under pathophysiological conditions.[91,92] Elastin fragments enriched in cross-linked regions resulting from elastase degradation also recruit leukocytes including monocytes into the lung *in vivo*. Monocytes could lead to the formation of other leukocytic factors.[93] It has been suggested that alveolar macrophages play a central role in modulating neutrophil migration to the lung by generating and releasing the lipophilic, low molecular weight chemotactic factor, AMCF.[94]

4) Elastase and Blood Coagulation - Elastase is one of the major fibrinolytic proteinases of the leukocyte. Early events in blood coagulation induce the active secretion of elastase from leukocytes.[95] It has been proposed that a human plasma enzyme, kallekrein, is primarily responsible for elastase's release during coagulation.[96,97] Elastase release occurs shortly after clot formation permitting coagulation to arrest bleeding before it participates in fibrinolysis. The fibrin degradation products generated by elastase are structurally distinct from plasmin-derived degradation products. Fragments from digestion of

fibrinogen (fibrinogenolysis) by elastase have been shown to possess anticoagulant activity, apparently due to their ability to competitively inhibit thrombin.[98]

Elastase is also capable of degrading and inactivating human plasma Factor XIII, the fibrin stabilizing factor,[99] and destroying the procoagulant activity of Factor VIII, a high molecular weight plasma glycoprotein which corrects the coagulation defect in hemophilia A.[100]

5) Alveolar Macrophage Internalization, Degradation and Release of HLE - Alveolar Macrophages (AM) may play a very important and complex role in modulating HLE injury to connective tissue in the lung, particularly in the lower respiratory tract. Endocytosed HLE, although slowly degraded in the AM, has an extended half-life of several days.[101] The slow release of elastase from the AM occurs primarily by active secretion and during cell death.[102] The internalization of HLE by AM appears to be receptor mediated. Human AM have surface receptors for the binding and internalization of HLE and with the use of high resolution autoradiography, radioiodinated HLE was shown to bind rapidly and specifically to AM *in vitro*.[103] Fucoidan is reported to be a competitive inhibitor of this receptor mediated endocytosis process.[104] It has been recently suggested that binding of HLE by AM triggers the release of AM derived chemotactic factors for neutrophils which results in further amplifying the inflammatory response.[105]

PATHOLOGY

Neutrophil elastase has been implicated as a pathogenic agent in a number of disease states in man, most notably, pulmonary emphysema. In this section we review the evidence for these implications.

1) Emphysema - The potential role of uninhibited proteolysis in the lung tissue destruction in pulmonary emphysema is the so called protease pathogenesis hypothesis and was recognized in 1962.[106] Several recent reviews detail the potential pathological role of leukocyte proteinases in emphysema.[120,121,122,123,124] Certain conditions may give rise to an excess of proteinase activity over proteinase inhibitory capacity.[107] Two major populations are at high risk for developing emphysema: (i) cigarette smokers[108] and (ii) genetically α_1-PI deficients.[109]

Cigarette smokers have increased numbers of PMNs and pulmonary alveolar macrophages (PAMs) which are recoverable by bronchoalveolar lavage.[110] PMN recruitment into lungs may occur as a result of elaboration of chemotactic factors by stimulated alveolar macrophages.[111] Cigarette smoke may serve in this stimulatory role.[110,111,112] Bronchopulmonary lavage fluids (BAL) from chronic human smokers show decreased α_1-PI inhibitory activity,[113,114,115] however these results have recently been disputed, and the physiological implications are unclear.[116,117] Experimental emphysema can be induced in a variety of animals by the administration of elastolytic enzymes, including HLE, oxidative inactivation of natural inhibitors and chronic administration of PMN chemoattractants. This is the subject of several recent reviews.[118,119]

2) Rheumatoid Arthritis - There are two principle means by which HLE could contribute to the pathogenesis of rheumatoid arthritis (RA): (i)

cleavage of IgG[43,125,126] and IgM[42,127] molecules and (ii) destruction of structural proteins of cartilage.[128-133] Immunoglobulins are known substrates for HLE.[41-43] The hydrolysis products of the reaction of HLE with these molecules appears to have in vitro and in vivo biological properties that include: (i) stimulation of oxidative metabolism and degranulation of neutrophils,[41] (ii) interaction with IgG and/or immune complexes at inflammatory sites,[126-127] and (iii) interference with the ability of IgG rheumatoid factor to bind to immune complexes.[42,127] Combining these factors could have an enhancing effect on inflammatory reactions in vivo.[42,43,125-127]

A more direct effect of HLE in RA is its ability to hydrolyze proteoglycan[35] and collagen,[28-31] the principle structural proteins in cartilage. Degradation of these proteins significantly reduces the mechanical strength of cartilage.[131-132] Furthermore, free- and inhibitor-bound HLE have been found in synovial fluids of patients with RA.[133]

3) Adult Respiratory Distress Syndrome (ARDS) - Bronchoalveolar lavage fluids from patients with ARDS contain elevated levels of catalytically active HLE and oxidants and decreased levels of functionally active α_1-PI.[134-136] It is supposed that oxidants are generated in the lung of ARDS patients and that these are responsible for the inactivation of α_1-PI. The high level of HLE activity presumably results in the lower level of its physiological inhibitor.

4) Glomerulonephritis - HLE is thought to play a causative role in glomerulonephritis due to: (i) its ability to degrade the structural proteins, type IV collagen and fibronectin, of glomerular basement membrane;[137] and, (ii) high concentrations of HLE being found in the urine of patients with this disease.[138] Studies have also demonstrated the degradation of the extracellular matrix of glomerular basement membrane by serine proteinases derived from neutrophils stimulated in situ by immune complexes deposited on this matrix.[36]

5) Cystic Fibrosis - The progressive pulmonary damage that accompanies cystic fibrosis is thought to involve destruction of lung connective tissue proteins mediated by bacterial proteinases and HLE.[139-140] The involvement of HLE is based on demonstration of its existence, in uninhibited form, in the sputum of patients with this disease.[139-140]

SUMMARY

In the past five years, the importance of human leukocyte elastase as a pathogenic agent in a variety of disease states in man has been recognized. This has resulted in a surge of interest in defining substrate specificity and designing potent synthetic inhibitors of HLE. The naturally occurring elastase inhibitor, α_1-PI, has recently received considerable attention as well. The expression of an oxidation resistant α_1-PI variant has been reported which may find use in the treatment of pulmonary emphysema.

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Chapter 25. Sickle Cell Anemia

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Introduction - Sickle cell anemia is an inherited genetic disorder arising from a point mutation in the beta-globin gene that leads to the replacement of Glu by Val at the sixth position of the beta chains of hemoglobin.[1] The mutant form, known as hemoglobin S, self-associates in the deoxygenated state into helical fibers that rigidify the red cells and distort their shape into the characteristic sickle-like appearance.[2-4] Individuals that inherit the mutation from both parents are homozygous for hemoglobin S. Such individuals experience, to varying degrees, the major disabilites of sickle cell anemia: acute susceptibility to infection in the early years of life, periodic painful vaso-occlusive crises, and a progressive degeneration affecting many organs of the body.[5] Because sickling cells have a greatly reduced lifespan compared to normal cells, a marked anemia generally accompanies their presence; hence the name sickle cell anemia. The anemia counteracts to some extent the circulatory difficulties caused by cell sickling which presumably would be exacerbated by higher hematocrits.

Sickle cell anemia occurs largely in individuals of African ancestory, because carriers of the mutation appear to have been genetically selected due to a modest resistance to malaria in the African tropics. As a consequence, carriers of the S mutation comprise approximately 10% of the American Black population. In Africa, large ethnic groups exist with an incidence of carriers of 25% of the population or more. Since the childhood period of susceptibility to infections can be controlled by regular administration of antibiotics, the population of homozygous SS individuals is on the increase in the United States and may also be expected to increase in Africa in the decades ahead. As a result, a significant population of individuals (on the order of 50,000 in the United States) confronted with the periodic painful crises and progressive degenerative aspects of the disease will be in need of treatment. In spite of concerted efforts, particularly since the initiation of the NIH Sickle Cell Disease Program in 1972, there is still no routine treatment for sickle cell anemia.

The lack of treatment for sickle cell anemia prevails even though virtually all of the molecular details of the disease are known. The appearance of sickle-shaped cells, first reported in 1910 by Herrick,[6] was related to an alteration of hemoglobin by Pauling and his colleagues in 1949.[7] Several years later, the single amino acid change was identified by Ingram.[1] In the last decade, the structure and assembly of the fibers formed by hemoglobin S have been studied extensively.[8-11] The fiber structure has been described in terms of a 14-strand helix, with the strands organized in 7 sets of double strands.[12] The description of a closely related crystal form of hemoglobin S composed of double strands[13] has permitted the organization of the hemoglobin molecules in the fibers to be specified in crystallographic detail.[14] An end-on view of the fibers depicting the postulated arrangement of the hemoglobin S

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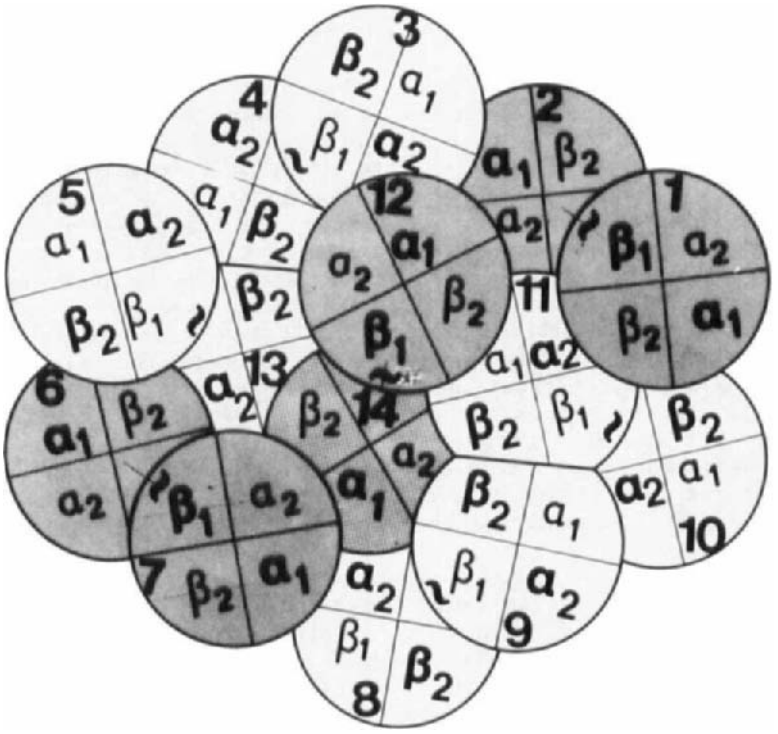
molecules is shown below.



The strands of molecules joined in the 7 double strands (1-2, 3-4, 5-13, 6-7, 8-9, 10-11, and 12-14) are related by a twofold screw axis indicated by the symbol (~) at the edge of one molecule near its contact with the molecule of the other strand of the pair. The shaded pairs have been proposed to occur with an opposite polarity with respect to the unshaded pairs.[14] The beta-6 Val responsible for fiber formation lies at the contact between molecules of opposite sides of the double strands.

As a result of the precise knowledge concerning the molecular basis for sickling, sickle cell anemia is a prime example of a disease that can be attacked by rational drug design. Nevertheless, sickle cell anemia has its own specific challenges, not the least of which is associated with the large quantity of hemoglobin (kilogram amounts) in the circulation. In principle, sickle cell anemia could be attacked at one of three levels: the beta-globin gene, the hemoglobin S molecule, or the red cell membrane. There has recently been considerable activity in the study of compounds believed to act at the level of the gene and other compounds believed to act at the level of the red cell membrane. While the studies on these compounds do not offer possibilities for rational drug design as clearly as compounds acting directly on the hemoglobin, there is moderate promise for some of them. Therefore, investigations dealing with compounds that act at the level of genes and those acting at the level of the membrane will be summarized briefly. Attention will then be turned to compounds acting on hemoglobin for which the stereochemical basis of the activity can be more readily visualized.

Gene modifications - Although a gene replacement that would substitute a normal beta-globin gene for the gene with the S mutation is far beyond current capabilities, treatment of sickle cell anemia would be possible by reversing the switch that normally occurs at birth from fetal hemoglobin to adult hemoglobin. Such an activity was first suggested for the leukemia drug 5-azacytidine on the basis of studies with adult baboons. The primates, rendered anemic by bleeding, showed increased levels of fetal hemoglobin after treatment with 5-azacytidine, thought to be related to hypomethylation of the fetal globin genes.[15] Subsequent studies with patients revealed marked increases in fetal hemoglobin levels upon treatment with 5-azacytidine and changes in methylation consistent with the initial hypothesis.[16-18] However, a number of concerns remain to be addressed before the potential of 5-azacytidine can be evaluated fully as an antisickling drug. The possible toxicity, including mutagenicity, of long term administration must be explored. The possible effects of altering the activity of genes outside the globin family must also be considered. In addition, recent studies with other drugs, such as hydroxyurea and cytosine arabinoside which mimic the increases in fetal hemoglobin induced by 5-azacytidine, have complicated the issue. These two drugs are thought to act by blockage of cell division rather than alteration of methylation patterns, suggesting that the elevated fetal hemoglobin levels for all three drugs may involve some subtle change in the pathways of red cell development.[19]

Membrane modifications - Changes in red cell membranes accompany the repeated cycles of sickling and unsickling, as the cells pass from oxygen-poor to oxygen-rich environments, leading to a leakiness for ions and a progressive dehydration that accompanies these repeated sickling events. Agents that alter membrane permeability or rheological properties may be successful in treating the disease and a substantial number of compounds acting at this level have been described. Unfortunately, most have been examined in only a limited way, either in vitro or in preliminary clinical trials that were not placebo-controlled, so that assessment of clinical efficacy is not possible in most cases. Among the drugs that have been subjected to such preliminary investigations are pentoxifylline, meclofenoxate, buflomedil, vincamine and pervincamine, ticlopidine, and gramacidin.[20-25] More extensive studies have been carried out with the drug cetiedil, both in vitro[26-29] and in double-blind multi-center clinical trials for the treatment of acute sickle cell crisis.[30] The first reports indicate some significant effects of the drug in shortening crises, decreasing the pain associated with the crises and reducing the number of painful sites.[30] A note of caution regarding hemolysis has been advanced for cetiedil, however, particularly at the higher range of the concentrations for which antisickling effects have been observed in vitro.[29]

Hemoglobin S modifications - By far the greatest activity concerned with antisickling agents has been directed towards the chemical modification of hemoglobin, either covalently or noncovalently. The goal of such modification is to reverse the self-association of hemoglobin S molecules into fibers and thereby eliminate the symptoms of sickle cell anemia. This reversal of fiber formation could take place either by a direct blockage of the intermolecular contacts responsible for stabilization of the fibers, or indirectly by increasing the affinity of hemoglobin for oxygen. Since it is the deoxygenated form of hemoglobin S that assembles into fibers, any increase in affinity would lead to a reduction in the fraction of the molecules in the deoxygenated state at partial saturations with oxygen and a corresponding reduction in fiber formation. Indeed, a large proportion of the antisickling compounds

that have been described to date act largely or entirely by this indirect mechanism. While a successful antisickling drug that acts only by raising the affinity of hemoglobin for oxygen is not out of the question, it would pose certain difficulties. For example, a patient treated with such a drug could experience a period of reduced sickling that would alleviate significantly the anemia normally associated with the disease. However, should the patient then experience a state of unusually severe hypoxia, appreciable sickling could occur (since at sufficiently low oxygen tensions extensive deoxygenation would still be produced) provoking a crisis that would be more serious than any that occurred before treatment due to the lessening of the anemia. Another consideration for any drug that modifies hemoglobin is the highly cooperative nature of the fiber assembly process. Since the rate of fiber formation is greatly dependent on the concentration of hemoglobin S[31] relatively small changes in the amount of hemoglobin available to assemble into fibers can have a marked effect on the time required for sickling.[32] As a result, a significant therapeutic effect may be achieved with the modification of only a fraction of the total hemoglobin S molecules. However, the situation is complicated by the existence of a wide spectrum of sickling cells of different densities in the typical patient,[33] with significant quantities of fibers present in the denser cells even at arterial oxygen tensions.[34] Nevertheless, since assembly kinetics are related to the solubility of the hemoglobin S molecules under a given set of conditions, the antisickling effects of any added agents are most commonly measured in terms of the extent to which they increase hemoglobin S solubility.

Historically, serious efforts to treat sickle cell anemia with a specific chemical agent began in the early 1970's when patients were treated with urea.[35] Use of urea as a chaotropic agent was based on the "hydrophobic hypothesis" to describe the nature of the interactions of hemoglobin S molecules responsible for fiber formation.[36] Although some success was initially reported, carefully controlled, double-blind studies failed to reveal a significant effect of urea in relieving crises.[37] Since crises will generally end even if untreated, it is particularly clear in the case of sickle cell anemia that studies without placebo controls are unlikely to provide reliable indications of clinical efficacy of a drug. Nevertheless, before urea was completely dismissed, it provoked the idea that the antisickling effects of urea may be due to cyanate, since urea slowly breaks down to form small amounts of cyanate which react covalently with hemoglobin by carbamylating alpha-amino groups. An antisickling effect was found for cyanate *in vitro*[38] and clinical trials were promptly initatiated with cyanate administered orally. Unfortunately, serious side effects, particularly peripheral neuropathy, forced the trials for oral cyanate to be abandoned,[39] although some investigations have continuted using cyanate with extracorporeal administration.[40] However, cyanate is not likely to be used as a general antisickling drug in the future, since (a) its clinical efficacy appears limited and arises principally from an increase in affinity of hemoglobin for oxygen (which, for the reasons noted above, is less desirable than a direct effect on fiber contacts);[41] and (b) administration requires the extracorporeal route which has obvious limitations in terms of the expenses and resources required. The use of urea also prompted the examination of a series of alkylureas for antisickling activity, with increasing potency observed as a function of alkyl chain length.[42]

Since the early 1970's, numerous other compounds reacting with hemoglobin, either covalently or noncovalently, have been tested for

antisickling activity. In general, the covalent compounds have been characterized more fully in terms of their sites of reactivity on the hemoglobin S molecule, although information for noncovalent reagents has also recently begun to emerge from studies by X-ray crystallography.[43] Two approaches can be seen in these studies: (1) selecting a target and tailoring reagents to improve their reactivity with the target, or (2) studying reagents that show some antisickling properties, with modifications of the reagent to improve efficacy attempted in some cases, and with the site of reactivity possibly identified subsequently.

Target-oriented modifications of hemoglobin S - In general, specific targets for chemical modification of a protein are usually associated with an "active site", since only the precise molecular architecture of such a site has the conformational detail necessary for specificity. For the regions of the hemoglobin S surface that participate in the various intermolecular contacts, there are no obvious distinguishing features that endow these sites with the qualities needed for specific targets.[14] The active site for the primary function of the hemoglobin molecule is the heme, but for practical purposes it does not appear to be a suitable target for chemical modifications that would have antisickling properties. However, hemoglobin also possesses a specific binding site for the effector molecule 2,3-diphosphoglycerate (DPG), which has the properties of an "active site".[44] One line of research that has exploited this site began with aspirin and various aspirin derivatives.[45] As the research evolved compounds were developed with a high degree of specificity for the DPG site, especially bis(3,5-dibromosalicyl) fumarate, which effectively displaces DPG.[46] Compounds of this type have potential as specific antisickling agents, although they produce an increased affinity of hemoglobin for oxygen that may complicate their utility.[46]

One other natural "target" on the hemoglobin molecule is the reactive SH group of the beta-93 Cys. Most thiol reagents react preferentially with this Cys residue, with more rapid kinetics in the oxygenated state of hemoglobin than in the deoxygenated state. Antisickling effects of thiol reagents have been described.[47-50] Characterization of 21 thiol reagents recently reported,[51] indicates that many have antisickling properties.[52] In most cases, the antisickling effects of thiol reagents are related to an increase in the affinity of hemoglobin for oxygen, although in some cases a direct inhibition of fiber formation occurs, perhaps related to a distortion of the "receptor pocket" on each hemoglobin S molecule that binds the beta-6 Val residue of the adjacent molecule across the double strand. This receptor site involves the beta-85 Phe and beta-88 Leu residues that occur a few turns of the F helix away from the beta-93 Cys.[13] While the beta-93 SH represents a highly specific target on the hemoglobin molecule for thiol reagents, other proteins of the body might also react well with any particular thiol reagent, leading to possible toxicity.

Other covalent modifications - Apart from the obvious targets, a number of sites have been "discovered" by the relatively specific reactivity of certain reagents. For example, mechlorethamine (nitrogen mustard) reacts at the His residue at the beta-2 position to inhibit sickling markedly.[53] Although the extreme toxicity of nitrogen mustard severely limits its usefulness as an antisickling drug, whatever properties have led to the special reactivity of this His residue may be exploited with other less toxic reagents. Another example is glyceraldehyde, which reacts preferentially at the alpha-16 Lys residue to inhibit sickling.[54] This residue lies at the intermolecular contact along individual strands

in the fibers.[13,14] Other aldehydes with antisickling properties include acetaldehyde[55], 5'-deoxypyridoxal,[56] and the bifunctional aldehyde 4-4'-diformyl-2-bibenzyloxyacetic acid.[57] The bifunctional reagent bis(N-maleimidomethyl) ether (BME), which reacts with hemoglobin with a high degree of specificity (at the beta-93 Cys and beta-97 His residues), displays antisickling activity.[58] Antisickling effects of mono- and bi-functional imidates have also been reported.[59,60] Significant antisickling effects have recently been reported for ethacrynic acid.[61] Acrylophenones such as ethacrynic acid can undergo Michael-type additions with sulfhydryl-containing molecules. However, preliminary chemical characterization indicated that the beta-93 SH of hemoglobin was still free after the hemoglobin had reacted with ethacrynic acid, although a covalent reaction does appear to have taken place.[61] Ethacrynic acid was selected for testing on the basis of earlier studies with halogenated (benzyloxy) acetic acid molecules which react at the alpha-14 Trp of hemoglobin.[43,62] It may therefore bind at the same region, although it is not clear what functional group in that region could participate in a covalent bond.

Noncovalent agents - Fiber formation can be inhibited by many agents, such as ethanol and other polar organic solvents,[8,45] but the major effort in the development of noncovalent antisickling agents has concerned amino acids, peptides, and related small molecules. The dominant effect indicated by the studies of many peptides and amino acid derivatives is hydrophobicity. For example, phenylalanine and phenylalanine-containing compounds exhibit relatively strong antisickling effects.[63,64] Recent investigations have emphasized the increased antisickling potency of halogenated derivatives or extended ring structures. The properties of 42 beta-aryl-substituted alanines were studied, with 5-bromotryptophan most effective.[65] Studies with other halogentaed aromatic acids have already been noted.[62] Coupling two aromatic moieties increased efficacy, as in the compounds N-phenylacetyl-L-phenylalanine and N-L-phenylalanyl pyridine amide.[66] Overall, improvements in the antisickling activity of compounds of the noncovalent class may lead to viable antisickling agents, but obstacles include achieving suitable concentrations in vivo (in the millimolar range) and causing deleterious effects on red cell properties.[67] Another compound of interest is DBA (3,4-dihydro-2,2-dimethyl-2H-1-benzopyran-6-butyric acid), an analog of a natural product with antisickling activity,[68] although its efficacy in vivo is uncertain.[69,70] The antisickling effects of certain gases that bind to hemoglobin have also been reported,[71] with special attention focused on dicholoromethane,[72] although hemolysis may complicate interpretation of the results.[73,74] Perfluorochemicals such as Fluosol-43 have also been studied.[75] Following the anomalous behavior reported for certain hemoglobins in citrate agar electrophoresis, glycosaminoglycans were investigated as potential antisickling agents, with some encouraging results.[76]

General conclusions - The summary of efforts to develop antisickling agents presented here reveals that a considerable background of activity exists, although strong candidates for antisickling agents of routine use have not yet emerged. The special problems of modifying a protein as abundant as hemoglobin have not been addressed explicitly, but it is apparent that the usual pharmacological requirements for specificity and safety will be especially acute due to the high doses expected for an antisickling drug. Actually achieving sufficiently high levels for a therapeutic effect may pose additional problems. One possible strategy to circumvent some of the difficulties associated with the abundance of hemoglobin would be to employ a combination of several agents, in order

to minimize toxicity and maximize the benefit from additive therapeutic effects. While agents that act at the beta-globin gene or the red cell membrane have also been considered, antisickling drugs at these levels may pose other problems. One possibility for achieving targeted delivery of antisickling agents is the use of liposome-mediated systems.[77] Another avenue to explore involves drugs that act directly on the enhanced adhesion to the endothelium reported for sickled cells.[78] Modification of DPG levels via inhibition of the enzymes that synthesize DPG could also affect sickling.[79,80] Comparative evaluation of diverse antisickling agents by standardized techniques in one laboratory should help to establish relative efficacies.[81] In conclusion, sufficient progress has been achieved in understanding the basic parameters of the sickling process, and sufficient experience has been obtained with a number of categories of antisickling agents, so that the next generation of antisickling agents developed may reasonably be expected to achieve markedly improved efficacies, perhaps suitable for therapeutic use. No efforts involving systematically applied computer-aided drug design have been reported, although hemoglobin would appear to be an ideal candidate for such activities.

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Chapter 26. Renin Inhibition

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Introduction - The renin-angiotensin system (RAS) is a multiregulated proteolytic cascade which produces two potent pressor and aldosteronogenic peptides: an octapeptide, angiotensin II (AII), and a heptapeptide, angiotensin III (AIII).[1,2] Although the exact role that the RAS plays in the maintenance of normal blood pressure is unclear, it has been demonstrated that the pharmacological interruption of the RAS can lower blood pressure in a majority of hypertensive patients.[3,4] Specifically, inhibitors have been developed of angiotensin converting enzyme (ACE, peptidyl dipeptidase A, kininase II [EC 3.4.15.1]), the enzyme which cleaves the inactive decapeptide angiotensin I (AI) to yield AII.[2,5] These angiotensin converting enzyme inhibitors (CEI's) may be a major advance in the treatment of hypertension and congestive heart failure.[6]

The first proteolytic step in the RAS is the renin reaction, in which the decapeptide AI is cleaved by the enzyme renin [EC 3.4.23.15] from the N-terminus of protein substrate, the α_2-globulin, angiotensinogen. Unlike ACE, which is a relatively non-specific peptidyl-dipeptide hydrolase,[7] renin is a uniquely specific enzyme, with only one known natural substrate.[2] Inhibition of the RAS by blockade at the renin reaction could be an extraordinarily specific pharmacological intervention which might be a valuable alternative to inhibition of ACE.[8] Despite almost two decades of effort, medicinally-useful inhibitors of renin have not been reported. However, recent demonstration of the broad efficacy of CEI's in the treatment of hypertension and congestive heart failure has accelerated renin inhibitor research.

Several recent reviews of renin inhibitors and CEI's have appeared.[2,5,9,10] This chapter will review recent progress in the design of renin inhibitors, focusing on *in vivo* evaluation of these compounds, and will seek to outline any differences between renin inhibitors and CEI's which either have been seen or might be uncovered upon further study of both of these classes of RAS inhibitors. Detailed consideration of other possibilities for interruption of the RAS, including inhibition of renin release,[11,12] inhibition of angiotensinogen synthesis or release,[13-15] inhibition of the activation of inactive renin[16-19] and antagonists of AII[20,21] is beyond the scope of this review.

Renin - Renin of the plasma RAS is synthesized in the juxtaglomerular cells of the kidney and released from storage granules into the bloodstream.[1] In studies of the biosynthesis of mouse submaxillary gland renin, a tissue renin identical to the kidney enzyme, it has been shown that a pre-pro-enzyme is processed to the active renin and either released directly into the circulation as an active one-chain form or from storage granules as an active two-chain form.[22] Renin release is regulated by a variety of factors, including renal perfusion pressure, a β-adrenergic receptor mechanism, sodium balance, and AII levels.[11] In addition, an inactive plasma renin component is present, although physio-

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logical activation of this material has not been demonstrated.[16-19] Exogenously administered plasma renin in rats has a mean half-time of clearance of 13 min and is cleared primarily by the kidney.[23] In addition to the "classical" RAS of the plasma, renin and other components of the RAS are synthesized and may act intracellularly in the kidney, brain, pituitary, adrenal, arterial wall, testes, uterus and heart.[24-28]

On the basis of active site chemistry[29] and primary sequence,[30-34] renin is classed as an aspartic proteinase, in the same mechanistic and structural class as pepsin, cathepsin D, gastricsin, and a variety of fungal proteinases, such as penicillopepsin, endothiapepsin, and rhizopuspepsin. Although the three-dimensional structure of renin has not been determined, the active site structures of several related aspartic proteinases show a long cleft, capable of binding a substrate peptide of eight amino acids or more.[35] In roughly the center of this enzyme cleft are the two catalytic aspartic acid residues. An understanding of the detailed interactions which give renin its unique specificity awaits completion of the X-ray structure of renin with substrate analogs bound.[36] Models of renin which might be useful for drug design have been described, based upon the known primary structure of renin and the X-ray structures of related aspartic proteinases.[37-41]

Human renin is a less specific enzyme than the other mammalian renins studied. The human substrate sequence at the scissile bond is ...Leu-Val..., while it is ...Leu-Leu... for other mammals.[42-45] While the human enzyme will cleave human and other mammalian substrates, only the human enzyme will cleave human substrate.[46]

Renin Inhibitors: In Vitro - Polyclonal, monoclonal, or FAB antibodies, prepared using purified renins, have given inhibitory antibodies with K_i = 0.01-1.0 nM.[47-50]

Competitive inhibitors based upon the fully active N-terminal 13- or 14-amino acids of the substrate sequence are the focus of most of the current efforts in renin inhibitor design, beginning either from the human substrate sequence, **1**, or the horse/dog/hog sequence, **2** (see Table,

#	Name	Structure	Renin Inhibition, 10^{-9}M Human	Dog
1	Human	Asp...Ile-His-Pro-Phe-His-Leu—Val-Ile-His	29000	13000
2	Dog	Asp...Ile-His-Pro-Phe-His-Leu—Leu-Val-Tyr-Ser	32000	74000
3	RIP	Pro-His-Pro-Phe-His-Phe—(R)Phe-Val-Tyr-Lys	2000	70000
4	H-77	D-His-Pro-Phe-His-Leu—(R)Leu-Val-Tyr	1000	24
5	H-142	Pro-His-Pro-Phe-His-Leu—(R)Val-Ile-His-Lys	10	10000
6	H-261	Boc-His-Pro-Phe-His-Leu—(OH)Val-Ile-His	0.70	20
7	SCRIP	Iva-His-Pro-Phe-His-Sta———Leu-Phe-NH_2	16	42
8		Iva-His-Pro-Phe-His-Sta———Ile-Phe-OCH_3	0.63	13
9	CGP-29287	Z-Arg-Arg-Pro-Phe-His-Sta———Ile-His-Lys(Boc)-OCH_3	1.0	200
10		Boc-Phe-His-Sta———Ala-Sta-OCH_3	27	NR
11	Pepstatin-A	Iva-Val-Val-Sta———Ala-Sta	22000	1300
12		Boc-Phe-Leu-Phe-al	2100	NR
13	RRM-188	Z-[3-(1-naphthyl)Ala]-His-Leu-al	80	3400

Table 1. Selected Renin Inhibitors. Boc = tert-butyloxycarbonyl; Iva = isovaleryl. Values given for substrates **1** and **2** are K_m. Others are either K_i or IC_{50} values. See references in text. NR = not reported.

K_m values from Ref. 51). Pioneering work was done by Kokubu and coworkers, using Leu-Leu-Val-Tyr and analogs as non-cleaved, but weak (mM), competitive inhibitors.[52] Burton and colleagues began from the minimum synthetic renin substrate octapeptide 6-13 (see Table),[43] substituting an uncleaved ...Phe-Phe... for ...Leu-Leu..., and extending to a decapeptide, **3**, called "RIP" (renin inhibitory peptide), with 2 μM inhibitory potency versus human renin.[53]

Szelke and coworkers replaced the scissile peptide bond in octapeptide substrate analogs with non-cleavable dipeptide analogs, giving compounds containing either a "reduced peptide bond" ($-CH_2NH-$) as in **4** or **5**, or a "hydroxyethylene" ($-CHOHCH_2-$) as in **6**, which are thought to be transition-state or intermediate analogs of the substrate.[54-56] The reduced peptide bond inhibitors are surprisingly species specific (see Table), but the hydroxyethylene-containing peptide **6** shows 0.7 nM potency versus human renin while also retaining high potency (20 nM) versus dog renin.[56]

The natural product pepstatin, **11**, isovaleryl-Val-Val-Sta-Ala-Sta, is an extraordinarily potent, competitive inhibitor of most aspartic proteinases (for example, K_i = 0.05 nM for pepsin[57]).[58] The central Sta, 4-amino-3-hydroxy-6-methylheptanoic acid, is thought to function as a transition state or intermediate analog.[59] Pepstatin however, is a relatively poor inhibitor of renin, with K_i = 22 μM versus human renin.[60] Nevertheless, soluble derivatives of pepstatin have been prepared and tested as renin inhibitors, the best of which was pepstatin-Glu, with IC_{50} = 5.8 μM versus human renin.[61] A semi-synthetic pepstatin analog, benzoyl-Val-Sta-Ala-Sta, gave improved hog renin inhibition (IC_{50} = 0.5 μM compared to 6.6 μM for pepstatin itself).[62]

On the basis of computer modelling, Boger and colleagues proposed that Sta could be a dipeptide analog, replacing both amino acids around the scissile bond, and giving more potent and specific inhibitors of renin.[60] Incorporation of Sta into the renin substrate octapeptide according to this hypothesis gave potent competitive inhibitors **7** (statine-containing renin inhibitory peptide, "SCRIP") and **8**, which are selective for renin versus related aspartic proteinases.[60,63] Potent human-renin-specific analogs of these Sta-containing compounds, such as **9**, have been described.[64] Incorporation of Sta as a single amino acid analog into a renin substrate analog gives less potent inhibitors.[60,65] Evin and coworkers reported a pentapeptide pepstatin analog, **10**, with IC_{50} = 27 nM versus human renin.[66] Similar pentapeptide Sta-containing inhibitors were reported independently by Boger.[63] Pepsin inhibitors incorporating statine analogs, including "statone"[67] and "phosphastatine",[68] have been described, and use of these statine analogs in renin inhibitors can be expected.

Recently, C-terminal amino acid aldehyde inhibitors of renin, **12**[69] and **13**,[70] a type first reported by Ito et al.,[71] were described. The potency of **13** is remarkable (IC_{50} = 80 nM, human renin), in light of the fact that the substrate sequence has been deleted to the right of the scissile bond. These are the smallest potent renin inhibitors yet reported, containing only three amino acids.

Renin is inhibited weakly by many hydrophobic compounds, which presumably interact with the hydrophobic enzyme cleft.[72,73] A putative endogenous phospholipid inhibitor has been isolated from kidney,[74] but its physiological relevance has been questioned.[75,76] Several synthetic

phospholipids have been prepared as renin inhibitors, but these have all been of only mM potency in vitro.[77-79] Although these compounds do inhibit renin, it has not been demonstrated that their antihypertensive action is due solely (or even primarily) to renin inhibition.

Recently, peptides related to the prosegment of pro-renin have been synthesized as competitive renin inhibitors, the best of which, Boc-Leu-Lys-Lys-Met-Pro-OCH_3, showed an IC_{50} of 2 μM.[80]

Renin Inhibitors: In Vivo - There are no reports of in vivo studies using renin inhibitors which would suggest that their use to block the RAS offers any advantages over ACE inhibition. Renin antibodies are effective blood pressure lowering agents in sodium depleted animals and in acute renovascular hypertension.[49,50,81-83] In one study with sodium depleted dogs, good blood pressure lowering was obtained but less of a renovasodilator effect was observed than with the peptidal CEI, teprotide.[84] In general, renin antibodies have not been effective in low renin models such as sodium replete animals or chronic low-renin hypertension. Concerns about anaphylaxis and the confinement of these potent inhibitors to the plasma compartment may limit their use as medicinal agents.

Studies with peptidal competitive inhibitors of renin have demonstrated effective blood pressure lowering in renin-dependent models, when administered either as infusions or as bolus injections. However, the duration of action of moderate intravenous doses has been only a few minutes. Blood pressure lowering by H-77 (4) and SCRIP (7) has been studied in sodium-deficient dogs, using infusions at 17-160 μg/kg/min, from 1 h to 2 days, giving 9-22 mm Hg decreases in mean blood pressure.[54,85,86] Infusion of SCRIP (7) at 30 μg/kg/min over 2 days steadily maintained a 22 mm Hg decrease in blood pressure from control levels, demonstrating no tachyphylaxis or escape from renin inhibition, despite a 3-4 fold increase in renin concentration.[85] Single iv doses of SCRIP (7) at 10-1280 μg/kg caused dose-related maximum blood pressure decreases of 5-34 mm Hg.[85] Compared to the CEI captopril, H-77 (4) gave a similar blood pressure floor in one study with sodium deficient dogs,[87] while another similar study found captopril to be slightly more effective.[88] Comparison of SCRIP (7) with the CEI enalaprilat (MK-422) in sodium deficient dogs showed that high doses of enalaprilat gave a larger blood pressure lowering, alone or in combination with SCRIP, compared to SCRIP alone.[89] Enalaprilat was associated with a modest tachycardia, not seen with SCRIP.[89] In a model of acute left ventricular failure in the dog, enalaprilat and SCRIP gave similar beneficial hemodynamic effects.[90]

Studies in primates show similar results to those in dogs. Renin inhibition in sodium deficient monkeys by iv injections of RIP (3) at 2 mg/kg or in acute renovascular hypertensive monkeys at 0.2-0.6 mg/kg lowered blood pressure similarly to 1 mg/kg of teprotide.[53] No effect on blood pressure was seen in sodium-replete animals. Infusions of H-142 (5) in conscious marmosets were compared with anti-renin antisera and with teprotide, and the decreases in blood pressure were similar.[91,92] A single large iv dose (10 mg/kg) of CGP-29287 (9) in mildly sodium-deficient marmosets lowered blood pressure 13 mm Hg for 2 h, while plasma renin activity was reduced to zero.[93] This compound was longer acting than a close analog lacking protecting groups on the Arg and Lys residues. The same blood pressure floor was obtained as when using enalapril.[93] Infusion of 9 at 0.1 mg/kg/min gave a 16 mm Hg blood pressure decrease which persisted for 60 min following cessation of the infusion.[64]

Only RIP (3) and H-142 (5) have been studied in humans, in normal volunteers. Infusion of RIP at 0.5 mg/kg/min in sodium depleted humans in supine position had no effect on blood pressure. However, at 1 mg/kg/min, a large drop in blood pressure was observed, followed by a decrease to undetectable levels when the subjects sat upright. The hypotension was accompanied by bradycardia. One subject was examined while on a high salt diet, and a similar infusion of RIP at 1 mg/kg/min lowered blood pressure from 118/68 to 66/0, and heart rate fell. No effect on blood pressure was noted at 0.5 mg/kg/min.[94] Clouding interpretation of these results is the fact that RIP is a weak CEI as well as a renin inhibitor.[95] In contrast to these dramatic results, infusions of a more potent renin inhibitor, H-142 (5), into normal sodium deficient volunteers at 17 μg/kg/min produced only a 10 mm fall in blood pressure without a change in heart rate.[96]

Initial evaluation in vivo of the new tripeptidal aldehydic renin inhibitors has been disappointing. Infusions of RRM-188 (13) in a rabbit model of acute renovascular hypertension showed blood pressure lowering only at high doses, similar to the antihypertensive doses of a much less potent alcohol analog of 13, suggesting that the aldehyde is metabolized rapidly to the less active alcohol.[97,98]

Angiotensin Converting Enzyme Inhibitors and the Renin-Angiotensin System - The mechanisms responsible for the antihypertensive and other cardiovascular actions of CEI's are still controversial. When they are administered at reasonable doses, no definitive evidence has been discovered for any mechanism of action other than inhibition of ACE, although it is becoming increasingly likely that inhibition of RAS's other than in plasma is important.[5,27,89,99,100] However, if mechanisms unrelated to inhibition of the RAS are involved in the antihypertensive effects of CEI's, then specific inhibitors of renin may not be medicinally useful.

Kininase II, one of the enzymes which inactivate the potent vasodilator bradykinin, and ACE, which produces the potent vasoconstrictor AII are the same enzyme.[7] Thus, CEI's could have dual actions, both of which would lead to lower blood pressure.[101-103] Potentiation by the CEI enalaprilat of the depressor response to intraarterial bradykinin in an acute renovascular hypertensive model, which persisted throughout a 24 h hypotensive period during which the pressor response to intraarterial or intravenous AI returned, has been cited recently in support of the kinin hypothesis.[104] Monitoring of bradykinin levels in plasma during CEI has provided mixed results.[105-110]

A proposal for a prostaglandin-mediated antihypertensive activity for CEI's, due to bradykinin-stimulated prostaglandin synthesis, has been reviewed recently.[111] In experiments suggesting this pathway, a prostaglandin synthesis inhibitor markedly attenuated the antihypertensive effects of captopril in a rat model[112] and the renovasodilator effects of the CEI, YS-980, in dogs.[113] There is also some evidence, from experiments in pithed rats, that high doses of CEI's may be related to inhibition of sympathetic function, but it is unclear that these effects would be important at pharmacologically reasonable doses.[114,115]

Angiotensin-Converting Enzyme Inhibitors: Non-Antihypertensive Effects - The low incidence and mildness of side effects observed clinically for enalapril and for low-dose captopril is remarkable, considering the many possible effects which might arise from inhibition of this non-specific enzyme.[3,4,116] It is likely, however, that a complete

picture of the side effects of CEI's will not emerge until more compounds are used for more years in many patients. Against this background of relative safety, it is difficult to advance the case for renin inhibitors on the basis of a theoretical argument for lesser expected side effects. Nevertheless, several areas of potential advantage of renin inhibition versus ACE inhibition can be outlined.

The role of kinins in the potentiation and mediation of inflammatory responses and tissue injury has been reviewed recently.[117] Local vasodilation and the promotion of permeability of capillary endothelium caused by kinins contributes to tissue injury in some situations, and these effects can be exacerbated by converting enzyme inhibitor potentiation of tissue kinins. Experiments with teprotide and YS-980 in carrageenin-induced rat paw swelling has confirmed their ability to exacerbate swelling and tissue damage.[118,119] One explanation proposed for the minor cutaneous side effects seen with captopril[3] and enalapril[4] is CEI-potentiation of kinins, which might uncover a subclinical inflammatory state.[117,120]

Although the genesis of cardiac pain is complex,[121] bradykinin has been suggested as the substance responsible for pain in angina pectoris.[122] In dogs, after ligation of the anterior descending artery, bradykinin levels in coronary sinus blood rose and remained elevated for 10 min.[123] Kininase levels also increased. These bradykinin levels were similar to those reported to cause pain when injected intraarterially in man,[124,125] and in dogs.[126,127] Application of extremely low doses of bradykinin to exposed ventricular surfaces of dog epicardium caused a reflex cardiovascular response interpreted as nociception.[128] In related studies, the non-specific kallikrein-inhibitor aprotinin was found to have a positive effect on survival of dogs in an acute myocardial infarction model.[129] If local bradykinin causes damage and pain in ischemic tissue, then inhibition of kininase II by CEI's may have deleterious effects. A clinical case has been described in which three episodes of angina and a suspected myocardial infarction were ascribed to captopril administration.[130] Renin inhibitors should not affect kinin levels.

Renin-Angiotensin System and the Brain - The RAS in the brain and its possible role in the control of blood pressure have been the subject of recent comprehensive reviews.[131-133] In addition to modulation of blood pressure, the brain RAS may be involved in control of sodium appetite and thirst. Intracerebroventricular (icv) administration of CEI's lower blood pressure in spontaneously hypertensive rats and can depress the drinking responses and salt appetite elicited by icv AI administration.[134,135] Peripherally administered CEI's can penetrate into the brain, and inhibition of brain ACE has been shown,[134,136] although this has not been observed in some acute experiments.[137] The degree to which peripherally administered CEI's exert their antihypertensive effects through inhibition of the brain RAS may depend upon the lipophilicity and other physical properties of the CEI used.[100]

Some reported side effects of ACE inhibition may be due to inhibition of related peptidyl-dipeptide hydrolases in the brain. Converting enzyme inhibitors have been reported to cause mood changes, particularly euphoria,[138,139] although one double-blind study found a significant depression due to CEI administration.[140] More severe neurological impairments have also been described.[141] The reported euphoria might be linked to the ability of CEI's to inhibit one of the enzymes of enkephalin metabolism.[142] While potentiation of morphine analgesia by capto-

pril has been reported,[143] no effect was observed due to enalapril in rats.[144]

Injections of AII into the brain can increase sodium appetite in experimental animals.[131,145] Paradoxically, oral doses of captopril in rats also can stimulate sodium appetite.[146] This phenomenon has been confirmed recently, using both oral and subcutaneous administration.[147] The sodium appetite due to peripheral administration of captopril was blocked by intracerebroventricular administration of captopril. The explanation proposed was that peripheral administration of CEI's, which leads to buildup of the inactive AI, may lead to spillover of AI into the brain, where conversion to AII can occur. At high peripheral doses of captopril, where it might also cross into the brain and inhibit the conversion, no paradoxical sodium appetite was observed.[147] No increased sodium appetite has been reported in clinical studies of CEI's, but modest effects would be difficult to document.

Summary - Potent inhibitors of renin have been developed, but none yet reported has the duration of action or oral effectiveness characteristic of medicinally useful agents. Thus, conclusions based upon available comparisons between renin inhibitors and angiotensin converting enzyme inhibitors are necessarily premature. Despite the potential for greater specificity for renin inhibitors, no advantages of renin inhibition over angiotensin converting enzyme inhibition have been demonstrated.

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Section VI - Topics in Chemistry and Drug Design

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Chapter 27. NMR Spectroscopy in Biological Systems

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Introduction - The application of the non-invasive technique of nuclear magnetic resonance (NMR) spectroscopy to the study of biological systems has flourished since the first medical application by Moon and Richards in 1973.[1] Intraerythrocytic pH of hemolysates, packed red cells and whole blood was determined by measuring the chemical shifts of organic phosphate ^{31}P resonance of 2,3-biphosphoglycerate (BPG) or of inorganic phosphate (Pi) inside and outside the cell. This in turn led Hoult to use ^{31}PNMR spectroscopy to study the enzyme activity of excised, but intact rat hind leg muscle as a function of time by monitoring the turnover of phosphocreatine (PCr) to Pi.[2] Subsequently, ^{31}PNMR spectroscopy in particular, and NMR spectroscopy in general has been applied to the *in vivo* study of a broad range of biological systems employing the NMR detectable nuclei of biological importance, which include ^{1}H, ^{13}C, ^{43}Ca, ^{17}O, ^{35}Cl, ^{14}N, ^{23}Na and ^{39}K. The NMR experiment together with the capabilities and relative merits of these nuclides in a biological setting has been reviewed recently.[3,4] *In vivo* studies have ostensibly focused on the nuclides ^{31}P, ^{1}H and ^{13}C, and have been subject of a large number of reviews.[5-28] The clinical, non-imaging aspects of NMR spectroscopy, which have long been recognized[29], have also been reviewed.[30-34] This review emphasizes the recent application of NMR spectroscopy to the study of metabolism as it occurs in *in situ* and perfused organs and in cellular suspensions.

Brain Metabolism - ^{31}PNMR spectroscopic studies have provided a wide variety of information on *ex vivo* brain tissue metabolism of the resting organ[35,36] and more recently on the effects of hypoxia on a mammalian system.[37] In the latter study, after incubation under the desired anaerobic or aerobic conditions, the brain tissue was rapidly frozen at liquid nitrogen temperature and subjected to an extraction procedure. ^{31}PNMR of the extracts gave high resolution spectra of the phosphatic metabolites which could be characterized and quantified. In this way interspecies variation in brain metabolism, together with species specific responses to oxygen deprivation and subsequent recovery could be studied[38]. A rat brain perfusion study involving induction of hypoxia by switching perfusion media from an artificial blood (hematocrit 30%) to a fluorocarbon preparation showed that hypoxia reduced only cerebral high energy phosphate levels (adenosine triphosphate (ATP) and PCr) with a concomitant increase in Pi and adenosine diphosphate (ADP) concentrations.[39] This was subsequently confirmed, but it was found that mainte-

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nance of high energy phosphates is less effective in the aged brain than in the mature cerebrum.[40] A further use of bloodless perfluorocarbon as a perfusion medium in a rat brain study [41] demonstrated that the erythrocyte metabolite BPG does not, as had been suggested,[42] contribute to and interfere with the ^{31}PNMR analysis.

The application of proton NMR (^{1}HNMR) inversion-recovery spin-echo pulse sequence to biological systems has been well documented.[43] It has recently been applied to the metabolism of homogenates of rabbit[44] and rat[45] brain. A comparison of metabolites showed the relative abundances of neurochemicals such as taurine, N-acetyl-L-asparate and 4-aminobutanoate. The enzymic activities of prolidase, acetylcholinesterase and glutaminase were calculated using this technique.

The advent of surface coils in NMR spectroscopy [46] has made possible the noninvasive, in vivo assessment of metabolism in intact animals.[12] However, in contrast to the high resolution studies described above, the in vivo ^{31}PNMR spectroscopy reveals only seven broad phosphorous spectral peaks: phosphomonoesters (PME), Pi, phosphodiesters (PD), PCr and the three peaks of ATP (α, β and γ). In brain metabolism, ^{31}PNMR can be utilized to ascertain the intracellular pH and the bioenergetic status of the organ (generally the ratio of PCr to β -ATP) and this can be correlated with changes in function, e.g. stress and subsequent recovery.[47,48] A study of drug (bicuculline) induced status epilepticus in a rabbit has been reported.[49]

A natural extension of this is the clinical application of NMR spectroscopy to study human, particularly neonate, cerebral function.[50,51] The number of techniques[52] used to suppress the background cranial bone resonances in these studies has increased,[52,53] including the use of chronically implanted surface coils.[54]

Recently, a ^{1}HNMR system has been developed for in vivo studies which describes lactate production in intact rat brain subjected to low-oxygen gas mixtures, and its clearance on reoxygenation.[55,56] Obscured amino acid resonances in these studies were resolved by an in vivo homonuclear ^{1}H double resonance difference spectroscopic technique.[57]

Skeletal Muscle Metabolism - In the same progression as in the case of cerebral metabolism, ^{31}PNMR spectroscopic studies have developed from characterization and quantification of phosphatic metabolites in tissue extracts, to monitoring metabolism in perfused systems, to metabolism in intact subjects. This, with an emphasis on the diseased state, has been reviewed recently.[28] Cumulatively, the main advantages of this non-invasive technique are the determinations of intracellular pH and the energetic competence of the muscle at a given time. At rest, the molar ratio of PCr to Pi of healthy muscle is approximately 10, decreasing to a value of about 1 for fully activated oxidative metabolism. At lower ratios than this, the activation of glycolytic energy metabolism causes severe lactic acidosis which may impair the recovery to resting concentrations of high-energy phosphates.[58] Several human inborn errors of metabolism have been studied by in vivo ^{31}PNMR spectroscopy including McArdle's disease[29] and phosphofructokinase deficiency.[58-60] A more recent case involved assessing the bioenergetic capacity of skeletal muscle in a subject with a severe defect in complex III of the electron transport chain.[61] The resting PCr/Pi ratio was 1.3-2.5, which can be

compared to control values of approximately 9 (Figure 1). The rate of recovery of the PCr/Pi ratio after exercise was 2.5% of the normal (control), but on administration of menadione and ascorbate, a 21 fold increase in the recovery rate relative to the pretherapy value was observed. Similarly, evaluations of skeletal muscle energy metabolism in patients with heart failure[62] and the effects of this on aging[63] have been carried out. This type of NMR spectroscopy is also amenable to quantification of phosphatic metabolites and has been shown to be more accurate than the more classical method of bioassay.[64]

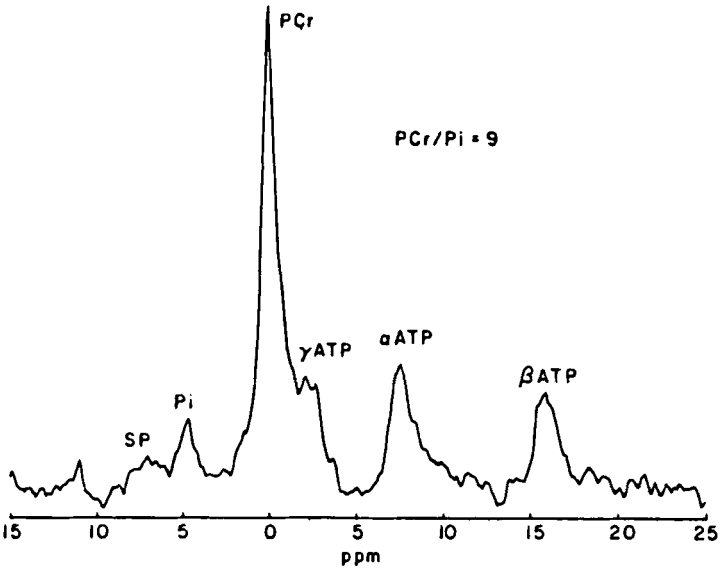

Figure 1. A ^{31}PNMR spectrum of the arm of a normal young female control at rest. Peak assignments are given in the text. Metabolite concentration is quantified by peak area. A low concentration of glycolytic intermediates (SP) is present. (From Reference 61 with permission.)

Although the clinical applications of *in vivo* ^{31}PNMR spectroscopy are far reaching, most basic research is still in the domain of the perfused animal model. For example, using a feline system, ^{31}PNMR was used to assess the use of hypothermia to prolong the viable life-time of ischemic skeletal muscle.[65] Perfusion studies on diseased tissue[66,67] and muscle energetics[68,69] are still providing new insights into metabolism at the cellular level.

^{1}HNMR spectroscopy is now being used to study muscle preparations and extracts. ^{1}H is more sensitive to the NMR experiment than ^{31}P, and because of its ubiquitous distribution, a more complete range of tissue metabolites becomes accessible. In this way healthy and diseased muscle have been compared [70] and intracellular pH and lactate production

measured.[70-72]

Heart Metabolism - In vivo ^{31}PNMR spectroscopy has been applied to determining the concentration and turnover of high-energy phosphates in the heart under various conditions. Although the in vivo studies for the superficial tissue, described above, allow surface coils to be utilized, internal organs, such as the heart, present difficulties with reduced sensitivity and spatial selectivity. Surgery is required to place the NMR transceiver on the internal organ[73] or studies are performed on isolated, perfused hearts.[74-77] More recently a catheter-coil has been placed inside a canine heart, without major surgery, and ^{31}PNMR spectra were recorded in various parts of the organ (Figure 2).[78]

High resolution ^{1}HNMR spectroscopic studies have been applied to describe a more complete array of heart metabolites.[79]

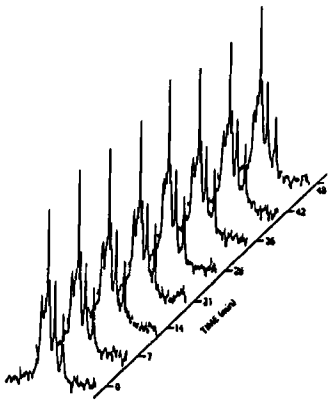

Figure 2. Stack plot of eight ^{31}PNMR spectra collected sequentially from the right chamber of the heart. See Figure 1 for peak assignments. (From Reference 78 with permission.)

In this review application of carbon-13 (^{13}C) NMR to in vivo studies has not been discussed. Carbon-13 is used relatively infrequently because of its lack of sensitivity to the NMR experiment and the need, for reasons of spectral simplification and increased signal to noise ratio, to be decoupled from any attached protons. This insensitivity usually requires that specifically ^{13}C-enriched substrates be used in a kinetic in vivo metabolic study.[80] A ^{13}CNMR time-course study of guinea pig myocardial glycogen synthesis from D-[1-^{13}C]-glucose has been carried out on the live animal.[81] This required the construction of a combination solenoidal receiver coil for ^{13}C and a saddle-shaped proton decoupler coil which was then placed around the heart.[82] Glycogen synthesis was also monitored by ^{13}CNMR spectroscopy on an isolated perfused rat heart system.[83]

In ^{13}CNMR spectroscopic studies, the flow of the enriched carbon can be followed through major and secondary pathways; for example, the production of [^{13}C]-enriched glutamate from [2-^{13}C]-acetate has been monitored in vivo.[84] A more extensive study involving perchlorate extracts of rat hearts perfused separately with [2-^{13}C]- acetate and [3-^{13}C]- pyruvate exemplifies another application of NMR spectroscopy to

biological systems.[85] It is possible to analyze the ^{13}C-^{13}C spin coupling constants of the end-products of metabolism in terms of the particular pathway involved, the rate of flux of ^{13}C label through it, and its branch points.

Liver Metabolism - Hepatic studies illustrate that ^{31}P is the observed nuclide of choice for studying the bioenergetics of a biological system, while ^{13}C is preferred when characterizing a particular metabolic pathway. Both have been used extensively in studies of hepatic metabolism.[86] ^{13}CNMR spectroscopy has monitored the in vivo metabolism of [4-^{13}C]-phenacetin by isolated perfused rat liver in a continuous flow apparatus. Metabolism of this drug to [4-^{13}C]-acetaminophen and an as yet unidentified secondary metabolite was monitored over a 4 hour period.[87]

Recent progress in the study of liver metabolism by NMR spectroscopy has seen the use of carbon-13 surface coils.[88-90] Rat hepatic glycogenesis was monitored in this manner (Figure 3) after injection of D-[1-^{13}C]-glucose.[91] This technique, therefore, provides a means to study the response of hepatic glycogen levels in vivo to a specific metabolic stimulus.

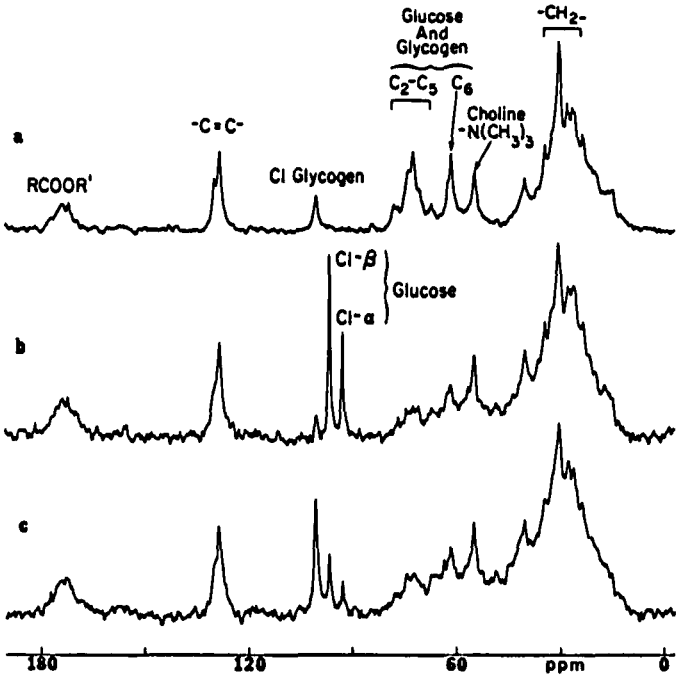

Figure 3. Proton decoupled ^{13}C surface coil NMR spectra at 90.56 MHz from rat liver in vivo.
a) Spectrum of liver from a rat fed ad libitum.
b) Spectrum of liver from a rat fasted for ~15 h.
c) Spectrum of liver as in b) showing hepatic conversion of glucose to glycogen over an ~30 min. period. Peak assignments as indicated.
(From Reference 91 with permission.)

Miscellaneous - The use of in vivo ^{31}PNMR techniques to study renal metabolism[26,92] and metabolism of the eye[28] have been reviewed recently. The high-energy phosphate metabolism of rat uterus,[93] pig adrenal[94] and canine mandibular[95] glands have been studied by in vivo ^{31}PNMR spectroscopy.

Erythrocyte Metabolism - The metabolism of the intact erythrocyte has been the subject of many NMR spectroscopic investigations which have been recently reviewed.[96,97] Investigators have explored the possibility of double-tuning a coil to detect signals from ^{31}P and ^{13}C sequentially.[98] This has been extended to the use of a probe tuned to three observable frequencies, ^{31}P, ^{13}C and ^{1}H with which erythrocytic metabolism of D-[1-^{13}C]-glucose was monitored.[99] A time-course of metabolic change was thus followed through three different nuclei at once (Figure 4).

In vivo ^{13}CNMR spectroscopy has been used to study the rate of glycolysis in mice red cells infected with Plasmodium berghei[100] and Babesia microti[101] and the effects of drug treatment thereon.

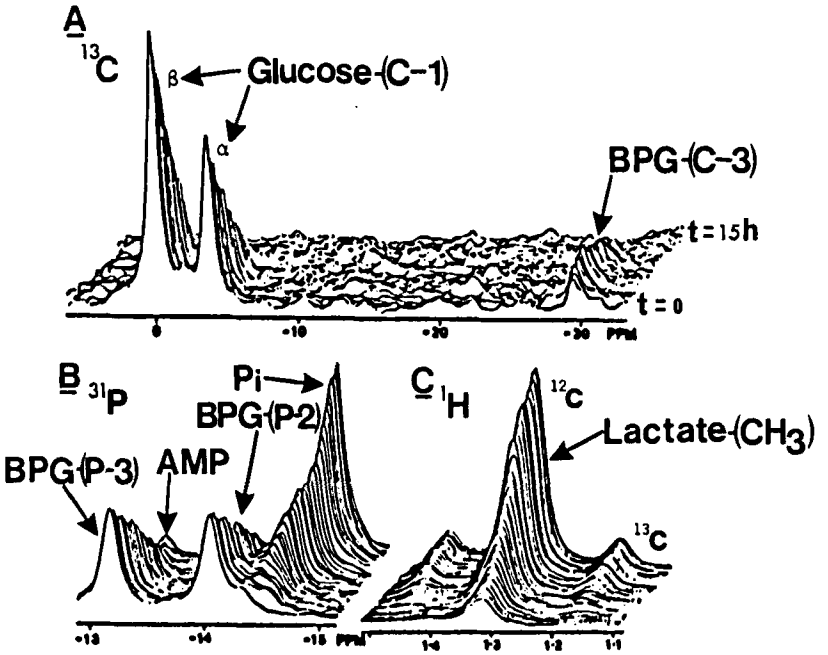

Figure 4. A simultaneous multinuclear NMR time-course from human erythrocytes metabolizing [1-^{13}C]-glucose. A 15 h time-course is represented by stacked plots of 40 spectra. A) 67.89 MHz proton-decoupled ^{13}C spectra. B) 109.28 MHz proton-decoupled ^{31}P spectra. C) 296.96 MHz ^{1}H spin-echo spectra. Peak assignments as indicated here and in text. (From Reference 99 with permission.)

Cancer Cell Metabolism - A recent review of the application of in vivo NMR spectroscopy to cancer covers metabolism by dispersed cells, excised tumors and in vivo tumors.[102] A ^{31}PNMR study of a transplanted brain

tumor has also appeared.[103] Furthermore, high resolution ^{1}HNMR spectroscopy has been applied to the analysis of metastatic cancer cells.[104] The previously reported[105] narrow ^{1}H resonances in spectra obtained from intact viable rat mammary adenocarcenoma cells have been identified by two-dimensional scalar-correlated spectroscopy[106] as arising from lipid acyl chains.[107]

Microorganisms - The ability to study metabolic processes of microorganisms by non-invasive NMR spectroscopy is well established.[6,11,20] Carbon is the favored nuclide for in vivo studies because administration of a ^{13}C-enriched substrate is experimentally easy, as is the observation of the fate of the label. In this way biochemical pathways can be delineated and the degree of cycling through these quantified.[108] Popular vehicles for study are the prokaryotic Escherichia coli and the eukaryotic Saccharomyces cerevisiae. A recent example of the use of these organisms involved a ^{13}CNMR comparison of the redox status of the diphosphopyridine nucleotide pool when both were cultured in media containing ^{13}C-enriched nicotinic acid. Large differences were seen when both systems underwent a variety of perturbations.[109] A novel use of two-dimensional proton-carbon chemical shift correlation to analyze the end-products of ^{13}C-enriched formate metabolism by E. coli has been reported, together with a deuterium NMR (^{2}HNMR) study of whole cells in deuterium depleted water, which demonstrated that dismutation of formaldehyde is not an important aspect of its detoxification.[110]

Conclusion - Although the majority of in vivo NMR spectroscopic investigations have been centered on the ^{31}P, ^{1}H and ^{13}C nuclides, others are used. For example, studies of intracellular metal ions in intact cells and tissue by direct observation or by indirect NMR methods have been recently reviewed,[111] while fluorine-19 (^{19}F) NMR spectroscopy has been used to measure intracellular free Ca^{2+} [112] and the transformation of the anaesthetic halothane (2-bromo-2-chloro-1,1,1-trifluorethane) in rabbit liver to trifluoroacetic acid.[113] This diversity of available nuclides in tandem with further improvements in probe design[114] and pulse sequences[115,116] will ensure the future of NMR spectroscopy as a viable technique for the study of biological systems.

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Chapter 28. Contrast Enhancing Agents in NMR Imaging

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Since the first observation of nuclear magnetic resonance, independently reported by physicists Felix Bloch[1] of Stanford and Edward Purcell[2] of Harvard in 1946, developments in NMR spectroscopy have progressed at a rapid pace. In four decades, NMR has become a powerful analytical method in chemistry and biochemistry, and most recently in the field of medicine. Magnetic resonance imaging (MRI) has shown exceptional promise as a useful diagnostic modality for human diseases. Unlike computerized x-ray tomography (CT), MRI does not use ionizing radiation, but yields two dimensional images of the body with quality often equal to and in some cases exceeding that of CT images.

A number of biologically relevant nuclei have a nuclear spin component (Chapter 28); however, the hydrogen proton (1H), because of its high sensitivity, isotopic abundance, and abundance in biological tissues, has received the vast majority of attention in nuclear magnetic resonance imaging. The protons giving rise to signal intensity in biological tissues are primarily those of cellular water and lipids. Protons of macromolecules, for example proteins and DNA, and solid structures, for example bone and gallstones, provide little contribution to the total signal. Contrast in NMR images is a direct result of inherent differences in the spin density, the spin lattice (T1), and spin-spin (T2) relaxation parameters of water in different tissues.[3,4]

To improve MRI diagnostic sensitivity and specificity for disease, the development of pharmaceutical contrast agents, capable of altering the intrinsic contrast between tissues, has been undertaken. Such pharmaceuticals may improve diagnostic confidence, eliminate the need for further procedures, and reduce medical costs by shortening the time for NMR examinations. A number of methods are available to alter image contrast; however the use of paramagnetic pharmaceuticals which directly affect the T1 and T2 relaxation parameters has been found to be the most effective. This chapter is intended as an introduction to nuclear magnetic resonance imaging with emphasis on the development and use of paramagnetic pharmaceuticals as contrast media. Several reviews on contrast media for NMR imaging have appeared in the last few years.[5-11]

IMAGING METHODS

In the production of NMR images, a variety of instrumental methods have been employed.[12-16] All of these methods have in common the use of magnetic field gradients to establish spatial localization, and a pulse sequence to define the timing and nature of the radiofrequency pulses that generate the transverse magnetization. To obtain spatial information, orthogonal magnetic field gradients are superimposed on the static magnetic field during the preparation, evolution, and detection of the

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signal. Since the frequency of the NMR signal (H_2O resonance) is proportional to the magnetic field strength, the nuclei residing in different locations of the specimen will resonate at different frequencies.

The most common MRI pulse sequences,[3,16-20] inversion recovery (IR) and spin echo (SE), have seen extensive application in NMR spectroscopy as methods to quantitatively determine relaxation times, T1 and T2. The signal intensities generated by the IR and SE pulse sequences are generally described by eqn. 1 and 2, respectively,

$$I = Nf(v)[1-2\ \exp(-TI/T1)+\exp(-TR/T1)] \quad (1)$$

$$I = Nf(v)[1-\exp-TR/T1]\exp(-TE/T2) \quad (2)$$

where I is the signal intensity of the transverse magnetization, N is the number of protons contributing to the signal, f(v) is a function of flow, TR is the delay time (under software control) between repetition of the pulse sequence, TI is the IR interpulse delay time, and TE is the spin echo delay time (under software control). The inversion recovery sequence, (180-t-90), where t = TI, produces images dependent primarily upon the T1 characteristics of the tissues. The signal intensity produced by the spin echo pulse sequence, (90-t-180), where t = TE/2, can be dominated by either T1 or T2 relaxation depending on the values selected for TR and TE.

Because image contrast can be altered through selection of pulse sequence and interpulse delay times, optimization of the pulsing parameters for different tissues has received extensive investigation.[21-23] Although the pulsing parameters may lend themselves to optimization, other experimental parameters, for example temperature, viscosity, and magnetic field strength, cannot be readily altered. Parameters optimal for one tissue may not work well for other tissues because of differences in the tissue variables N, T1, and T2. Thus, optimal parameters must be determined empirically for all tissues and for various pathologies. On a clinical level, time constraints on patient setting do not permit the use of all possible software parameters. Selection of TR and TE must be diagnostically efficient, yet not be a burden on patient cooperation or rapid throughput.

PRINCIPLES OF CONTRAST ENHANCEMENT

Pharmaceutical intervention with contrast media provides a means to change the relative contrast of different tissues by alteration of the intrinsic tissue parameters N, T1, and T2. These parameters as well as flow are the primary determinants of image contrast between native tissues. For example, spin echo signal intensity (eqn. 2) can be enhanced by increased proton density, decreased T1, and increased T2. The characteristics of flow or proton motion within tissues and vascular spaces can also greatly influence image intensity. Considerable effort has been directed to assess the effects of flow on image contrast; however there is little need or opportunity for pharmaceutical intervention.[24-27]

Proton density plays a relatively minor role in tissue contrast. Soft tissues, with comparatively high proton density, provide the major contribution to image signal intensity. The proton density variation between soft tissues is small, however, and it is not always altered by the presence of disease. Manipulation of signal intensity by altering

proton density has been approached by using lipid solutions,[28,29] ethanol,[30] furosemide,[31] olive oil,[31] and clomiphene.[31] Changes in renal tissue hydration, through voluntary dehydration or administration of parenteral diuretic agents, have also been demonstrated.[32] These methods of contrast enhancement all have the disadvantage of lacking the sensitivity to effect large changes in image contrast at safe dosages.

Development of contrast media for NMR imaging has focused on the use of paramagnetic agents which directly affect the T1 and T2 relaxation times of the tissue water. The predominant mechanism of proton relaxation is through dipole-dipole interaction of the proton with the surrounding magnetic spin lattice. Random rotational and translational motions of the adjacent magnetic moments (nuclei or electrons) within the lattice generate fluctuating local magnetic fields thereby promoting relaxation. Molecular motions that occur at a rate approximating the resonance (Larmor) frequency of the proton are the most effective at inducing this spin-lattice relaxation. Similarly, the fluctuating local magnetic fields augment the loss of phase coherence thus shortening T2. The magnitudes of the relaxation rates, (1/T1 and 1/T2), are proportional to the squares of the magnitudes of the interacting magnetic moments. Paramagnetic agents, with unpaired electrons, can greatly increase the relaxation rate since the magnetic moment of the electron is 657 times larger than the magnetic moment of the proton.

PARAMAGNETIC CONTRAST AGENTS

In the evaluation of pharmaceutical contrast agents for NMR imaging, several criteria must be considered. A useful pharmaceutical contrast agent should be: 1) chemically stable and easily stored in a form suitable for clinical administration; 2) readily available and inexpensive; 3) water soluble; 4) well tolerated in diagnostic doses and free from adverse physiological responses (high blood pressure, myocardial irritability, etc.); 5) quickly excreted; 6) highly paramagnetic, thereby altering tissue contrast with relatively low doses; and 7) chemically versatile so it can be bound to biological probes, if necessary, to permit selective tissue targeting. With these criteria as a guide, evaluation of the efficacy of the major classes of paramagnetic contrast agents, transition and lanthanide metal ions, and organic free radicals, for use in NMR imaging can be undertaken.

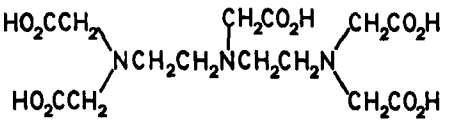
First row transition metal ions contain partially filled 3d electron shells, and are therefore potentially attractive contrast agents for NMR imaging. By virtue of Hund's rule (maximal spin angular momentum), the five degenerate 3d orbitals are filled such that each orbital must contain one unpaired electron before two electrons (spin paired) may occupy the same orbital. Since the magnitude of the T1 relaxation is, in part, dependent on the magnetic moment, the metal ions most favored are Mn^{2+}, Cr^{3+}, and Fe^{3+} which have 5, 3, and 5 unpaired electrons, respectively, in their 3d orbitals. At concentrations of 10^{-5} to 10^{-1} M, these metal ions significantly reduce solvent proton T1 relaxation values *in vitro*.[33,34,35] For example, a 10 mM solution of $Fe(NO_3)_3$ reduces T1 values from 3 sec to 10 msec. Lanthanide metal ions have partially filled 4f electron shells and, like the transition metals, may also be paramagnetic. Proton T1 relaxation measurements of several lanthanides, including Gd^{3+}, Dy^{3+}, Ho^{3+}, Eu^{3+}, have shown Gd^{3+} to be the most effective at enhancing proton relaxation.[34,36] The other lanthanides, Dy^{3+}, Ho^{3+}, Eu^{3+}, have very short

electron spin relaxation times and are therefore good NMR shift reagents, but poor spin relaxing agents.

A number of investigations in NMR imaging have employed "uncomplexed" metal ions as contrast media. Small phytate- and hydroxy-colliods of insoluble gadolinium were shown to be phagocytosed by the recticuloendothelial system and produced contrast enhancement of the liver and spleen.[37] Insoluble gadolinium oxalate has been administered orally and rectally to produce contrast enhancement of the stomach and large bowel, respectively.[38] Iron containing contrast agents have also been evaluated as oral and rectal pharmaceuticals for enhancement of the gastrointestinal tract. Oral administration of $FeCl_3 \cdot 6H_2O$ successfully produced gastric opacity in a single human volunteer; however this reagent can produce marked gastrointestinal irritation.[39] In more recent work, dietary iron supplements containing ferric ammonium sulfate and ferric ammonium citrate provided enhanced contrast of the alimentary canal in both animals and human volunteers.[40] Dilute solutions of ferric ammonium citrate have subsequently been evaluated in selected patients and shown good tolerance at effective doses.[41]

Following intravenous injection in mice, $MnCl_2$ was rapidly cleared from the blood and concentrated in the heart, liver, spleen, and kidney.[42-44] In vitro measurements revealed enhanced T1 relaxation of these tissues at doses as low as 8-16 mmoles/kg body weight. In vivo injections of $MnCl_2$ in doses of 0.05-0.1 mmoles/kg have been used in acute canine infarct models.[45-47] In vitro NMR images of the hearts revealed a significant decrease in normal myocardial tissue T1 relaxation relative to the poorly perfused ischemic or infarcted myocardial tissue. Without manganese ion, differences in image intensity between regions of normal, ischemic, or infarcted myocardium were not observed.

Unmodified metal ions remain relatively too toxic to be considered as parenteral NMR contrast agents for human use.[48,49] Metal ions are typically metabolized by the liver and display slow biological clearance. Below acutely toxic doses, metal ions often have adverse effects as well. For example, doses of 0.01 mmoles/kg $MnCl_2$ exhibit moderate cardiovascular toxicity which includes hypertension, heart rate changes, and electrocardiographic changes. At a dose of 0.2 mmoles/kg, ventricular fibrillation occurs.[50] Because of this inherent toxicity of free transition and lanthanide metal ions, chelated metal complexes have been explored as NMR contrast agents. A major concern for the intravenous use of metal chelates is the potential for in vivo dissociation of the complexes to afford toxic free metal ions. Therefore, preparation of metal complexes having a high stability constants is requisite. Stabilities of metal chelate complexes vary greatly, depending on the choice of both metal and chelating ligand.[51] To date, the polyaminocarboxylate ligands, ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) (1), have received wide attention due to their relatively high affinity for most metal ions.



1

To assess metal complex efficiency for proton relaxation enhancement, *in vitro* T1 relaxation measurements have been recorded for Cr-EDTA,[52,53] Fe(II)-EDTA,[54] Gd-EDTA,[54] and Gd-DTPA,[53] and for complexes between the metals Gd^{3+}, Mn^{2+}, Fe^{3+}, Cr^{3+}, Cu^{2+}, and V^{2+} and the ligands DTPA, desferrioxamine, and glucoheptonic acid.[34,37] The relaxation enhancement of these complexes varied according to the metal ion, the concentration, and the ligand. The metal complexes were less effective at reducing T1 than the corresponding hydrated ions, but the loss in efficacy was smaller than one order of magnitude. This reduction in T1 relaxation observed with the metal complexes, relative to hydrated ions, is a direct result of chelation which reduces the number of coordination (hydration) sites through which the metal interacts with the tissue water.[55,56]

In vivo studies with animals have shown that the metal-polycarboxylate complexes, injected intravenously, are rapidly excreted through the kidneys by glomerular filtration. Chromium-EDTA has an *in vivo* half-life of 51 min. and has provided contrast enhancement of the normally functioning kidney, ureter, and bladder at a dose of 0.075 mmoles/kg.[52] Similar results have been demonstrated for Fe(II)-EDTA[53] and Gd-EDTA.[54] The LD_{50} (I.V.) of Cr-EDTA in mice is 0.5 mmoles/kg.[53] Using Cr-EDTA as a contrast agent, renal hydronephrosis and renal ischemia were readily characterized by changes in normal T1 relaxation values.[52,53] Contrast enhancement of a human tumor model implanted in mice was also demonstrated with Cr-EDTA.[52]

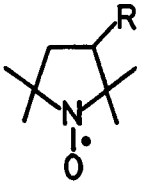
The most promising metal complex to date for NMR imaging is Gd-DTPA. Gadolinium has the largest magnetic moment (7 unpaired electrons) of all metal ions, and a sufficiently slow electron spin relaxation to be useful in NMR imaging. The gadolinium-DTPA complex has a high formation constant (pK 22-23),[51] and is stable *in vivo*. Additionally, gadolinium has a coordination number of 9, in contrast to 6 for most transition metals; thus the complexed metal is more effectively exposed to the bulk solvent than transition metal complexes. Reduction in proton relaxation times has been demonstrated at a dose of 0.01 mmoles/kg.[57] The half-life of Gd-DTPA *in vivo* is 20 min. with predominant renal excretion. The LD_{50} dose in rats is 10 mmoles/kg, much lower than the LD_{50} of 0.4 mmoles/kg for free gadolinium ion. Diagnostically relevant doses of 0.1-0.5 mmoles/kg, cause no demonstratable blood pressure or ECG changes.[58]

Gadolinium-DTPA has been used to provide contrast enhancement of normal kidney, and to assess renal and splenic infarctions in animals.[59] Additionally, contrast enhancement of sterile edematous inflammatory lesions in rat[59] and human tumors models in mice[60] has been demonstrated. Although metal chelates normally do not cross the blood brain barrier, accumulation of Gd-DTPA at focal disruptions of the blood brain barrier has been demonstrated.[59] From these studies it has been postulated that intravenously administered Gd-DTPA is distributed in the vascular and extracellular spaces and thus produces contrast enhancement in tissues having high interstitial fluid content. The distribution of Gd-DTPA in normal, infarcted, and ischemic myocardium has been determined as a function of time.[61,62] The rapid, early distribution of Gd-DTPA into normal myocardium results in significantly reduced T1 and T2 relaxation relative to poorly perfused ischemic or infarcted tissues. The contrast agent is, however, rapidly washed out of the normal tissues. For ischemic and infarcted tissues having impaired perfusion, the small amount of Gd-DTPA accumulated in these tissues is retained longer than for normal

myocardium. Thus contrast enhancement of infarcted and ischemic regions can be observed at latter imaging times, a reversal of the early contrast enhancement.

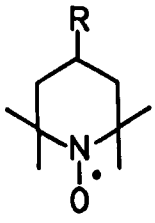
Development of contrast agents in NMR imaging has reached the point of clinical trials using Gd-DTPA in Europe and the USA. Cerebral tumors, hepatic tumors, and transitional cell carcinoma of the bladder have demonstrated contrast enhancement in humans using Gd-DTPA at a dose of 0.1 mmoles/kg.[63,64] In all but two of the reported 32 cases, NMR images obtained using Gd-DTPA afforded tumor contrast equal to or better than contrast produced by CT. No significant changes in electrolyte concentration, liver function, blood coagulation, or urine were observed in these studies, and no short term side effects were noted. The efficacy of Gd-DTPA to enhance contrast of a variety of other body tumors has been assessed as well.[65]

Nitroxide spin-labels (NSL) are stable free radicals which have one unpaired electron localized in a nitrogen-oxygen bond. For several decades, NSL have been used for in vitro proton relaxation enhancement in biological studies of protein conformation and dynamics.[66,67] These paramagnetic compounds are secondary amine N-oxides that generally bear no hydrogen atoms on the carbons adjacent to the nitroxide nitrogen. Incorporation of methyl groups at the alpha carbons renders the nitroxide stable toward disproportionation, a major decomposition pathway of radical centers. The two major classes of nitroxides tested for NMR imaging are the five-membered ring pyrrolidine nitroxides (2) and the six-membered ring piperidine nitroxides (3). These nitroxides may have additional functional groups (R) which can be used to alter solubility properties and provide a method for chemical modification. Nitroxides have prolonged stability at varying pH and temperature, reasonable availability and shelf life, chemical versatility for conjugation to biomolecules, long electron spin relaxation times in comparison to paramagnetic inorganic cations, and relatively low toxicity.



2

2a, R = CO_2H



3

3a, R = $NHCCH_2CH_2CO_2H$

In vitro spectroscopic measurements on a variety of nitroxides have demonstrated greater relaxation (per mole contrast agent) with increased nitroxide molecular weight, with six-membered ring piperidine nitroxides relative to five-membered ring pyrrolidine nitroxides, and with compounds containing two nitroxides per molecule.[68-70] Additionally, relaxation enhancement by nitroxides is more effective at low magnetic field strengths, and in plasma compared to water. Following intravenous injection, the nitroxides 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PCA)

(2a) and 4-[(3-carboxy-1-oxopropyl)amino]-2,2,6,6-tetramethyl-1-piperidinyloxy (TES) (3a) are rapidly excreted through the kidneys at the rate of glomerular filtration.[71] The *in vivo* half-lives of PCA and TES are approximately 38 minutes. The pharmacokinetics and metabolic fate of nitroxide free radicals *in vivo* have been thoroughly investigated.[72-74] Contrast enhancement of normally functional kidney has been demonstrated with TES and PCA at a dose of 1.8 mmoles/kg. The nitroxide TES has also been used to characterize experimentally induced hydronephrosis, renal artery and vein ligations, and sterile subcutaneous abscesses.[71] Additionally, contrast enhancement of pyrogenic cerebral abcesses and radiation cerebritis has been demonstrated.[75] The dynamics of perfusion in acutely infarcted canine myocardium has been studied using the nitroxide PCA.[76,77] PCA has also been used to improve tumor/soft tissue contrast in a nude rat model of human renal cell carcinoma.[78]

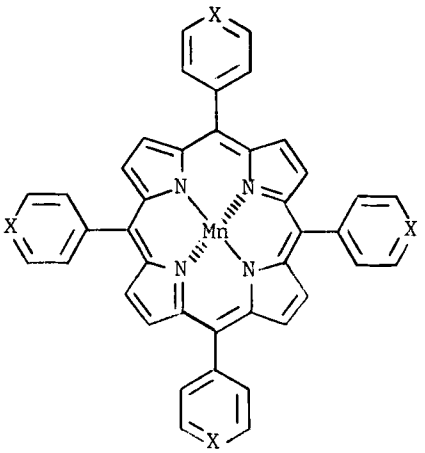
Nitroxide compounds have been shown to be susceptible to reduction *in vivo*, by both chemical (ascorbate) and enzymatic processes.[79,80] The resultant N-hydroxylamine compounds are diamagnetic and no longer contribute to proton relaxation enhancement. The propensity of the nitroxide to reduce *in vivo* is variable, to some extent, and depends on both the nitroxide and the tissue. The five-membered ring pyrrolidine structure is more resistant to reduction than the six-membered ring piperidine nitroxide, and cationic nitroxides are more easily reduced than anionic nitroxides. Reduction occurs most rapidly in the liver and to a lesser extent in the blood and kidneys. Development of nitroxide contrast agents less susceptible to reduction is currently being investigated.[70,72]

As a new class of pharmaceutical contrast media, nitroxides must be thoroughly evaluated for toxicity. The nitroxides PCA and TES have an LD_{50} of 15.5 mmoles/kg or greater in rats, a concentration 100 times greater than the least effective diagnostic dose of 0.15 mmoles/kg.[81] Toxicity tests on the nitroxide agents PCA and TES for possible carcinogenicity have been performed.[81] A sensitive measure of mutagenesis and chemical carcinogenisis is the sister-chromatid exchange test.[82] Using this method, no evidence of mutagenicity was observed for the nitroxides PCA and TES, or for their reduced metabolites, the N-hydroxylamine and the amine. In addition, metabolic activation failed to evoke any carcinogenic activity from PCA or TES.

Current research interests are directed toward the development of tissue or organ specific paramagnetic contrast agents. This goal has been addressed through several different approaches utilizing methodology presently employed in other pharmaceutical fields, particularly radiopharmaceuticals. One possibility currently under investigation is development of paramagnetic immunoimaging agents. A variety of monoclonal antibodies have become available; these immunoglobulins allow for the potential of specific tissue targeting with paramagnetic agents. Recent advances in the development of bifunctional chelate compounds have made possible the derivatization of proteins with chelating agents which can subsequently bind metal ions.[83-85] Bovine immunoglobulin has been derivatized with EDTA and DTPA by reaction of the protein with the cyclic anhydrides of EDTA and DTPA, respectively.[86] *In vitro* relaxation measurements with protein bound chelates, containing Mn^{2+} and Gd^{3+}, demonstrated a 5-10 fold improvement in the efficacy of T1 relaxation relative to similar concentrations of free metal chelates.

Immunoimaging agents have been used successfully to demonstrate in vivo contrast enhancement in animals. Antimyosin monoclonal Fab fragments, consisting of the antibody variable regions (mw = 50,000 daltons) obtained through proteolytic digestion with papain, were covalently conjugated to DTPA in a 2.5:1 (DTPA:protein) ratio and subsequently complexed to Mn^{2+}.[87] Experimentally induced canine myocardial infarction demonstrated shortened relaxation time and image enhancement using the Fab-DTPA-Mn contrast agent. In a similar experiment, monoclonal antibody was conjugated to DTPA and complexed to gadolinium.[88] This immunoimaging agent produced contrast enhancement of human Burkett's lymphoma in nude mice. The implications of conjugating metal chelates to proteins in terms of metal-chelate stability, biodistribution, and metabolism have yet to be addressed. Nevertheless, these preliminary works show promise for further development of immunoimaging agents.

Porphyrins are endogenous compounds capable of chelating transition metals. Imaging studies have shown metalloporphyrins to produce in vivo contrast enhancement. The manganese complex of protoporphyrin has produced enhanced relaxation of liver tissues.[89] Porphyrins also tend to accumulate in tumorous tissues and thereby provide a method to improve contrast of tumors. Manganese complexes of tetra(4-sulfonatophenyl)-porphyrin (TPPS4) (4), and tetra(N-methyl-4-pyridyl)porphyrin (TMPyP) (5), in 10 mg doses, have effected a 50% decrease in T1 relaxation of human colon carcinoma implanted in mice.[90]



4, X = C-SO_3H

5, X = $\overset{\oplus}{N}$-CH_3

An additional potential methodology for targeting specific tissues employs the liposome. Following intravascular administration, liposomes are rapidly removed from the blood by the reticuloendothelial system. Manganese-DTPA, entrapped in multilamellar vesicles smaller than 1.2 microns, was selectively accumulated in the liver and spleen, and effected significant relaxation enhancement of these two organs.[91]

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Chapter 29. Solid State Organic Chemistry and Drug Stability

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Introduction - Solid state organic chemistry in the pharmaceutical sense is an unusually broad area. It includes x-ray crystallography, mechanistic organic chemistry, physical pharmacy, drug stability, kinetics of solid state reactions, and formulation and dosage form design.[1] Solid state organic chemistry attempts to explain the rates of solid state reactions and the products of these reactions in terms of the chemical process and the crystal structure. The crystal structure and the restricted motion provided by the solid are, of course, unique aspects of solid state organic chemistry. The objectives of this review are to outline the basic principles of solid state organic chemistry and to illustrate how this knowledge has been brought to bear on pharmaceutical problems such as drug stability.

The Solid State

Crystals are ordered arrangements of molecules held together by hydrogen bonding or noncovalent attractive forces. Crystals of a compound may exist in habits, polymorphs or solvates. Crystal habits have different external shapes but the same internal structure, and they can either be solvated or nonsolvated. Crystal polymorphs have both a different external shape and a different internal structure, while crystal solvates have different external shapes and can either have the same or different internal structure. Crystal solvates in the strictest terms are different from polymorphs. However, from a chemical point of view, both polymorphs and solvates contain the same chemical entity and therefore in this respect are quite similar.

Amorphous solids are a second important class of solids. Amorphous solids have no long range internal order and, while solid in their appearance, they are in some respects similar to a solution in that the arrangement of molecules is more random. They are generally more reactive than their crystalline counterparts,[2] and thus processes which produce amorphous solids can often lead to an unstable product. Amorphous solids are also often quite hygroscopic and are sometimes produced by lyophilization.

Solid State Organic Chemistry

Solid state organic chemistry generally emphasizes the study of crystalline solids because the geometric arrangement of molecules in the reacting solid can be determined. It is this ability to determine the specific geometry of the reacting molecules that has stimulated much of the work in solid state organic chemistry. Many reactions have been thought to be solid state reactions but upon closer scrutiny have been found to occur in the liquid, in a melt, or in a liquid layer on the surface of the crystal. A reaction occurs in the solid when at least one of the following conditions is met:[3,4]

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(1) when the liquid reaction does not occur or is much slower than the corresponding solid reaction;

(2) when there are pronounced differences in the reactivity of chemically related compounds in the solid;

(3) when different reaction products are formed in the solid and liquid states;

(4) when different crystalline modifications (polymorphs or solvates) have different reactivity or lead to different reaction products;

(5) if it occurs at a temperature below the eutectic of a mixture of the starting material and the products.

The general steps in a solid state reaction are different from those of a solution reaction.[4] The first step in a solid state reaction is molecular loosening. Solid state reactions begin at one or more nucleation sites in the solid and spread through the crystal. Nucleation sites are developed either during the crystallization process or can be produced by mechanical deformations. At these sites, the molecules that are going to react obtain enough freedom of motion to undergo the second step, the molecular change step. This step is similar to the solution reaction in that covalent bonds are broken and made; it is usually slower than the corresponding solution reaction because of the restricted molecular motion in the solid. However, in some cases solid state reactions can be accelerated due to favorable orientation.[5] The third step involves the formation of a solid solution of the product crystal in the reactant cyrstal. This solution exists until in the final step the product is no longer soluble in the reactant crystal and the product crystal separates. These latter two steps are of lesser significance because the first two steps control the chemical process.

An important aspect of the reactivity of crystals is summarized in the topochemical postulate, which states that reactions in the solid state proceed with a minimum of molecular motion.[6-8] This postulate assumes that there is a reaction cavity or defined area in which solid state reactions can occur, and that this cavity is much more restrictive with respect to molecular motion than the corresponding reaction cavity in solution. This postulate explains a number of solid state photochemical reactions in which the structure of the product is related to the structure of the reactants in the crystal.[6-9]

There are a number of equations for treating solid state reactions in terms of nucleation processes and the progress of fronts through crystals.[10-12] These equations are quite important in stability prediction; however the interpretation of kinetics of solid state reactions is still in its infancy, and much more needs to be understood about the nucleation process and the physical factors influencing solid state reactions.

Solid State Organic Reactions

The known solid state organic reactions can be classified as either physical transformations or chemical reactions.

Physical Transformations

Physical transformations are solid state reactions in which no new covalent bonds are formed or broken, but instead the nature and structure of their crystal is altered. There are two types of physical transformations: polymorphic transformations and desolvations.

Polymorphic Transformations - In polymorphic transformations the crystal form and internal structure of the crystal changes. These transformations are important because different polymorphs often have different stabilities and different bioavailabilities. In general, these reactions involve only three steps; the molecular change step does not occur because no reaction is involved. The literature is replete with examples of polymorphic transformations. For example, tulobuterol hydrochloride, a phenethylamine derivative and bronchodilator, exists in three polymorphs and a pseudopolymorph (hydrate).[13] No interconversions of the four forms were found when they were ground in a mortar or compressed; however, polymorph I was converted to polymorph II upon heating.[14]

Desolvation Reactions - A number of pharmaceuticals crystallize with solvent of crystallization. When the solvent of crystallization is lost, a solid state reaction of the type solid to solid + gas occurs. Such reactions involve no breakage or formation of covalent bonds; however, they involve the breaking of the bonds which hold the solvent in the crystal. During this reaction the crystal structure usually changes; however, there are a small number of transformations called pseudopolymorphic transformations in which desolvation does not result in the change of crystal structure. This has resulted in the description of two types of solvates: polymorphic solvates and pseudopolymorphic solvates.[15] For polymorphic solvates the crystal structure changes upon desolvation. An example of a polymorphic desolvation is shown in Figure 1.

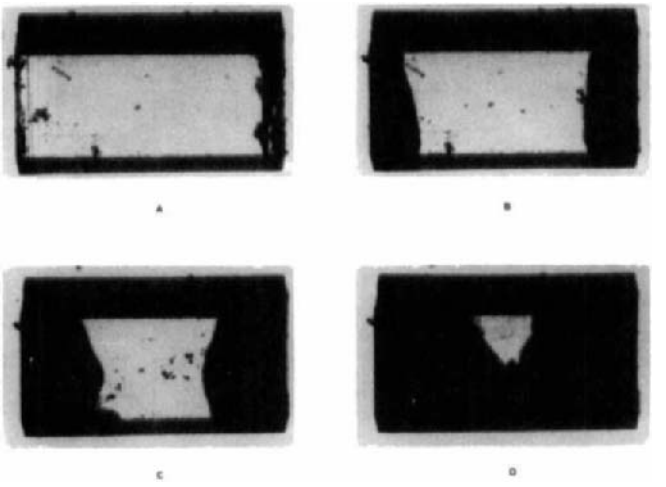


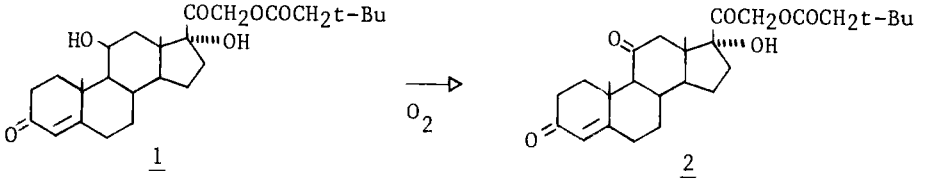
Figure 1. The desolvation of cytosine hydrate in air at 60 degrees: (a) at start; (b) after 8 min.; (c) after 15 min.; and (d) after 24 min.

In this reaction cytosine hydrate loses solvent of crystallization and transforms to the anhydrous form. The loss of solvent of crystallization occurs in a front as shown in the figure. This front can be explained in terms of the crystal packing of cytosine hydrate in which there are solvent tunnels running parallel to the needle axis of the crystal. As the solvent exits the crystal, a front moves from the ends of the crystal towards the center.[16]

In pseudopolymorphic transformations, the solvent simply exits the crystal leaving a crystal of the same structure. Thus in this reaction only two steps are involved, molecular loosening and breaking of the host-solvent bonds. Examples of pseudopolymorphic desolvations include the loss of ethanol from the hexagonal crystal form of hydrocortisone tert-butylacetate,[18] and the interconversions of the solvated crystal forms of cephalosporin antibiotics.[15]

Chemical Reactions

Solid-Gas Reactions - The most common solid-gas reactions involve atmospheric oxygen. Solid-gas reactions which are well known include oxidations of dihydrophenylalanine,[17] dialuric acid,[19] vitamin C,[21] vitamin D_2,[20] reserpine,[22] and phorbol esters.[23] One of the best examples of the solid-gas reaction is the oxidation of the above mentioned hydrocortisone tert-butylacetate (1) to cortisone tert-butylacetate (2).[46,47] This reaction occurs with the hexagonal crystal form, but does not occur with other crystal forms, including



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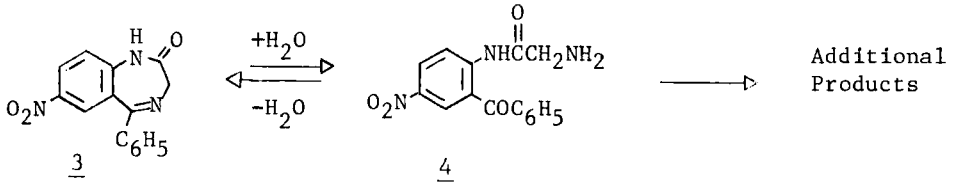
the monoclinic and orthorhombic crystal forms. The hexagonal crystals undergo a pseudopolymorphic desolvation as mentioned above, leaving a "tunnel" by which oxygen can penetrate the crystal.

A second type of solid state reaction involving the addition of gases to solids is best exemplified by solid state hydrolyses. Since these reactions involve water and reactions in thin layers or pools of water are known, it is often difficult to completely verify that they are true solid state reactions. However, the slow hydrolysis of aspirin to salicylic acid is probably a good example of this type of reaction,[24,25] as is the ring opening of nitrazepam (3) via the intermediate (4).[26] The rate of this reaction was found to be dependent on both temperature and relative humidity.



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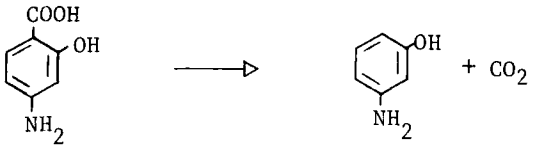
Solid State Decompositions of a Solid to a Solid + Gas - These reactions are exemplified by the dehydration of tetracyclines,[27-29] dehydrochlorination reactions of hydrochloride salts,[30] and decarboxylation reactions,[31-33] such as the decomposition of the antituberculosis drug p-aminosalicylic acid (5) to form m-aminophenol (6).[30-32]



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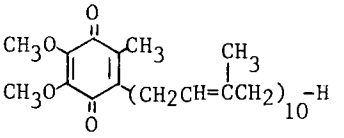
Solid State Photochemical Reactions - Solid state photochemical reactions are relatively common for pharmaceuticals.[34-36] However, because of the ability to prevent these by storing in amber bottles, few of these reactions have been carefully studied. Recently, the photostability of ubidecarenone (7), a benzoquinone derivative used to treat angina, has been studied.[37]



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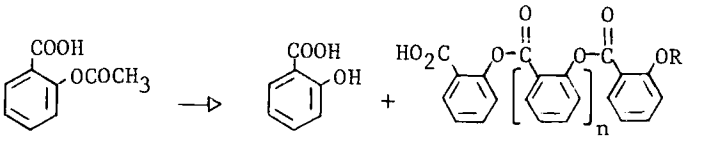
Solid State Thermal Reactions - Solid state thermal reactions in which some type of rearrangement occurs are also known for pharmaceuticals.[38-42] The best example of these is possibly the solid state thermal reaction of aspirin anhydride.[41] In this reaction several acyl migrations occur to form new acyl products. Another example of a solid state thermal reaction is the decomposition of aspirin (8) to form salicylic acid, oligomeric salicylate esters (9), and their acetate derivatives.[43]



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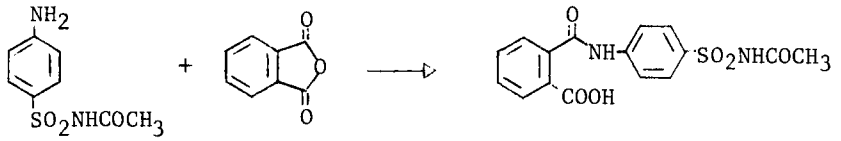
Solid-Solid Reactions - Finally it is important to point out that solid-solid reactions can occur. These reactions are, of course, the basis for physical incompatability of solids. An example of this type of reaction is the neutralization of the hydrochloride salt of p-aminosalicylic acid with sodium carbonate, a solid state acid base reaction.[30] Codeine phosphate in the presence of aspirin undergoes a solid state acylation reaction to form acetylcodeine phosphate.[44] This observation along with other data indicate that aspirin may be able to acylate other drugs via solid-solid reactions.

Solid-solid reactions of sulfa drugs and phthalic anhydride have been observed. For example, sulfacetamide (10) and phthalic anhydride (11) react to form phthalylsulfacetamide (12). The reaction rate increased with increasing temperature and compaction pressure up to a point. However, further increases in compaction pressure resulted in a decrease in the rate of reaction possibly due to decreased surface area and/or rate of migration.[45]



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Methods for the Study of Solid State Organic Chemistry

A variety of methods are available for the study of solid state organic chemistry and the solid state chemistry of drugs. These include x-ray crystallography, light microscopy, thermal gravimetric analysis, differential thermal analysis and infrared spectroscopy among others. The use of these techniques has been reviewed.[1]

Solid state nmr spectroscopy is a powerful new method which has the potential to allow major new developments in the field of solid state organic chemistry.[48,49] Solid state nmr spectroscopy is not an averaging technique like x-ray diffraction and thus offers the potential for analysis of mixtures of crystalline or amorphous forms. In addition this technique can be used to examine conformational and dynamic changes which occur in solid drugs during solid state transformations and reactions.

Classical carbon-13 nmr spectra of solids obtained by FT techniques are characterized by broad featureless lines that obscure detailed chemical shift information. However, a new experimental technique called CP/MAS (cross polarization/magic angle spinning) can overcome this problem.[50] This technique involves using a cross-polarization pulse sequence on a sample which is spun at the magic angle. This technique allows the observation of carbon-13 solid state spectra with line widths of a few hertz. Atalla et al.[51] have used this technique to show that the different polymorphs of cellulose have different solid state nmr spectra and other work shows that polymorphs of drugs show different solid state carbon-13 nmr spectra.[52] Shiau et al. have used solid state nmr to show that solid naphthazarine B exists in the hydroquinone-quinone tautomer in the solid state at both 25° and -160°.[53]

Other potential applications for solid state nmr spectroscopy include the study of amorphous forms, solid state reactions and drug-polymer interactions.[52]

Future Directions

The future directions of the field of solid state organic chemistry and drug stability mostly involve the careful investigation of other solid state reactions in terms of the molecular details of the reaction and the principles outlined in this review. It is interesting to note that attempts to place the fields of biology and chemistry on a molecular basis have led to rapid developments in these fields. It is expected that a similar rapid development of the field of solid state organic chemistry can also occur, especially with the application of useful new techniques such as solid state nmr. It is anticipated that such studies will put the investigation of drug stability on a more rational basis and allow rapid, efficient development of more stable pharmaceuticals.

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Chapter 30. Altered Drug Action in the Elderly

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Introduction - Thirty-one percent of all drugs are prescribed for the elderly (11.7% of the population), two-thirds of whom also use non-prescription drugs and home remedies. On the average, elderly receive 1.5 prescriptions per office visit. However, one-third of elderly with mental complaints receive four or more, while in nursing homes, many of the residents receive eight or more drugs daily.[1-3]

The high usage of drugs in the elderly is accompanied by a disproportionately high rate of adverse drug reactions. The Royal College of Physicians estimates that 20-25% of all elderly admitted to acute care hospitals are admitted because of adverse drug reactions.[4-6] Comparable United States data are estimated at 12-17%, three to four times the adult rate. Other data indicate that 30% of all elderly admitted to acute care hospitals from nursing homes suffer from adverse drug reactions, and 40% of community living elderly are probably affected by adverse drug reactions. The cost of these undesirable reactions has been estimated at $3 billion annually.[7]

What are the reasons for adverse drug reactions? Most studies to date are cross-sectional rather than longitudinal studies.[7] Thus, more is known about age differences in drug action than changes of drug action with age. But it is clear that reasons for altered drug action, and perhaps for the increased incidence of adverse drug reactions with age, are multifactorial. Physiologic changes with age interact with pathophysiologic changes and sociogenic changes, resulting in altered and more unpredictable drug action in the aged. The physiologic scatter in response to drugs exhibited by older adults is much wider than in younger adults.[8]

Genetic factors certainly play a role.[9] While inherited traits are not peculiar to the elderly, older adults may lack sufficient reserve capacity and, therefore, the influence of these factors on drug action may be more pronounced. Furthermore, several well-established or suspected dietary factors interact and may influence drug response in man.[10,11] Among these are the presence in foods of agents with pharmacologic action, the excessive presence or absence of specific nutrients or trace metals, and a direct or indirect effect of drugs on a person's nutritional status. Many patient-related factors, such as anemia, cardiac failure, degenerative vascular disease, the patient's state of hydration, and others will also affect drug action. It is, therefore, probably most effective to explain altered drug action with age by using the pharmacodynamic and pharmacokinetic hypotheses of altered drug action with age.

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Pharmacodynamics

Of major interest in the management of the chronic diseases of elderly with drugs are the physiologic, psychologic, and possible toxicologic responses, i.e., pharmacodynamics. It is still difficult to differentiate between normal aging effects and pathophysiologic effects and their influence on drug pharmacodynamics. Advancing age heightens the interplay of the aging process and chronic degenerative diseases,[12] and perhaps the most serious physiologic change with age is the body's decreased ability to maintain homeostasis.[13,14] Older people are less able to regulate blood glucose levels, blood pH, pulse rate, blood pressure and oxygen consumption, but to varying degrees. These variations preclude clinically useful generalizations.[15] Importantly, some variables change with age, but others, such as the hematocrit, do not. Thus, the influence of aging on clinically relevant variables is not clear in many instances, nor is the possible effect of normative changes.

It has been known for some time that elderly are more "sensitive" to the therapeutic and toxic actions of some drugs,[16] such as the barbiturates,[16] methyldopa,[17] benzodiazepines,[18-20] warfarin,[20,21] heparin,[22] and morphine.[23] Drugs may induce changes themselves, for example changes in the cholesterol/phospholipid ratio.[24,25] An increase in that ratio in erythrocytes decreases the furosemide-sensitive cotransport of sodium and potassium, while a decrease in cholesterol increases ouabain-sensitive transport of sodium.[24] In general altered drug action with age can be ascribed to changes in the brain and central nervous system, the cardiovascular system, receptors, and endocrine changes.[26-30]

With age, one would expect a decreased cellular brain mass sensory conduction time, decreased cerebral blood flow and, possibly, increased permeability of the blood-brain barrier.[26] These changes may result in decreased coordination, prolongation of reaction time and impairment of short-term memory. These effects manifest themselves as increased frequency of confusion, increased number of falls, particularly among elderly women, and an increased frequency of urinary incontinence. There is also a decline in the homeostatic response. All of these can be exaggerated by drug therapy, particularly if drugs are used in the usual dose or if multiple drugs are used. The antidepressants, antihistamines, digitalis, levodopa, and the phenothiazines cause an increased incidence of confusion, delirium and/or disorientation in the elderly.[1]

Changes in the cardiovascular system involve changes in blood vessel consistency,[31] leading to increased arterial blood pressure and changes in the functioning of the baroreceptors. Major electrical, mechanical, and biochemical properties change with age. The overall result of these changes is a decreased response of the heart to stress and catecholamines and an increase in sensitivity to the toxic effects of drugs such as digoxin. However, the inotropic effect of digitalis decreases with age.[1] Baroreceptors, located in the carotid sinus and aortic arch, function by recognizing changes in arterial pressure. Arterial baroreceptor reflexes respond to changes in blood pressure by changes in sympathetic and parasympathetic outflow,[32] decreasing arterial pressure via vasodilation and a decrease in both the rate and force of cardiac contractility. Altered action

of antihypertensives in the elderly would involve changes in the sympathetic and parasympathetic pathways, as well as the afferent and central connections of the baroreceptor reflex arc, the hypothalamic neural afferent pathways, and hypothalamo-pituitary effector systems.[32] Baroreceptor sensitivity is significantly and progressively decreased with age, regardless of whether the patient is hypertensive or normotensive. This decrease is responsible for the high incidence of drug-induced orthostatic hypotension.[33] The risk of orthostatic hypotension is increased in elderly with volume depletion due to salt and/or water depletion, or circulatory changes due to infections or fever.[34]

Virtually all drugs bind to receptors, and thus, their action is initiated.[35] Perhaps age-related altered drug action is also related to altered receptor-drug interactions. The literature, though, is sometimes difficult to evaluate, since reports often do not differentiate between studies of binding sites and true receptors. It has been postulated that a given receptor site/drug concentration produces a greater pharmacologic effect in elderly than in younger persons. Generalizations, however, cannot be drawn. One reason for this is that the number of receptors is not fixed, but is regulated by a number of factors, including disease states and drugs.[35,36] Adrenergic agonists and antagonists, glucocorticoids, thyroid hormones, estrogens and progesterone, ischemia, hypertension, heart failure, cardiac hypertrophy, as well as aging can all influence the number of cell receptors.[35] Certain receptors or their responsiveness change with age, while others, such as the alpha-adrenoceptors do not.[37-38] Therefore, the antihypertensive action of clonidine and methyldopa, which is thought to be mediated through brain alpha-adrenoceptors, is not affected by aging.[39] There may be receptor changes with age in certain parts of the body, but not in others. The CNS cholinergic receptors decline in the basal ganglia, but probably not in other brain regions. Receptors also can become either supersensitive or desensitized in adaptation to drug therapy or its withdrawal.[40-42] For example, alterations in insulin receptors account for some forms of insulin resistance[43] and the apparent inability of some of the nitroglycerin patches to provide therapeutic action for 24 hrs. may be related to receptor changes.

Age-related changes have been documented for brain benzodiazepine receptors[44] and for several hormone receptors.[45] Most studies, however, have concentrated on the beta-adrenergic, the cholinergic, and the dopamine receptors. Elderly patients are less responsive to beta-adrenergic agonists and more responsive to beta-adrenergic antagonists.[46] The decreased responsiveness to beta-adrenergic bronchodilators is clinically significant. Until recently, it was thought that age differences in beta-adrenergic responsiveness were due to alterations in receptor number and affinity,[47,48] a theory which was used to explain that plasma norepinephrine levels correlate positively with age,[49] and that the resistance of the heart to the chronotropic response of isoproterenol correlates positively with age.[50] The dose of isoproterenol needed to increase the heart rate by 25 beats per minute is four to six times higher in older than in younger persons.[47] Newer studies indicate that neither beta-receptor density nor affinity change with age, but that the altered action of beta-adrenergic blockers observed with age may well be due to lower levels of cyclic AMP and reduced adenylate cyclase activity.[51,52]

The pharmacologic effects of cholinergic agonists increase with age, but the effects of parasympathetic antagonists (e.g. atropine) produce smaller increases in the heart rate of elderly people. The beneficial effects of anticholinergics are decreased, while the side effects are more hazardous to the elderly. Both the changes in the beta-adrenergic receptors and the cholinergic receptors lead to changes in the sympathetic and parasympathetic control of the heart and changes in response to autonomic drugs. With age, there is a progressive depletion of dopamine in the extrapyramidal centers of the brain, increasing the risk of drug-induced Parkinson's disease. Age-related changes in the endocrine system have been documented[53] and specific age-related disturbances in the extrahepatic hormonal regulatory mechanism have also been proposed.[54] As a result, one would expect a modification in the control of circulating levels of insulin, glucagon, corticosteroids, and thyroid hormones. The reduced availability of hormones results in diminished endocrine regulatory mechanisms with age, as well as deficiencies in hormonal feedback control mechanisms. It also leads to decreasing binding affinities and receptor numbers with age.[41]

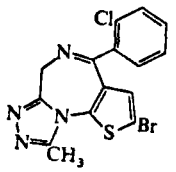
Alterations in pancreatic and adrenal hormone levels result in decreased glucose tolerance with age and an increased susceptibility of elderly to drug-induced hypoglycemia.[26] Some elderly suffer from a decreased release of insulin, while others have a decreased number insulin receptors and/or post-receptor abnormalities.[55] There is also a decreased production of sex hormones.[26] In females, reduced estrogen levels have been correlated with a greater incidence of osteoporosis.[26] Women are also more susceptible to orthostatic hypotension because of these hormonal changes.[26] Decreased thyroid hormone levels make elderly people less sensitive to the effects of sympathetic nervous stimulation and activation of beta-adrenerigc receptors by catecholamines and other drugs (phenylephrine, ephedrine) which stimulate these receptors.[56] On the other hand, decreased thyroid levels make elderly people more sensitive to the action of digitalis and increase the risk to drug-induced hypothermia.[57]

Pharmacokinetics

Many comparative pharmacokinetic studies fail to isolate age as a variable and as a result, differences in phamacokinetics apparently due to age are often confounded by differences due to environmental, disease or drug factors between the study groups. The most important age-related changes in pharmacokinetics are those associated with metabolism and excretion, since these processes markedly influence the accumulation and pharmacological effects of drugs. Changes in absorption and distribution are generally of lesser clinical importance. There have been many excellent general reviews of the effects of age on pharmacokinetic processes.[58-64] Several overviews of age effects on the pharmacokinetics of specific drug classes, including benzodiazepines,[65-67] psychotherapeutic agents[68,69] and beta-blockers[70] have been published. Pharmacokinetic aspects of drug-drug interactions in the elderly have also been reviewed.[71] A recent review examined the interrelationships between age, smoking and pharmacokinetic changes.[72] Smoking is an important determinant of pharmacokinetics of theophylline in the elderly,[73] but age itself apparently is more important than smoking in the case of beta-blockers.[74]

Absorption - A recent review of theoretical aspects of drug absorption from the intestinal tract as it relates to the aging process has been published.[75] With advancing age there are functional and morphological changes in the intestinal tract which may affect the rate and extent of absorption (i.e., bioavailability). Mechanisms of age-related changes in absorption have been studied using D-xylose as a marker.[76] With advancing age intestinal absorption of D-xylose slows but because of a longer residence time in the gut, absorption is as complete as in the young. The use of D-xylose as a test of gastrointestinal absorption in the elderly has been reviewed and in some ways criticized.[77] For many drugs aging has little effect on absorption, but for some drugs there are age-related changes in the extent of bioavailability.[56]

Age-related changes in bioavailability are most likely to occur with drugs that have a first pass effect, i.e., drugs which are partially metabolized in the liver before reaching the systemic circulation. Propranalol was reported to have higher bioavailability in elderly because of a reduced first pass effect,[78] but the bioavailability of metoprolol was 39% in the elderly and 55% in the young,[79] and the rate of metoprolol absorption was slower in the elderly.[80] No changes in absorption were observed for oxprenolol.[81,82] Absorption kinetics of some psychotherapeutic agents widely used in the elderly have been assessed. No significant changes were found for amitriptyline,[83] alprazolam,[84] azapropazone,[85] brotizolam (1),[86]



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clobazam,[87] mianserin,[88] trazodone[89] or triazolam.[90] No significant changes in absorption were found for antimicrobial agents (pipemidic acid,[91] rifampicin,[92] sulfisoxazole,[93]) analgesics-antiiflammatory agents (acetaminophen,[94] ketoprofen,[95] salicylates[96]) or other drugs acting on the central nervous system (caffeine,[97] diphenhydramine,[98] theophylline,[99] valproic acid[100]).

Distribution - The accumulation and extent of distribution of drugs in the body are influenced mainly by regional blood flow, body composition and plasma protein binding. Changes with advancing age in the latter two factors may alter drug pharmacokinetics in the elderly in comparison to younger control groups. The extent of distibution is described by the pharmacokinetic parameter known as the volume of distribution (V_d) and plasma protein binding is described by the parameter, unbound fraction (f_u). Often V_d is directly proportional to f_u. The percentage of body weight represented by adipose tissue is generally higher in the elderly and the V_d of lipid-soluble drugs is therefore greater. Conversely V_d for water-soluble drugs is often less in the elderly. The major binding proteins for drugs in plasma are albumin and alpha-1-acid glycoprotein (AAG). Weakly acidic drugs bind to the former and weakly basic drugs bind to the latter protein. In the elderly there are usually age-related decreases in plasma albumin concentrations and increases in AAG.

Increases in V_d considered to be significant have been reported for drugs from divergent therapeutic classes: amikacin,[101] amitriptyline,[83] cephalothin,[101] cefoxitin,[102] clobazam,[87] salicyate,[103]

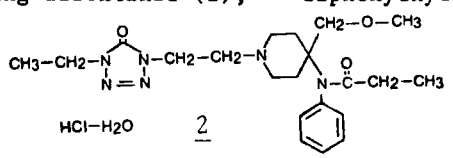
theophylline[99] and thiopental.[104] These increases in V_d are not unequivocal in that in other studies no changes in the V_d of salicylate[96] and theophylline[105] were found. Conflicting pharmacokinetic data usually reflect differences in the patient populations studied. In some cases increases in V_d with constant plasma clearance result in increases in elimination half-lifes ($t_{1/2}$) of drugs in the elderly, such as in the case of amitriptyline.[83] Decreases in V_d are significant in calculating appropriately reduced doses for single dose or chronic dose administration. Not many studies reported a reduction of V_d for drugs in the elderly. Drugs with lower V_d are caffeine,[97] lorazepam,[106] metronidazole[107] and morphine.[108] No age-related changes in V_d have been found for alfentanil,[109] azapropazone,[85] brotizolam,[86] diphenhydramine,[98] furosemide,[110] ketoprofen,[95] lidocaine,[111] metoprolol,[79] or valproic acid.[100]

Changes in plasma protein binding in the elderly are difficult to predict *a priori* because the presence of diseases and/or drugs modulate the age-related changes in protein binding. Significant increases have been reported for diazepam[112] and valproic acid,[100] while no age-related differences in protein binding were found for brotizolam,[86] furosemide,[110] haloperidol[113] or salicylate.[96]

Metabolism - On theoretical grounds the elderly should have reduced rates of metabolism of drugs because of age-related physiological changes such as reduced liver weight/body weight ratio, reduced microsomal enzyme activity and decreased hepatic blood flow. However, changes in pharmacokinetics due to age are unpredictable because of the large variety of factors other than age which can influence drug metabolism. Among these factors are smoking habit, alcohol intake, previous drug exposure, disease states, gender and diet. There have been only a few studies of age-related differences in the magnitude of drug-drug metabolic interactions. Two studies reported that the effects of cimetidine on antipyrine metabolism were similar in young and elderly volunteers.[114,115] Pharmacokinetic measures of drug elimination, which includes metabolism and renal excretion, are the elimination half-life ($t_{1/2}$) and clearance. Changes in CL reflect differences in intrinsic metabolic capability, while changes in $t_{1/2}$ may reflect changes in CL or V_d since these parameters are interrelated through the equation, $t_{1/2} = (\ln 2 \times V_d)/CL$.

For many benzodiazepines there are age-related changes in their metabolism if the primary pathway of metabolism is oxidation (phase I metabolism). Conjugation pathways (phase II metabolism) are not affected by advancing age. Brotizolam,[86] triazolam,[90] clobazam,[87] and alprazolam[84] all have reduced clearances in the elderly. For the latter two drugs, gender is important because reduced clearances were observed only in elderly men.

Age-related decreases in metabolic capacity have been reported for antidepressants (amitriptyline,[83] imipramine,[116] maprotiline[116] and mianserin[88]) and many other individual therapeutic agents including alfentanil (2),[109] diphenylhydantoin,[117] ketoprofen,[95]



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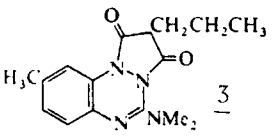
lidocaine,[111] metronidazole,[107] morphine,[108] propranalol,[78] ranitidine[118] and spironolactone.[119] In con-

trast, other investigators were unable to find decreases in metabolism with respect to age for acetaminophen,[94] caffeine,[97] diphenhydramine,[98] metoprolol,[79,80] rifampicin,[92] salicylate,[96] theophylline[105,73] and thiopental.[104]

There have been very few studies of metabolite kinetics in the elderly. One study reported that the clearance of desmethyldiazepam, a major active metabolite of diazepam, was reduced in elderly men but not elderly women.[120] For some drugs decreases in metabolism in the elderly are not detectable when total (bound and unbound) serum drug concentrations are evaluated, but they become evident when free (unbound) drug concentrations are measured. Such was the case for the anticonvulsant, valproic acid.[121]

Excretion - There is an average 35% decline in kidney function in the elderly as measured by creatinine clearance which reflects the functional state of the kidney. Decreases in renal clearances of drugs excreted principally unchanged by the kidney often correlate well with creatinine clearance. Elderly with normal creatinine clearances typically have no change in the pharmacokinetics of drugs excreted unchanged. No age-related changes in renal excretion were observed in elderly with normal renal function for azapropazone, (3)[85]



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furosemide,[122] pipemidic acid[91] or sulfisoxazole.[93] In elderly with renal insufficiency, creatinine clearances correlated with the reduced clearances of amikacin,[101] benoxaprofen,[123] cephalothin,[101] metronidazole[107] and rifampicin.[92]

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Chapter 31. Strategies for Delivery of Drugs Through the Blood-Brain Barrier

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Introduction - The vertebrate brain, unlike peripheral organs, is endowed with capillaries having unique morphologic characteristics that make up the blood-brain barrier (BBB). Due to the presence of the BBB, which acts as a system-wide cellular membrane separating blood and brain interstitial space, hydrophilic drugs that readily gain access to other tissues are barred from entry into brain. It is likely that many potential new drugs with unique properties in in vitro screening systems are ineffective in vivo due to the negligible transport through the BBB. Because of the traditional view that the BBB plays a more or less passive role in brain function, many investigators subscribe to the notion that the problem of the BBB cannot be circumvented, and thus, potentially useful agents that have poor distribution in brain are not studied further. This chapter will review some of the recent approaches that have been used to circumvent the BBB, with the view that an understanding of the basic physiology of BBB transport processes can lead to yet further improvement of these strategies.

Blood-Brain Barrier versus Blood-CSF Barrier - There are two barrier systems in brain, the BBB and the blood-CSF barrier. Because the BBB has a surface area that is 5,000-fold greater than that of the blood-CSF barrier, the BBB constitutes the principal diffusion barrier separating brain interstitial space and blood (Figure 1).[1] The unique morphologic characteristics of the brain capillaries which make up the BBB are: (a) epithelial-like high resistance tight junctions which literally cement all endothelia of brain capillaries together, and (b) scanty pinocytosis or transendothelial channels, which are abundant in endothelia of peripheral organs.[2] Owing to the presence of the BBB, circulating substances may gain access to brain interstitial space only via one of four possible mechanisms: (a) lipid-mediation, which allows for the free diffusion of lipid-soluble substances such as the steroid hormones through the BBB;[3] (b) carrier-mediation, which allows for the transport of circulating water-soluble nutrients through the BBB via the action of nutrient-specific carrier systems localized in the lumenal and antilumenal membranes of brain capillaries;[4] (c) pore-mediation, which allows for the transport of water through water-specific pores in the BBB;[5] and (d) receptor-mediated transcytosis of circulating peptides via peptide-specific receptor systems localized on both the lumenal and antilumenal membranes of brain capillaries.[6]

About a half-dozen tiny areas surrounding the ventricular system in brain make up the circumventricular organs (CVOs), which constitute the blood-CSF barrier.[7] These areas include the median eminence at the base of the third ventricle, the subfornical organ at the roof of the third ventricle, the organ vasculosum of the lamina terminalis (OVLT) near the preoptic area of the hypothalamus, the area postrema at the base of the fourth ventricle, and the choroid plexus. The CVOs are perfused by porous capillaries with active pinocytosis, and thus act as

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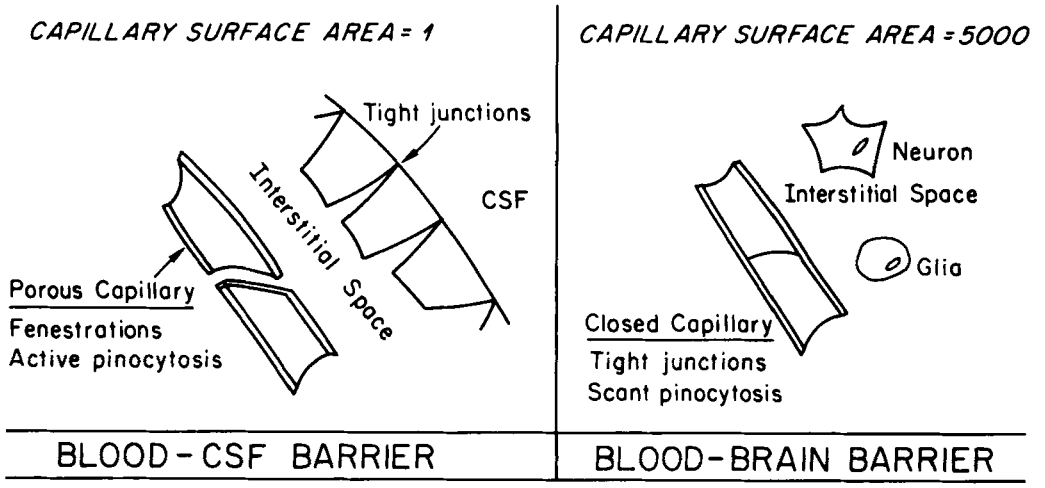


Figure 1. Blood-cerebrospinal fluid (CSF) barrier and blood-brain barrier (BBB) comprise the two major membrane systems segregating brain extracellular space from systemic extracellular space.

tiny windows of the brain where circulating substances readily distribute into the interstitial space in these regions.[2] The CVOs are adjacent to the ventricles, and the ependymal cells lining the ventricular surface at the CVO are characterized by tight junctions of moderate resistance that are believed to prevent the diffusion of substances from the brain interstitial space of the CVOs to the ventricular fluid. However, substances taken up by the CVOs may distribute laterally into adjacent brain areas. About half of the cerebrospinal fluid made per day arises from the CVOs, and the other half arises from the endothelia comprising the BBB. Thus, there appears to be a slow bulk flow of interstitial fluid through brain in the direction of capillaries to CSF to the CSF outflow tracts at the superior sagittal sinus.[8]

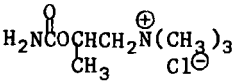
The following sections of this review will discuss three basic strategies for the delivery of drugs to the CNS: (a) invasive procedures, (b) pharmacologic-based strategies, and (c) physiologic-based (transport-directed) strategies (Table I).

Table I. Strategies for Pharmaceutical Delivery Through the BBB

Category	Strategy
Invasive	Intraventricular catheter Carotid injection of hypertonic media Transplant peripheral nervous tissue (artificial CVO)
Pharmacologic	Drug latentiation (prodrugs) Liposomes
Physiologic (transport-directed)	Neutral amino acid drugs Chimeric peptides

Invasive Procedures - Paradoxically, the strategies for selective drug delivery to the CNS that have been used most widely to date are procedures that are invasive and potentially harmful to patients. Such procedures are clearly of lesser direct interest to medicinal chemists. They do, however, serve to underscore the magnitude of the problem of drug delivery to the brain in terms of the heroic methods employed.

The most common procedure is the implantation of a catheter into the ventricular system for the delivery of drugs directly to brain, bypassing the BBB.[9] This procedure is particularly suited for the treatment of brain diseases which have a predilection for the meninges, e.g., leukemic involvement of brain,[10] because administration of drugs directly into the ventricular system allows for distribution of the drug only to the superficial areas of brain tissues, and not to the structures deep within the brain.[11] This is because the rate of absorption of CSF at the arachnoid villi of the superior sagittal sinus is much faster than the rate of diffusion of drugs from the ventricles into brain substance.[12] The intraventricular infusion of quaternary ammonium cholinergic drugs that do not cross the BBB, e.g., bethanechol (1), has been attempted recently for the treatment of Alzheimer's



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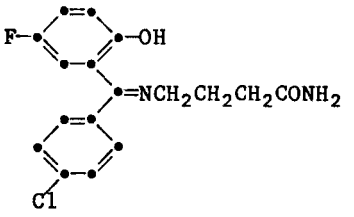
disease.[13] One application of intrathecal drug administration that has been proven to be efficacious is the epidural infusion of morphine sulfate for the treatment of pain in patients with cancer.[14,15]

Another invasive procedure that has been used on an experimental basis is the intracarotid infusion of hypertonic solutions of mannitol and other substances which cross the BBB poorly, and thus are osmotically active. The infusion of 1.5-2.0 M mannitol solutions into the carotid artery allows for shrinkage of the brain endothelia and opening of the BBB for very brief periods. This procedure has been used to treat brain tumors with intracarotid methotrexate,[16] and to deliver enzymes such as α-mannosidase[17] or hexosaminidase A to brain.[18] The latter enzyme is deficient in Tay-Sachs disease.

The transplantation of peripheral nervous tissue, e.g., the superior cervical ganglion, to brain to create an artificial CVO is another invasive approach.[19] However, this method only allows for drug distribution to the small area adjacent to the artificial CVO.

Pharmacologic-Based Strategies - A powerful pharmacologic-based approach for drug delivery to brain is drug latentiation, or the conversion of hydrophilic drugs into lipid-soluble drugs. Majority of the latentiation approaches for making drugs more lipid-soluble involve masking of three primary functional groups: hydroxyls, carboxyls, and primary amines. Hydroxyl groups, for example, must be blocked since BBB permeability to a drug decreases one log order for each hydroxyl.[20] The classic example of drug latentiation is the conversion of the highly polar morphine to diacetyl morphine (heroin), which is less water-soluble and diffuses through the BBB approximately 100-fold faster than morphine.[21] Once within the brain, pericapillary pseudocholinesterase allows for deacetylation and reconversion back to the parent morphine.[22]

Exogenously administered gamma-aminobutyric acid (GABA), a putative neurotransmitter, does not easily cross the BBB. Latentiation as the Schiff base-amide progabide (2) has provided a useful



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drug which crosses the BBB and releases GABA.[23,24] Pyrroline (3) has also been reported to be a centrally acting GABA prodrug.[25] Similarly, the propyl esters (4) and (5) readily penetrate the BBB where they release GABA agonists isoarecaidine and isoguavacine respectively.[26]

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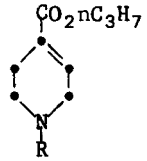
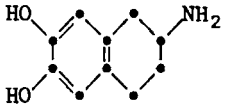


4, R=CH_3
5, R=H

Benzoyl and acetyl ester derivatives have been reported to be useful in the delivery of dopamine agonist ADTN (6) to the CNS,[27] as has the methylenedioxy analog (7) in the delivery of n-propylnorapomorphine.[28]



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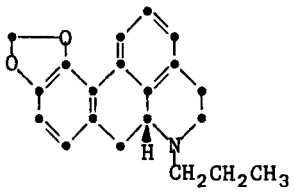

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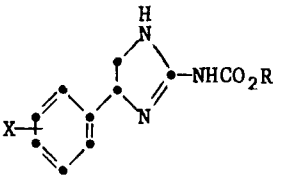
Numerous esters of the benzodiazepine oxazepam have been synthesized in an attempt to modify the activity/brain distribution/duration of action of the parent drug. It would appear, however, that the activity of most of these analogs parallels their hepatic hydrolysis rate, although some differences could be attributed to differing tissue localization.[29,30] Numerous peptidobenzophenones have also been reported to be metabolic precursors of benzodiazepines with somewhat modified profiles.[31]

Imidazoline carbamates (8) are described as prodrugs for the corresponding antidepressant aminoimidazolines.[32] Interestingly,



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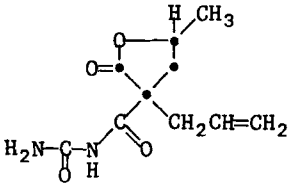
peripheral metabolite (9) of proxibarbal (10) is reported to penetrate the BBB more readily than its parent to which it is reconverted in the brain.[33]



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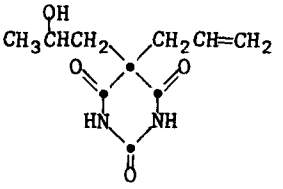

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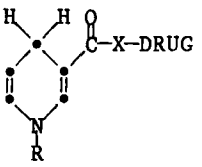
One of the most novel recent approaches to delivery of drugs across the BBB is the work of Bodor and colleagues.[34] Their concept, called "chemical delivery systems", is based on a dihydropyridine-pyridinium salt redox system, designed to yield a positively charged drug-pyridinium salt product (12) within the brain subsequent to diffusion into brain of the dihydropyridine drug precursor (11). The product (12) being charged, is trapped in the brain where it slowly releases the drug via enzymatic hydrolysis. This technique has been applied to the delivery of phenethylamine and dopamine to the brain.[34]



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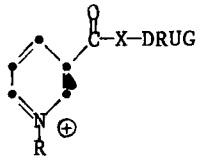

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Transport of di- and tripeptides through the BBB has been achieved via the formation of diketopiperazines. These compounds are formed when a dipeptide is cyclized by condensation of the N-terminal amino group and the C-terminal carboxyl group. The closure of the dipeptide into a cyclic compound releases several hydrogen bonds formed by the polar terminals of the dipeptide, and this increases the permeability of the BBB to the compound by at least 1-2 log orders of magnitude. The diketopiperazines of the oxytocin C-terminal leucine-glycine dipeptide[35] or the thyrotropin releasing hormone (TRH) C-terminal histidine-proline dipeptide[36] are compounds that are readily transported through the BBB. Conversely, uncyclized dipeptides such as glycylphenylalanine are poorly, if at all, transported through the BBB.[37]

Apart from condensation of dipeptides to form the corresponding diketopiperazine, latentiation of peptides for the purposes of increasing peptide distribution into brain is a strategy that has not been widely used. Moreover, preliminary studies with one naturally lipid-soluble peptide, cyclosporin, indicate that all latentiated peptides may not readily cross the BBB. Virtually all the polar, hydrogen bond-forming functional groups on the cyclosporin molecule are naturally masked.[38] Despite these features, the transport of ^{3}H-cyclosporin through the BBB is disappointingly slow. For example, in the presence of serum proteins, the first pass extraction of ^{3}H-cyclosporin through the rat brain is on the order of 2%, which approximates the extraction of the poorly diffusible substance, ^{3}H-inulin.[39]

The encapsulation of a water-soluble drug or peptide within a lipid-soluble liposome has been proposed for the delivery of peptides such as TRH to the brain.[40] Liposomes have proven to be a useful method for selectively delivering drugs to cells which take up liposomes, i.e., cells making up the reticulo-endothelial system (RES) in liver and spleen.[41] Apart from the RES system, there is poor distribution of liposomes into tissues, and it appears that this approach will not prove profitable in the general delivery of pharmaceuticals to the brain.

Physiologic-Based Strategies - Additional strategies for delivery of drugs to the brain arise from an understanding of the basic physiology of transport processes at the BBB for nutrients and peptides. Water-soluble nutrients cross the BBB via carrier-mediation,[4] and some circulating peptides may cross the BBB via receptor-mediated transcytosis of the peptide through the endothelial cytoplasm.[6] Neutral amino acids in blood are transported in brain interstitium via a specific neutral amino acid carrier system localized in both the lumenal and antilumenal membranes of the BBB.[4] This neutral amino acid transport system mediates the bidirectional movement of neutral amino acids between blood and brain. Neutral amino acid drugs such as α-methyldopa, L-dopa, α-methyltyrosine, and phenylalanine mustard or melphalan gain access to brain interstitium despite being very water-soluble, because they are transported through the BBB by the neutral amino acid carrier. All four of these drugs are probably also transported into peripheral tissues by carrier-mediated transport.

An understanding of the unique physiology of BBB neutral amino acid transport leads to new insights into how the distribution of such drugs may be selectively enhanced relative to the uptake by peripheral tissues. The K_m of the BBB neutral amino acid transport system is near the plasma level of amino acids in the 50-100 μM range,[4] while the K_m of neutral amino acid transport in virtually all other tissues of the body is in the 1-10 mM range. Since competition effects *in vivo* do not occur unless the physiologic concentration of plasma amino acids is near the K_m of the transport system, tissues other than brain will not be sensitive to competition effects in the physiologic range.[4] For example, Udenfriend and co-workers[42] have shown that the uptake of a neutral amino acid by brain is inhibited by other neutral amino acids, whereas the uptake of neutral amino acids in skeletal muscle is not inhibited. More recently Fernstrom and associates have shown that the brain uptake of α-methyldopa is inversely related to the existing concentrations of neutral amino acids in blood.[43] Hypoaminoacidemia, induced by insulin or a carbohydrate meal, results in enhanced brain uptake of α-methyldopa. Conversely, a protein meal depresses the

uptake of drugs such as L-dopa, and this dietary-induced decrease in the brain uptake of L-dopa may underlie the on-off phenomenon in the treatment of Parkinson's disease.[44] Similarly, it might be predicted that melphalan therapy for brain tumors,[45] in conjunction with insulin hypoaminoacidemia, would allow for a selective increase in melphalan distribution to the brain tumor without enhancing the distribution of melphalan into peripheral tissues. Thus, the drug efficacy/toxicity ratio can be selectively enhanced for brain in states of insulin-induced hypoaminoacidemia.

With regard to the delivery of neuropeptides to brain after systemic administration, it is well known that many peptides that are potent by the intraventricular route are ineffective even when administered in high concentrations by the intravenous route. This is because neuropeptides, such as somatostatin, TRH, or the enkephalins, which are all water-soluble, do not cross the BBB due to the absence of specific peptide transport systems.[46] Recent sudies have shown that peptides such as insulin or the insulin-like growth factors (IGF)-I and II may be transported through the BBB via receptor-mediated transcytosis through the brain capillary endothelial cytoplasm.[6,47] The presence of receptor-mediated transcytosis of peptides such as insulin or IGF-II explains the high concentrations of these peptides in brain and in CSF.[48,49] Aside from insulin or IGF-II, transferrin,[50] prealbumin[50] and prolactin[51] are other peptides that are present in high concentrations in CSF due to transport through either the BBB or through the blood-CSF barrier.

The presence of specific peptide receptor transport systems in the BBB suggests a new strategy for peptide delivery to brain, i.e., the synthesis of chimeric peptides. Work over the last several years has shown that coupling peptides[52-54] or even enzymes[55] to insulin results in the uptake of the chimeric peptide by cells via the insulin receptor-mediated uptake system. A side effect of the insulin-chimeric peptide approach is insulin-induced hypoglycemia, since the pharmaceutical-insulin complex will also activate insulin receptors in the periphery.[55] Such side effects can be eliminated either by (a) infusing glucose simultaneously, or (b) coupling the neuropeptide of choice to another peptide that also enjoys receptor-mediated transport through the BBB, but has a minimum of peripheral side effects. The production of chimeric peptides provides a novel approach to the synthesis of new pharmaceuticals that will have enhanced distribution in brain. Moreover, a convenient *in vitro* model system for BBB peptide transport is now available using isolated human brain capillaries,[6] and this approach allows for rapid screening of the novel chimeric peptides for transport through the BBB *in vivo* in man. Although much work needs to be done in this field, it becomes clear that an understanding of the basic physiology of BBB transport processes may lead to new strategies for the delivery of pharmaceuticals to brain.

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Section VII. Worldwide Market Introductions

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Chapter 32. To Market, To Market - 1984

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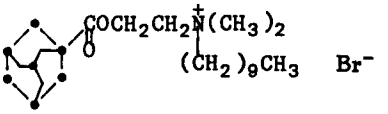
The new chemical entities (NCEs) for human therapeutic use introduced into the world marketplace for the first time during 1984 are similar in therapeutic breadth, albeit fewer in number than the 1983 introductions.[1] Interestingly, in a year when most countries showed a decline, Japan had a >50% increase in first time NCE introductions. As was the case last year, Japan and the United States, respectively, top the list of NCE originators.

The decline in the first time worldwide NCE introductions appears to be paralleled by a similar decrease in general NCE introductions into the US marketplace in 1984.[2] Of these latter agents, three (cefonicid, ceforanide and naltrexone) are represented in the following compilation of first time worldwide entries.

Amantanium Bromide (antiseptic)[3,4]

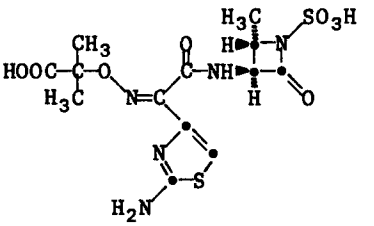
Country of Origin: **Italy**
Originator: **Rotta Research**
First Introduction: **Italy**
Introduced by: **Rotta Research**
Trade Name: **AMANTOL**



Amantanium bromide is a quaternary ammonium antibacterial/antifungal agent useful as a surgical antiseptic and for the topical therapy of mucous and cutaneous infections. It appears somewhat more effective than cetylpyridinium chloride and benzalkonium chloride.

Aztreonam (antibiotic)[5,6]

Country of Origin: **USA**
Originator: **Squibb**
First Introduction: **Italy**
Introduced by: **Squibb; Menarini**
Trade Name: **AZACTAM; PRIMBACTAM**



Aztreonam is the first member of the monobactam class of antibiotics to be introduced into the world market. It possesses high β-lactamase stability and moderately good activity against gram negative aerobes such as E. coli, S. marcescens, Proteus, Providencia, Salmonella, H. influenzae, N. gonorrhea, and K. pneumonia. While somewhat less potent against Pseudomonas aeruginosa, it is nonetheless one of the better β-lactams against this species. It has poor activity against gram positive organisms.

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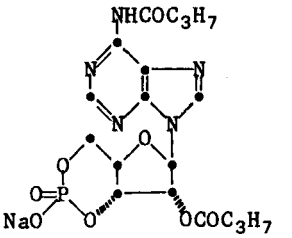
Bucladesine Sodium (cardiostimulant)[7]



Country of Origin: **Japan**
Originators: **Daiichi Seiyaku; Seishin Seiyaku**
First Introduction: **Japan**
Introduced by: **Daiichi Seiyaku**
Trade Name: **ACTOCIN**

Bucladesine sodium is a derivative of cyclic-AMP with cardiac stimulant and peripheral vasodilatory properties. It is reported to be useful in cardiac insufficiency, especially after heart surgery.

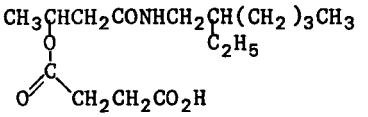
Buserelin Acetate (hormone)[8-10]

5-oxoPro-His-Trp-Ser-Tyr-D-Ser(1,1-diMeEt)-Leu-Arg-Pro-NHEt ·CH_3COOH

Country of Origin: **W. Germany**
First Introduction: **W. Germany**
Trade Name: **SUPREFACT**
Originator: **Hoechst AG**
Introduced by: **Hoechst AG**

Buserelin acetate is a potent analog of the hypothalamic hormone, luteinizing hormone-releasing hormone (LH-RH). Chronic administration of higher doses of buserelin desensitizes the pituitary, resulting in a decreased production of luteinizing hormone (LH), follicle stimulating hormone (FSH), and eventually sex steroids (testosterone in males and estradiol in females). Initial approval is for use in achieving medical castration in the treatment of advanced prostate cancer. It has also been investigated for the treatment of endometriosis, leiomyoma, precocious puberty, advanced breast cancer and infertility.

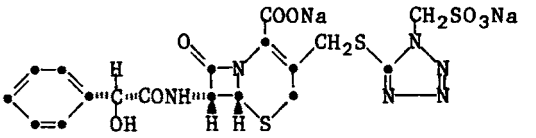
Butoctamide Hydrogensuccinate (hypnotic)[11,12]



Country of Origin: **Japan**
Originator: **Lion Dentifrice**
First Introduction: **Japan**
Introduced by: **Banyu**
Trade Name: **LISTOMIN S**

Butoctamide hydrogensuccinate is a structurally novel hypnotic agent useful in the treatment of insomnia. Unlike other marketed hypnotics, butoctamide appears to increase REM sleep.

Cefonicid Sodium (antibiotic)[13,14]

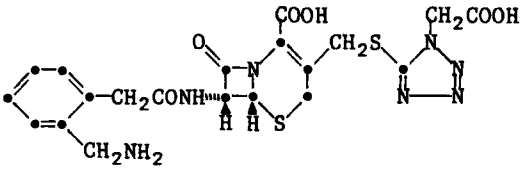


Country of Origin: **USA**
Originator: **Smith Kline & French**
First Introduction: **USA**
Introduced by: **Smith Kline & French**
Trade Name: **MONOCID**

Cefonicid sodium is the first of the second-generation cephalosporin antibiotics with a long enough serum half-life (~4.5 hr.) to allow once-daily i.v. administration. Cefonicid is resistant to β-lactamases produced by S. aureus, H. influenzae, N. gonorrhea, and related organisms. As with other drugs of this class, it is useful in the treatment of urinary tract and lower respiratory tract infections, skin and skin structure infections, and bone and joint infections, as well as in surgical prophylaxis.

Ceforanide (antibiotic)[15,16]

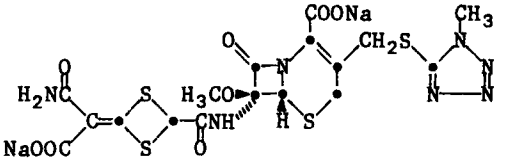
Country of Origin: **USA**
Originator: **Bristol**
First Introduction: **USA**
Introduced by: **Bristol**
Trade Name: **PRECEF**



Ceforanide is a second generation cephalosporin with good β-lactamase resistance. It has a serum half-life of about three hours, allowing twice-daily dosing.

Cefotetan Disodium (antibiotic)[17,18]

Country of Origin: **Japan**
Originator: **Yamanouchi**
First Introduction: **Japan**
Introduced by: **Yamanouchi**
Trade Name: **YAMATETAN**



Cefotetan disodium is a β-lactamase resistant, second generation cephalosporin with a serum half-life of about three hours, permitting twice-daily dosing.

Divistyramine (hypocholesterolemic)

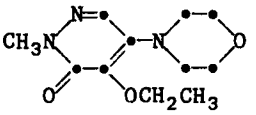
Country of Origin: **Switzerland**
First Introduction: **Switzerland**
Trade Name: **IPOCOL**

Originator: **Lagap**
Introduced by: **Lagap**

Divistyramine is a hydrophilic anion exchange resin useful in the treatment of primary hypercholesterolemia. In a similar manner to cholestyramine, it sequesters bile acids, preventing their reabsorption and thus depleting cholesterol stores. A recent study of cholestyramine in asymptomatic Type II hyperlipidemics suggests dramatic morbidity and mortality benefits from drugs of this type.[19,20]

Emorfazone (analgesic)[21,22]

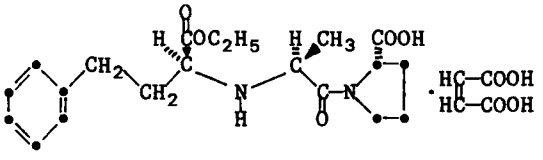
Country of Origin: **Japan**
Originator: **Morishita**
First Introduction: **Japan**
Introduced by: **Morishita**
Trade Name: **PENTOIL**



Emorfazone is an analgesic agent with a potency similar to aminopyrine, useful in the treatment of low back pain and similar musculoskeletal disorders.

Enalapril Maleate (antihypertensive)[23-25]

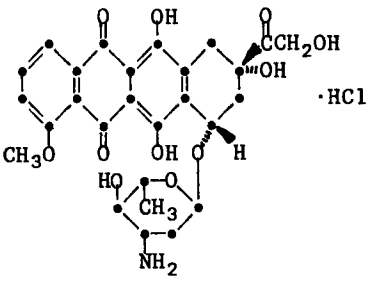
Country of Origin: **USA**
Originator: **Merck**
First Introduction: **Switzerland**
Introduced by: **Merck**
Trade Name: **RENITEN**



Enalapril maleate is the second angiotensin converting enzyme inhibitor to reach the marketplace. Like captopril, the first entry in this area, enalapril is useful in the treatment of hypertension and congestive heart failure. It has a longer effective half-life than captopril, allowing once or twice-daily dosing, and appears to have a somewhat lower incidence of side effects.

Epirubicin Hydrochloride (antineoplastic)[26,27]

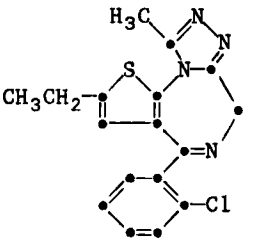
Country of Origin: **Italy**
Originator: **Erbamont**
First Introduction: **South Africa**
Introduced by: **Erbamont**
Trade Name: **FARMORUBICIN**



Epirubicin hydrochloride is an antitumor antibiotic which is epimeric with doxorubicin at the 4'-position of the amino sugar moiety. It has shown utility in the treatment of mammary, gastric, colorectal, pulmonary and ovarian carcinomas, as well as malignant lymphoma and melanoma and soft tissue sarcoma. It is reported to be less cardiotoxic than doxorubicin.

Etizolam (anxiolytic)[28,29]

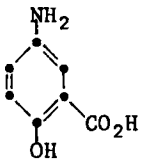
Country of Origin: **Japan**
Originator: **Yoshitomi**
First Introduction: **Japan**
Introduced by: **Yoshitomi**
Trade Name: **DEPAS**



Etizolam is a potent thienodiazepine anxiolytic closely related to brotizolam. Pharmacologically, however, its profile most resembles that of diazepam.

Fisalamine (intestinal antiinflammatory)[30-32]

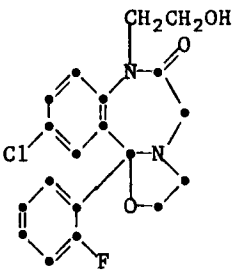
Country of Origin: **United Kingdom**
Originator: **Radcliffe Infirmary**
First Introduction: **W. Germany**
Introduced by: **Dr. Falk GMbH**
Trade Name: **SALOFALK**



Fisalamine is an intestinal metabolite of sulfasalazine useful in the treatment of ulcerative colitis and to a lesser degree in the management of Crohn's disease. Administered as a suppository, it appears to lack the hypersensitivity-type side effects of sulfasalazine.

Flutazolam (anxiolytic)[33,34]

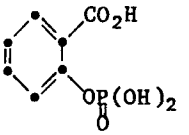
Country of Origin: **USA**
Originator: **Hoffmann-LaRoche**
First Introduction: **Japan**
Introduced by: **Mitsui**
Trade Name: **COREMINAL**



Flutazolam is a benzodiazepine anxiolytic closely related to mexazolam. It is useful in the treatment of anxiety, stress and depression in hypersensitive colon syndrome, chronic gastritis, and gastric and duodenal ulcers.

Fosfosal (analgesic/antiinflammatory)[35,36]

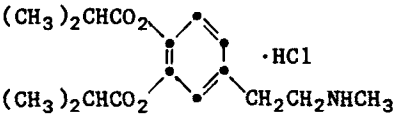
Country of Origin: **Spain**
Originator: **Uriach**
First Introduction: **Spain**
Introduced by: **Uriach**
Trade Name: **DISDOLEN**



Fosfosal is a highly water soluble salicylate useful in the treatment of musculoskeletal and arthritic pain. Unlike aspirin, it does not inhibit prostaglandin synthesis or platelet aggregation.

Ibopamine Hydrochloride (cardiostimulant/diuretic)[37,38]

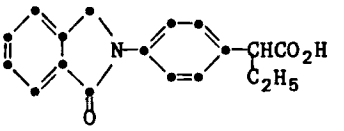
Country of Origin: **Italy**
Originator: **Simes**
First Introduction: **Italy**
Introduced by: **Simes; Zambon**
Trade Name: **INOPAMIL; SCANDINE**



Ibopamine hydrochloride is an orally active prodrug of dopamine with positive inotropic, vasodilatory and diuretic properties. It is effective in the treatment of congestive heart failure.

Indobufen (antithrombotic)[39,40]

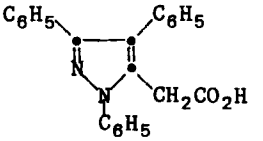
Country of Origin: **Italy**
Originator: **Erbamont**
First Introduction: **Italy**
Introduced by: **Erbamont**
Trade Name: **IBUSTRIN**



Indobufen is a platelet aggregation inhibitor related to the antiinflammatory agent indoprofen. It has shown clinical efficacy in peripheral artery disease, in prevention of deep vein thrombosis after myocardial infarction, and in blocking excercise-induced increase in platelet aggregation in angina patients.

Isofezolac (antiinflammatory)[41,42]

Country of Origin: **France**
Originator: **Pharmuka**
First Introduction: **France**
Introduced by: **Spret Mauchant**
Trade Name: **SOFENAC**



Isofezolac is a nonsteroidal antiinflammatory agent indicated for use in various forms of rheumatoid arthritis and osteoarthritis.

Leuprolide Acetate (hormone)[8,10,43]

5-oxoPro-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt ·CH_3COOH

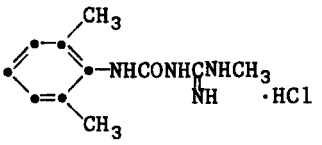
Country of Origin: **Japan**
First Introduction: **W. Germany**
Trade Name: **CARCINIL**

Originator: **Takeda**
Introduced by: **Abbott**

Leuprolide acetate is a potent analog of LH-RH useful in achieving a medical castration in the treatment of advanced prostate cancer. It has also been investigated for the treatment of advanced breast cancer.

Lidamidine Hydrochloride (antiperistaltic)[44-46]

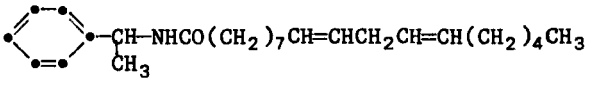
Country of Origin: **USA**
Originator: **Rorer**
First Introduction: **Mexico**
Introduced by: **Rorer**
Trade Name: **LIDARRAL**



Lidamidine hydrochloride is an antiperistaltic agent which appears to act by interfering with the availability of calcium necessary for excitation-contraction coupling in smooth muscle. It is useful in the treatment of diarrhea of various origins.

Melinamide (hypocholesterolemic)[47,48]

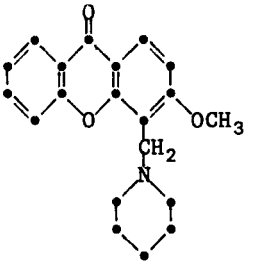
Country of Origin: **Japan**
Originator: **Sumitomo**
First Introduction: **Japan**
Introduced by: **Sumitomo**
Trade Name: **ARTES OIL**



Melinamide is an amide of linoleic acid useful in the treatment of hypercholesterolemia. The mechanism of action of melinamide appears to involve the inhibition of cholesterol absorption from the gastrointestinal tract.

Mepixanox (analeptic)[49-51]

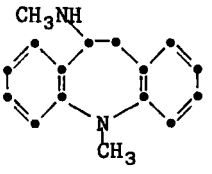
Country of Origin: **Italy**
Originator: **Univ. of Pisa**
First Introduction: **Italy**
Introduced by: **Formenti (Boots)**
Trade Name: **PIMEXONE**



Mepixanox is a respiratory stimulant useful in the treatment of chronic pneumopathy and other cardiorespiratory insufficiencies, including those induced by general anesthesia.

Metapramine (antidepressant)[52,53]

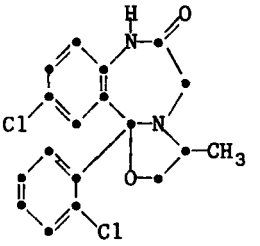
Country of Origin: **France**
Originator: **Rhone-Poulenc**
First Introduction: **France**
Introduced by: **Specia**
Trade Name: **TIMAXEL**



Metapramine is an antidepressant with a pharmacological profile similar to the tricyclics and an apparent rapid clinical onset of action. Metapramine differs biochemically from the tricyclics in that it markedly enhances norepinephrine turnover with minimal inhibition of reuptake. Like the tricyclics, however, it does down regulate β-receptors.

Mexazolam (anxiolytic)[54,55]

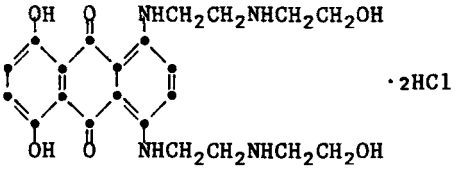
Country of Origin: **Japan**
Originator: **Sankyo**
First Introduction: **Japan**
Introduced by: **Sankyo**
Trade Name: **MELEX**



Mexazolam is a benzodiazepine anxiolytic closely related to flutazolam. It is reported to be particularly effective against psychosomatic essential hypertension, peptic ulcer, irritable colon and autonomic dystonia.

Mitoxantrone Hydrochloride (antineoplastic)[56,57]

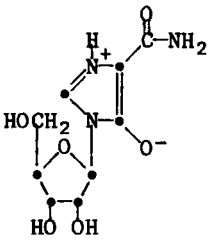
Country of Origin: **USA**
Originator: **Lederle**
First Introduction: **Canada**
Introduced by: **Lederle**
Trade Name: **NOVANTRONE**



Mitoxantrone hydrochloride is the first of the synthetic anthracenediones related to doxorubicin to reach the marketplace. Mitoxantrone is useful in the treatment of advanced localized and metastatic mammary carcinomas. It is reported to be less cardiotoxic than doxorubicin.

Mizoribine (immunosuppressant)[58,59]

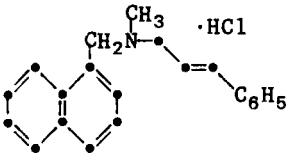
Country of Origin: **Japan**
Originator: **Toyo Jozo**
First Introduction: **Japan**
Introduced by: **Toyo Jozo**
Trade Name: **BREDININ**



Mizoribine is an orally active immunosuppressant isolated from Eupenicillium brefeldianum. It is reported to inhibit both cell-mediated and antibody mediated immune responses. Mizoribine is approved for use in the prevention of rejection of kidney transplants.

Naftifine Hydrochloride (antifungal)[60,61]

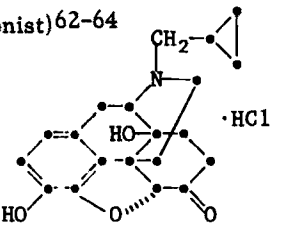
Country of Origin: **Switzerland**
Originator: **Sandoz**
First Introduction: **Malaysia/Singapore**
Introduced by: **Biochemie**
Trade Name: **EXODERIL**



Naftifine hydrochloride is a topical antifungal agent which acts via inhibiting squalene epoxidase. It demonstrates good activity against Trichophyton, Epidermophyton, Microsporum, Aspergillus, and Candida species. It is the prototype of a significantly improved series of antifungal agents, exemplified by SF 86-327 in which the phenyl group of naftifine has been replaced by t-butylacetylene.

Naltrexone Hydrochloride (narcotic antagonist)[62-64]

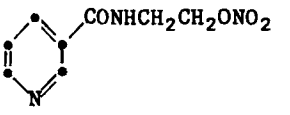
Country of Origin: **USA**
Originator: **Endo**
First Introduction: **USA**
Introduced by: **DuPont**
Trade Name: **TREXAN**



Naltrexone hydrochloride is a potent, long-acting, orally-effective narcotic antagonist useful in the management of narcotic addiction.

Nicorandil (coronary vasodilator)[65,66]

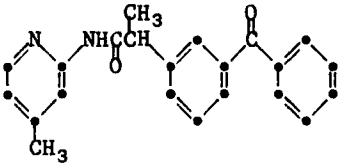
Country of Origin: **Japan**
Originator: **Chugai**
First Introduction: **Japan**
Introduced by: **Chugai; Mitsubishi Yuka**
Trade Name: **SIGMART; PERISALOL**



Nicorandil is a coronary vasodilator useful in the treatment of angina pectoris. Decreases in left ventricular end diastolic pressure and systemic vascular resistance also appear to contribute to the anti-ischemic effects of the drug.

Piketoprofen (topical antiinflammatory)[67,68]

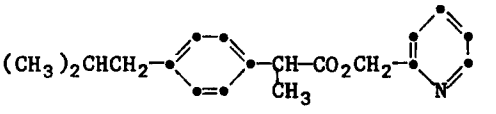
Country of Origin: **Spain**
Originator: **Labs. Almirall**
First Introduction: **Spain**
Introduced by: **Labs. Almirall**
Trade Name: **CALMATEL**



Piketoprofen is an amide derivative of the nonsteroidal antiinflammatory agent ketoprofen. Available in aerosol form, it is indicated for the topical treatment of inflammatory and painful musculoskeletal disorders.

Pimaprofen (topical antiinflammatory)[69,70]

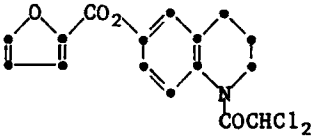
Country of Origin: **Japan**
Originator: **Hisamitsu**
First Introduction: **Japan**
Introduced by: **Hisamitsu; Torii**
Trade Name: **BESICUM; STADERM**



Pimaprofen (ibuprofen piconol) is an antiinflammatory agent useful in the topical treatment of acute and chronic eczema, and contact and atopic dermatitis.

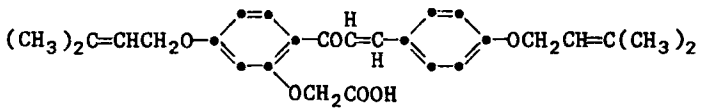
Quinfamide (amebicide)[71-73]

Country of Origin: **USA**
Originator: **Sterling-Winthrop**
First Introduction: **Mexico**
Introduced by: **Sterling-Winthrop**
Trade Name: **AMENOX**



Quinfamide is an amebicide indicated for the one-day treatment of subacute or chronic intestinal amebiasis. It acts by rendering the trophozoites of E. histolytica incapable of propagation.

Sofalcone (antiulcer)[74,75]



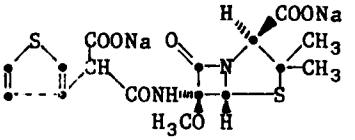
Country of Origin: **Japan**
First Introduction: **Japan**
Trade Name: **SOLON**

Originator: **Taisho**
Introduced by: **Taisho**

Sofalcone is a synthetic antiulcer agent related to sophoradin, the active principle of the Chinese medicinal plant, Sophora subprostata. It is suggested to act via a strengthening of the mucosal barrier through an increase in blood flow and stimulation of the synthesis of sulfated mucopolysaccharides.

Temocillin Disodium (antibiotic)[76,77]

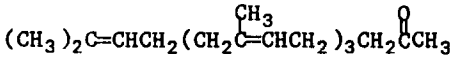
Country of Origin: **United Kingdom**
Originator: **Beecham**
First Introduction: **W. Germany**
Introduced by: **Beecham**
Trade Name: **TEMOPEN**



Temocillin disodium is a broad-spectrum, β-lactamase resistant, injectable penicillin. High serum levels and a five hour half-life allow once or twice-daily dosing.

Teprenone (antiulcer)[78,79]

Country of Origin: **Japan**
Originator: **Eisai**
First Introduction: **Japan**
Introduced by: **Eisai**
Trade Name: **SELBEX**

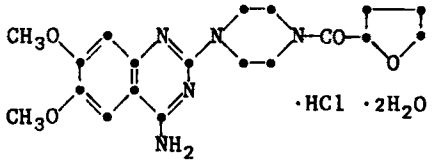


(5E,9E,13E):(5Z,9E,13E)=3:2

Teprenone (geranylgeranylacetone) is an antiulcer agent whose synthesis was inspired by the gastric protecting properties of Coenzyme Q_{10}. Teprenone is reported to act via enhancement of gastric mucosal glycoprotein secretion.

Terazosin Hydrochloride (antihypertensive)[80-83]

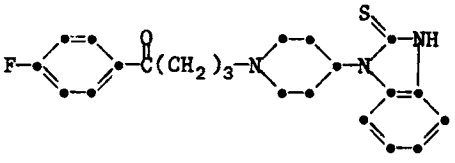
Country of Origin: **USA**
Originator: **Abbott**
First Introduction: **W. Germany**
Introduced by: **Abbott**
Trade Name: **HEITRIN**



Terazosin hydrochloride is a highly water-soluble antihypertensive agent with a structure and profile closely related to that of prazosin.

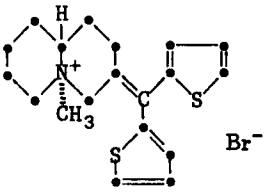
Timiperone (neuroleptic)[84,85]

Country of Origin: **Japan**
Originator: **Daiichi Seiyaku**
First Introduction: **Japan**
Introduced by: **Daiichi Seiyaku**
Trade Name: **TOLOPELON**



Timiperone is a butyrophenone neuroleptic agent closely related to benperidol.

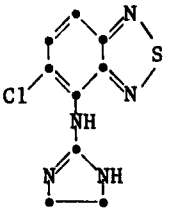
Tiquizium Bromide (antispasmodic)[86,87]



Country of Origin: **Japan**
Originator: **Hokuriku Seiyaku**
First Introduction: **Japan**
Introduced by: **Hokuriku Seiyaku**
Trade Name: **THIATON**

Tiquizium bromide is an anticholinergic agent useful in the treatment of various gastrointestinal syndromes including irritable colon.

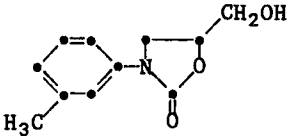
Tizanidine (muscle relaxant)[88,89]



Country of Origin: **Switzerland**
Originator: **Sandoz**
First Introduction: **Switzerland**
Introduced by: **Sandoz**
Trade Name: **SIRDALUD**

Tizanidine is a centrally-acting muscle relaxant useful in the treatment of muscle spasms and a variety of spastic conditions.

Toloxatone (antidepressant)[90-92]



Country of Origin: **France**
Originator: **Delalande**
First Introduction: **France**
Introduced by: **Delalande**
Trade Name: **HUMORYL**

Toloxatone is a selective, reversible and competitive inhibitor of type A monoamine oxidase (MAO-A) which shows clinical utility as an antidepressant. It is reportedly free of the "cheese effect" in man, possibly due to the reversible nature of its inhibitory effect on intestinal MAO-A in the presence of high concentrations of tyramine.

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