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Editor-in-Chief: **HANS-JÜRGEN HESS**

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Volume 17

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PFIZER INC.
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PREFACE

The present volume of Annual Reports in Medicinal Chemistry consists of 33 chapters, which are organized in six sections following the format of previous volumes. In addition to the traditional updates of the literature in current areas of active drug research, this volume covers a wide range of subjects relevant to areas of potential new drug development or of general interest. Topics presented for the first time include models of serotonin receptors, mechanisms of antibiotic resistance, hemorheologic agents, protein growth factors, platelet-activating factor, polyamine metabolism, and strategies for the discovery of drugs from natural sources. Several chapters discuss in detail the mechanistic aspects pertaining to the development of new drugs for the treatment of rheumatoid arthritis and other diseases where a state of immune dysfunction is implicated. The rapid expansion of the literature during the past year on arachidonic acid-derived lipoxygenase products warranted a follow-up chapter on their biological actions, and a separate chapter discusses the chemistry of these interesting substances. The growing importance of peptides in the modulation of physiological functions is reflected by the inclusion of two chapters on neuropeptides. Also of interest to medicinal chemists will be reviews on artificial sweeteners and herbicides and insect control agents, the development of which requires concepts and techniques not unlike those employed in drug research. Finally, we have succeeded in including a review on the basic elements of recombinant DNA research, which should be helpful to those following the specialized literature in this rapidly moving field.

Sincere thanks are again due to the many individuals who contributed their time and effort to make this volume possible, particularly to Mary Heinold and Carol Urso, whose help was invaluable in preparing the manuscript for photoreproduction.

Groton, Connecticut
May 1982

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Section I - CNS Agents

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Chapter 1. Biochemical Models for Serotonin Receptors

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Over the last seven years, three different in vitro biochemical models supposedly related to serotonergic receptors have been described: (i) an adenylate cyclase which is stimulated by serotonin (5HT),[1] (ii) high affinity binding sites for serotonin-like compounds detected in brain tissue preparations with ^{3}H-serotonin[2] and recently designated as 5-hydroxytryptamine$_1$ ($5HT_1$) or as serotonin-1 (S_1) receptors;[3,4] and (iii) high affinity binding sites for serotonin antagonists detected in frontal cortex tissue preparations with ^{3}H-spiperone[5,6] and later designated $5HT_2$ or serotonin-2 (S_2) receptors.[3,4] These three biochemically detected sites appear to be distinct molecular entities. In this review we will discuss the specificity of various ligands used for the characterization of these sites, the distribution of the sites in brain tissue, the activities of a wide variety of pharmaca at the sites and correlations with various in vitro and in vivo pharmacological activities, some structure-activity relationships, the in vivo regulation of the sites, and their hypothesized involvement in human diseases.

Specificity of Various Ligands and Occurrence of the Serotonin Receptor Sites

Serotonin-Stimulated Adenylate Cyclase - This enzyme activity is best detectable in colliculli of young animals, because the activity is found to decrease rapidly with age.[1,7] In particulate preparations of colliculi of newborn rats, 50 μM serotonin reportedly stimulated cAMP formation by 60-70 %; a K_m-value of 0.5 - 1 μM for serotonin was found. This stimulation was further enhanced 2-3-fold in the presence of 10^{-5}M GTP.[8,9,10] Various structural analogues of serotonin and tryptamine also stimulated the enzyme; 5-methoxy derivatives were found to be the most active (maximal stimulation of 80 % by 5-methoxytryptamine) and N-methylated indoles appeared to be the least active.[1] Serotonin agonists with a piperidinyl-indole structure were totally inactive.[11] In the colliculli, noradrenaline and dopamine produced only half as much adenylate cyclase stimulation as serotonin.[1,8,9] Conversely, serotonin produced much lower stimulation of the enzyme in the striatum compared to dopamine and in the cortex compared to noradrenaline.[8] Hence, a specific serotonin-stimulated adenylate cyclase was believed to exist.

The distribution of the serotonin-stimulated adenylate cyclase in the brains of newborn rats, shown in Table I, resembles the distribution of endogenous serotonin in adult rat brains. However, the distribution

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of endogenous serotonin in brains of newborns was reported to be dissimilar[8,9]. The distribution of serotonin-stimulated adenylate cyclase is different from that of the S_1-binding sites in adult rat brains,[2,7] but in the brains of newborns they are similar.[7] No relationship exists with the regional distribution of the S_2-receptor binding sites. In subcellular fractions obtained from brain tissue homogenates, the serotonin-stimulated cyclase is particularly enriched in the mitochondrial fraction.[7] The enzyme was further reported to be present in glial cell preparations from horse brain striatum[12] and in a particulate fraction of the central nervous system of the snail Helix Pomatia.[13]

TABLE I. Regional Distribution of Endogenous Serotonin, Serotonin-Stimulated Adenylate Cyclase, S_1-Binding Sites and S_2-Receptor Binding Sites in Rat Brain

	Endogenous serotonin (adults) (Ref. 8,9) Relative concentration	Serotonin stimulated adenylate cyclase (newborns) (Ref. 8,9) Relative activity	S_1-binding sites (adults) (Ref. 2) Relative density	S_2-receptor binding sites (adults) (Ref. 25) Relative density
Brain areas				
Hypothalamus	1.00	1.00	-	N.S.
Spinal cord	0.76	0.74	-	N.S.
Brain stem	0.74	0.67	N.S.	0.10
Colliculli	0.71	0.98	-	-
Striatum	0.57	0.50	0.96	0.45
Hippocampus	0.42	0.54	1.00	0.13
Cerebral cortex	0.24	0.39	0.72	0.60
Frontal cortex	-	-	-	1.00
Thalamus	-	-	0.76	0.11
Cerebellum	0.08	0.09	N.S.	N.S.

N.S.: not significant

S_1-Binding Sites - They are labelled with high affinity by ^{3}H-serotonin and are detected in membrane preparations from rat brain. For the specific binding of ^{3}H-serotonin, defined by inhibition with 10 µM unlabelled serotonin, assayed at 37° C, K_D-values of 1.4 nM[14] to 8nM have been reported. Brief incubation of the membrane preparations at 37° C in the presence of 4 mM Ca^{++} in the assay medium was reported to enhance the binding affinity.[15] Guanyl nucleotides at rather elevated concentrations were found to decrease the ^{3}H-serotonin binding affinity to rat brain membranes.[10,4] Some authors have inferred the existence of multiple ^{3}H-serotonin binding sites from the observation of curvilinear Scatchard plots[18] or of biphasic inhibition curves for certain serotonin antagonists.[19] However, the meaning of multiple binding sites is not known, and a relationship with subclasses of physiological receptors has not been shown. Moreover, it has been extensively discussed[20] that in vitro interactions between ^{3}H-ligands and membrane micelles in aqueous suspensions are subject to complex physico-

chemical phenomena such as surface effects. These phenomena may affect the shape of binding curves. Therefore the causes of curvilinear Scatchard plots or multiphasic inhibition curves are difficult to assess, and such observations are not a reliable basis for subclassification of binding sites.

Binding sites labelled by ^{3}H-lysergic acid diethylamide (LSD)[16,17] were first presumed to be related to the sites labelled by ^{3}H-serotonin, although differences in the binding characteristics using both ligands were already noted in the early experiments.[2,18] Recently, it was shown that ^{3}H-LSD labels both the S_1- and S_2-binding sites.[3] In addition to that, ^{3}H-LSD interacts also with dopamine receptors.[21] Hence, ^{3}H-serotonin appears to be the most selective ligand for the S_1-binding sites. As shown in Table I, these sites occur predominantly in the hippocampus and the striatum. In subcellular distribution studies the sites were found to be mostly enriched in the microsomal fraction,[2,7] in contrast to the subcellular distribution of the serotonin stimulated adenylate cyclase.

S_2-Receptor Binding Sites - These were first labelled using ^{3}H-spiperone in rat frontal cortex membrane preparations.[5,6] Although spiperone shows high binding affinity for the sites ($K_D \sim 1$ nM), it is not an ideal ligand for studying the distribution and the properties of the sites, since ^{3}H-spiperone also binds to dopamine receptors with an affinity which is 10 times higher than its affinity for the S_2-receptor.[6,22] Later investigations using ^{3}H-mianserin revealed that this was neither a selective ligand for the S_2-receptor binding sites because of its concomitant labelling of histamine-1 receptor sites.[23] The recently introduced serotonin antagonist, ketanserin,[24] was found to be the first selective ligand.[25] Specific binding can clearly be defined by inhibition of the ^{3}H-ketanserin binding with methysergide. Analysis of Scatchard plots of the specific ^{3}H-ketanserin binding to a rat prefrontal cortex membrane preparation (37° C, Tris-HCl buffer, pH 7.5) revealed linear curves yielding a K_D of 0.4 nM and a maximal number of sites of 31 fmoles/mg tissue. Physiological concentrations of electrolytes or ascorbic acid in the assay medium were found to reduce the binding affinity and the number of binding sites. Using ^{3}H-ketanserin, a typical brain regional distribution of S_2-receptor binding sites was found (see Table I), which appeared to be similar in 4 mammalian species.[26] The highest density of S_2-receptor binding sites occurs in the frontal cortex and substantial amounts are also detected in the so-called dopaminergic brain areas (striatum, nucleus accumbens and tuberculum olfactorium). The S_2-receptor binding site distribution is clearly distinct from the distribution of S_1-binding sites and the serotonin-stimulated adenylate cyclase. Subcellular distribution studies revealed a biphasic distribution of the S_2-receptor binding sites in heavier and light subcellular fractions, but the highest enrichment is found in the microsomal fraction.[26,27]

Binding Affinity of Drugs for the Putative Serotonin Receptor Sites and Relationship with Pharmacological Activity

The receptor concept [28] requires that binding sites (or acceptor sites on enzymes) must only be considered to be receptors when a relationship with a pharmacological or physiological effect is established. The problem can be approached by investigating the relative binding affinities of large series of compounds belonging to various chemical classes and by comparison with activities in pharmacological tests.

TABLE II. Potencies of Various Compounds in Biochemical Models of Serotonin Receptors

Compounds, grouped by type*	1. Adenyl Cyclase Colliculus (5HT-stimulated) (Ref. 10)	2. S_1-Binding Hippocampus (^{3}H-Serotonin) (Refs. 24, 29)	3. S_2-Binding Frontal Cortex (^{3}H-Spiperone) (Refs. 6, 25)	4. S_2-Binding Frontal Cortex (^{3}H-Ketanserin) (Ref. 25)
	IC_{50}, nM	K_i, nM	K_i, nM	K_i, nM
(i)				
pirenperone	-	>> 1 000	2.0	0.28
ketanserin	-	>> 1 000	2.1	0.39
(ii)				
pizotifen	68 300	1 500	6.5	0.28
metitepine	11 900	62	1.9	0.39
cyproheptadine	11 500	700	6.5	0.44
mianserin	27 600	1 100	13	1.4
chlorpromazine	-	3 100	20	3.3
amitryptiline	-	1 200	21	4.2
clomipramine	-	>> 1 000	82	9.8
imipramine	-	>> 1 000	260	37
desimipramine	-	>> 1 000	>> 1 000	78
(iii)				
cinanserin	21 500	3 500	41	2.0
(iv)				
spiperone	3 800	160	1.2	0.53
pipamperone	-	5 000	5.3	0.78
haloperidol	-	>> 1 000	48	22
(v)				
metergoline	18 100	20	0.9	0.28
methysergide	-	99	12	0.94
LSD	-	20	8.2	2.5
(vi)				
bufotenin	-	62	518	118
serotonin	-	7.8	1 033	296
tryptamine	-	197	5 420	1 500
(vii)				
quipazine	no activation	1 600	-	235
(viii)				
mescaline	-	70 000	-	5 900
(ix)				
dopamine	-	20 000	82 000	93 500
epinephrine	-	-	-	47 000
norepinephrine	-	200 000	-	>> 100 000
histamine	-	-	-	>> 100 000
acetylcholine	-	>> 100 000	-	120 000

Spearman rank correlation between the four models (N.S. = not significant, Student's t-test)			
	1 versus 2 (n=7)+ r_s=0.571, N.S.		
	1 versus 3 (n=7)+ r_s=0.523, N.S.	2 versus 3 (n=21)+ r_s=0.167, N.S.	
	1 versus 4 (n=7)+ r_s=0.09, N.S.	2 versus 4 (n=23)+ r_s=0.073, N.S.	3 versus 4(n=33)++ r_s=0.963, $p<0.01$

* Compound types: (i) 4-(p-fluorobenzoyl)piperidines; (ii) tri- and tetracyclic compounds related to cyproheptadine; (iii) cinanserin; (iv) butyrophenones; (v) ergolines; (vi) indole derivatives; (vii) quinoline derivative; (viii) phenylethylamine derivative; (ix) neurotransmitters. Groups (i) through (iv) are antagonists; group (v) includes putative mixed agonist-antagonists; groups (vi) through (viii) are agonists.

+ Calculated for compounds in groups (i) through (viii).

++Calculated for a larger series of compounds reported in ref. 25.

Table II shows K_i-values of compounds for inhibition of serotonin-stimulated adenylate cyclase in colliculllar tissue of newborn rats, of ^{3}H-serotonin binding in rat hippocampal tissue and of ^{3}H-spiperone and ^{3}H-ketanserin binding in rat pre-frontal cortical tissue. Data are presented for various serotonin antagonists, for drugs with putative mixed serotonin antagonist-agonist effects, for serotonin-mimetic compounds, and for various neurotransmitters.

All the IC_{50}-values for inhibition of adenylate cyclase in Table II are above micromolar, and it is quite unlikely that such high drug concentrations are attained in vivo. No correlation exists between activities of the compounds on the cyclase and their binding affinities for S_1- or S_2-receptor binding sites. Neither could a correlation be demonstrated between the serotonin-stimulated adenylate cyclase and the ^{3}H-serotonin binding when both were assayed in identical conditions using the same tissue preparation of newborn animals.[7] Based on the subcellular and regional distribution studies described above and on a well-controlled investigation of drug interaction with the cyclase and the S_1-binding sites, Nelson et al.,[7,10] furnished clear evidence that the S_1-binding sites and the serotonin-stimulated adenylate cyclase are unrelated. A supposed coupling[30,31] between the two systems was therefore refuted. Table III shows that the potencies of compounds to inhibit the cyclase do not correlate with the potencies of the compounds to antagonize serotonergic behavioural agitation, such as tryptamine-induced clonic seizures in rats[32] or mescaline or 5-hydroxytryptophane-induced head twitches in rats or mice.[33] Neither is a correlation found with antagonism of serotonergic effects on isolated peripheral tissues such as serotonin-induced contractions in arteries[34] or in rat fundus strips.[34,35] Thus, a physiological role for the enzyme has not yet been established.

Amongst the series of compounds presented in Table II, serotonin revealed the highest binding affinity (of nanomolar order) for the S_1-binding sites. Furthermore, only compounds with an indole nucleus (serotonin and ergoline derivatives), and exceptionally also metitepine, show binding affinities below 100 nM. Many of the potent serotonin antagonists are totally inactive. No relationship exists between the affinities of the compounds for S_1-binding sites and S_2-receptor binding sites (see Table II). The lack of correlation between the binding affinities of compounds for S_1-binding sites and their potencies in pharmacological behavorial tests precludes involvement of S_1-binding sites in serotonin-induced behavioural agitation. S_1-binding sites seem neither to play a role in serotonin-induced vasoconstriction (see correlation coefficients in Table III). There is an apparent relationship between the rank order of S_1-binding affinities of compounds and the rank order of potencies to antagonize serotonin-induced contractions in isolated rat fundus strips. The latter was thought to be a model for the D-receptors,[35] which were classified by Gaddum[36] as a subclass of peripheral serotonergic receptors. However, although the correlation is apparently significant, the result must be interpreted with reservation. Indeed, on the fundus preparation only a few compounds are active below micromolar concentrations. For half of the compounds which are considered for the Spearman rank correlation, the exact rank order could not be established, since they have an activity score greater than 20 µM. A relationship between S_1-binding sites and the pharmacological D-receptors is therefore unlikely. On the other hand, it was tentatively suggested by Ennis et al. that S_1-binding sites would play a role in the inhibition by serotonin of dopamine release in the striatum.[37] The hypothesis was based on the relative order of potency: methysergide >

metergoline > metitepine > cinanserin > cyproheptadine > mianserin, for the antagonism of the inhibition by serotonin of potassium-evoked dopamine release in striatal slices. That order of potency was considered to be similar to the relative order in S_1-binding affinities of the compounds. However, calculation of the rank correlation using S_1-receptor binding data from Table II reveals an $r_s = 0.657$, $n = 6$, which is not significant according to the Student's t-test. Hence,a physiological role for S_1-binding sites has not irrefutably been demonstrated. Moreover, the argument that the nanomolar binding affinity of serotonin itself for the binding sites is an indication for the specificity or physiological relevance of the sites is not in accordance with the neurotransmitter concept. In fact, neurotransmitters attain high concentrations (micromolar order) in synaptic clefts. If the neurotransmitters were to have nanomolar binding affinities for receptors on the synaptic membranes (post- or pre-synaptic) then the receptor sites would constantly be maximally occupied. Moreover, it would be nearly impossible to displace in vivo such a high affinity receptor occupation of a neurotransmitter by systemically administered antagonists, since the latter would never reach a competitively high concentration at the vicinity of the receptor. It is to be remarked that nanomolar receptor binding affinities of endogenous substances are only to be reconciled with endocrine functions such as known for hormones. The balance of available S_1-binding data indicates that the sites are to be considered as mere binding sites until further proof for a receptor function is furnished.

TABLE III. Correlation between Activities of Compounds in Biochemical Models and in Pharmacological Models for Serotonin Receptors

	Spearman rank correlation coefficient (r_s), number of compounds involved (n),significance (Student's t-test)		
	Serotonin-stimulated adenylate cyclase	S_1-binding sites	S_2-binding sites
In vivo behavorial tests			
tryptamine-induced clonic seizures[5,6,25]	$r_s = 0.393$ n = 7, N.S.	$r_s = 0.474$ n = 16, N.S.	$r_s = 0.831$ n = 19, $p < 0.01$
mescaline-induced head twitches[25]	$r_s = 0.143$ n = 7, N.S.	$r_s = 0.233$ n = 16, N.S.	$r_s = 0.875$ n = 19, $p < 0.01$
5-hydroxytryptophane-induced head twitches[31]		$r_s = 0.43$ n = 12, N.S.	$r_s = 0.98$ n = 13,$p < 0.001$
Effects on isolated peripheral tissues			
serotonin-induced contraction in rat caudal arteries[25,29]	$r_s = 0.071$ n = 7, N.S.	$r_s = 0.335$ n = 17, N.S.	$r_s = 0.912$ n = 25, $p < 0.01$
serotonin-induced contraction in rat fundus strip[25,29]	$r_s = 0.036$ n = 7, N.S.	$r_s = 0.638$ n=19,$p < 0.01$	$r_s = 0.368$ n = 25, N.S.

The binding affinities of the compounds for S_2-receptor binding sites, labelled either by ^{3}H-spiperone or ^{3}H-ketanserin, are totally different from the binding affinities for the S_1-binding sites. It is apparent from the data in Table II that S_2-receptor binding sites and S_1-binding sites are unrelated. On the other hand, the strong correlation between the drug binding affinities measured in assays using ^{3}H-ketanserin and ^{3}H-spiperone in frontal cortex tissue indicate that these ligands label the same S_2-receptor binding sites. Serotonin antagonists with various chemical structure show high binding affinity (nanomolar order) for the S_2-receptor binding sites. Highly significant correlations are found between drug binding affinities for these sites and the potencies of the compounds to antagonize serotonergic behavioural excitation, measured in three different tests (See Table III). This suggests that S_2-receptor binding sites play a role in these effects. Furthermore, it is also shown that S_2-receptor binding affinities of a very large series of compounds correlate virtually perfectly with the potencies of the compounds to antagonize serotonin-induced contractions in rat caudal arteries. Hence, serotonin-induced vasoconstriction is likely to be mediated by S_2-type receptors. S_2-receptor sites and the pharmacological D-receptors measured in the isolated fundus strips are unrelated. The binding affinities of serotonin agonists for the S_2-receptor binding sites are about two orders of magnitude lower than those of the most potent antagonists. This is quite in agreement with the ratio of the *in vivo* potencies of the compounds. An intravenous dose of 100 µmoles mescaline is completely antagonized by 0.07 µmoles subcutaneously administered cyproheptadine.[25] Also, the K_1-value of micromolar order of serotonin itself is as expected for a neurotransmitter function, such as explained above. Moreover, it is found that amongst biogenic amines only serotonin and its congener tryptamine exert physiologically relevant binding affinities for the S_2-receptor binding sites, whereas the other neurotransmitters are virtually inactive. It has thus been demonstrated that S_2-receptor binding sites mediate various actions of serotonin, and that the binding affinities of serotonin, serotonin-like compounds and serotonin antagonists are completely compatible with the anticipated features of a serotonin neurotransmitter receptor.

Structure-Activity Relationships at the S_2-Receptor Binding Sites

Metergoline, ketanserin, cyproheptadine, pipamperone and spiperone all bind very potently to the S_2-receptor sites, but they do not show any structural relationship as far as their crystal structure conformation (see Fig. 1) is concerned. Moreover, metergoline and LSD on the one hand, and cyproheptadine, amitryptiline, imipramine and desipramine on the other hand, are structurally related and yet they exhibit rather large potency differences at the S_2-receptor site. Also the fact that the butyrophenone derivatives spiperone and haloperidol show a 40-fold potency difference whereas pipamperone is almost as potent as spiperone, and that the structurally unrelated molecules LSD and cinanserin are almost equipotent, further add to the complexity of the SAR of the S_2-receptor binding. There is no overall structural kinship between serotonin and its antagonists. It appears that the indole group is an entirely optional feature for antagonists at the S_2-binding site, since pirenperone and pizotifen, which lack the indole moiety, are as potent as the indole derivative, metergoline.

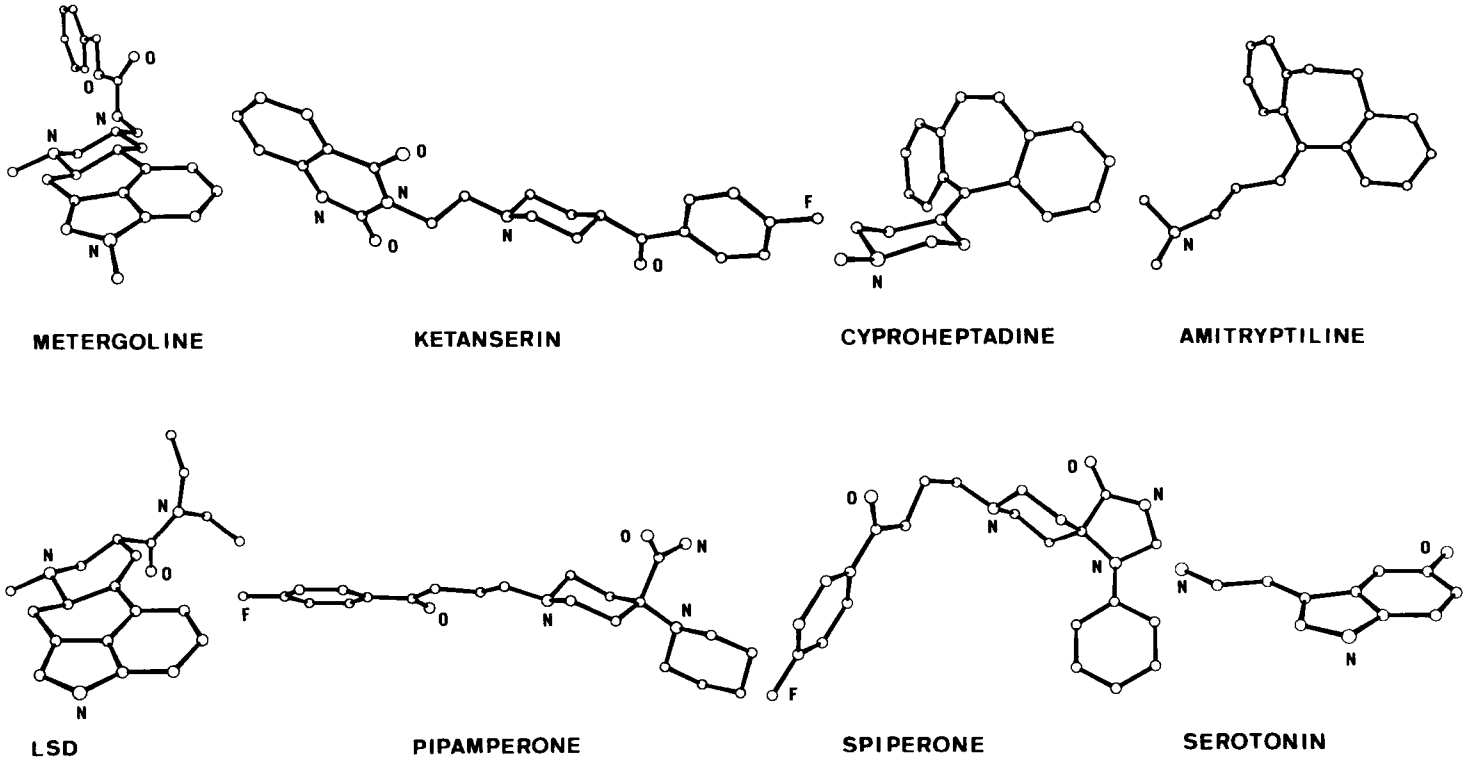

Figure 1. Perspective Drawings[38] Based on X-Ray Crystallographic Data of Some Structural Types of Serotonin Antagonists and Serotonin

In vivo Regulation of Serotonin Receptor Sites, Relationship with Disease States?

A number of investigators have studied alterations in ^{3}H-serotonin binding (S_1-binding sites) after various types of neuronal lesions and after in vivo treatment of the animals with various kinds of drugs.[29] Lesioning of serotonergic neurones by 5,7-dihydroxytryptamine[15] or 5,6-dihydroxytryptamine[39,2,17,18,40] did not affect S_1-binding sites, and neither did 6-hydroxydopamine lesions of the noradrenergic and dopaminergic neurons of the medial forebrain bundle.[41] Chronic drug treatment with 5HT releasing agents[42], monoamine re-uptake blockers,[43,44,45] or ergoline derivatives[46,42] caused no alteration of ^{3}H-serotonin binding (S_1-receptors). In contrast, a number of recent studies showed that S_2-receptor binding sites are decreased in number after chronic treatment of animals with antidepressant agents which block serotonin-reuptake.[47,48,49] The time course of the S_2-receptor site decrease was thought to match the slow onset of the therapeutic effect of the compounds. On the other hand, repeated electroconvulsive shocks were reported to increase the S_2-receptor site density.[47,50] Finally, S_2-receptor binding sites were reported to be decreased in postmortem brains of patients with Huntington's disease.[51] Hence, there are promising leads which suggest a role for S_2-receptor alterations in several disease states.

Conclusion

Amongst the three proposed biochemical models of serotonin receptors, only the S_2-receptor binding sites are abundantly shown to be related to pharmacological actions of serotonin. Hence, only this model can be indicated as a receptor, whereas the other models should be considered only as enzyme or binding sites of unknown relevance. Nothing is known as yet about the mechanism by which the S_2-receptor occupation is transduced in the cell membrane to lead to the physiological or pharmacological response. The study of the involvement of the S_2-receptor in diseases and in the therapeutic actions of drugs has only just started and leaves much to be explored. The availability of specific S_2-receptor antagonists is anticipated to stimulate this research.

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Chapter 2. Anti-Anxiety Agents, Anticonvulsants, and Sedative-Hypnotics

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Introduction - A greatly intensified interdisciplinary research effort concerning the mechanism of action of the benzodiazepines (BZs) and other anxiolytics has led to the rapid accumulation of data concerning the functional, biochemical, and physiological nature of the BZ receptor complex. Significant findings have been reported concerning receptor interactions, regional and cellular localization, heterogeneity, therapeutic agents other than BZs that interact with this complex, and the presence (or absence) of endogenous ligands. The discovery of specific CNS BZ receptors and ensuing research have provided a significant impetus to the search for new anxiolytics. It is hoped that this accumulating wealth of data will lead to a better understanding of the underlying biochemical mechanisms of action of anxiolytics, anticonvulsants and sedatives, and to a better understanding of the causes and nature of the disorders for which they are used.

Benzodiazepine Receptors - Strong evidence continues to support a functional link between BZ, GABA, and chloride recognition sites, possibly as part of a single macromolecular complex[1-3] that may also include barbiturate binding sites.[4] While the BZ binding site appears to be physiologically relevant to at least some of the clinical actions of these drugs, the exact role of GABA in these actions is less clear.[5] It appears that some of these pharmacological actions of BZs may be mediated by an activation of GABAergic transmission.[6] There is evidence that the sedative and anticonvulsant effects, but not the antianxiety effects, of BZs may be mediated by GABA.[7] This may be consistent with the proposal of two types of BZ receptors: Type I, which is not coupled to GABA receptors and which could mediate the anxiolytic actions, and Type II, which is coupled to the GABA receptor and is responsible for pharmacological effects other than anxiolytic actions.[8] While early studies indicated the presence of only a single homogeneous class of BZ binding sites, considerable evidence now exists for such BZ receptor heterogeneity.[9-12] Multiple binding sites appear to exist for both GABA and BZs, with some GABA and BZ sites functionally related, while others are independent of each other.[13] All BZ receptors may, however, be coupled to GABA receptors indirectly through anion binding sites.[14] At least two pharmacologically distinct types of BZ binding sites, which are located on different cell types, have been detected in mammalian central nervous tissue.[15] Electron microscopic autoradiography has been used in conjunction with immunocytochemistry to confirm that there are BZ receptors localized in GABAergic synapses. However, other BZ receptors may be localized in non-GABAergic synapses.[16] Light microscopic radiohistochemistry has also provided evidence for the existence of at least two types of BZ receptors as well as their distribution in various brain areas.[17] Molecular weights of approximately 57,000 daltons for brain BZ receptor and 34,000 for kidney BZ binding sites have been calculated.[18] Purification of BZ receptor from rat brain by affinity chromatography has been reported.[19] Chronic administration of relatively large doses of clonazepam to mice induced BZ receptor subsensitivity through a decrease in the number of BZ binding sites in the forebrain.[20]

Drugs such as picrotoxin which act on GABA receptor associated chloride ion channels have potent modulatory effects on BZ receptor binding.[21] It appears, however, that only the bicuculline-sensitive chloride-linked GABA receptors, and not the chloride-independent GABA receptors, are coupled to BZ receptors.[22] The complex interrelationship of these receptors has been the subject of numerous papers.[5,6,23,24]

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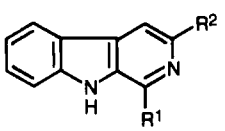
Both BZs and barbiturates potentiate chloride dependent GABA mediated responses; the barbiturate receptor, like the BZ receptor, appears to be coupled to the GABA postsynaptic receptor-ionophore complex.[25] Ethanol and barbiturates also enhance ^{3}H-diazepam (DZ) binding at the BZ-GABA receptor-ionophore complex, via the picrotoxinin sensitive site.[26-28] Certain anticonvulsants, convulsant BZs, and pyrazolopyridine anxiolytics also appear to interact at this barbiturate-picrotoxinin site.[29-34] Several reviews on the BZ receptor complex and agents that interact with this complex have been published.[35-39]

Endogenous Ligands - Efforts to isolate and identify an endogenous substance in the brain interacting specifically with the BZ receptor continue.[40] Various fractions from brain extracts have been shown to alter ^{3}H-BZ binding, suggesting the presence of one or more endogenous protein modulators.[41-45] Numerous studies continue to implicate certain purines (e.g., inosine, hypoxanthine) as possible low affinity endogenous ligands or modulators for the BZ receptor.[46-48] The finding of a pronounced potency correlation between inhibition of BZ binding, inhibition of adenosine uptake, and clinical and pharmacological actions further supports the proposal that inhibition of adenosine uptake may play an important part in the anxiolytic actions of the BZs.[49,50] Other studies have also implicated a "purinergic mechanism" for at least some of the central effects of the BZs,[51,52] although one study failed to show a correlation between compounds active in ^{3}H-DZ and ^{3}H-2-chloradenosine (A_1 adenosine receptor) binding assays.[53] Adenosine receptors in the CNS and the effects of adenosine have been reviewed.[54] Other possible endogeneous mediators involved in the neural mechanisms of anxiety and/or anxiolytics may include serotonin,[55-57] noradrenaline[58] and opiate peptides.[59]

Although it does not appear that ethyl β-carboline-3-carboxylate (β-CCE, 1a) itself is present as an endogenous ligand as originally thought, the question as to whether or not a related congener may function as such is under intensive investigation. β-CCE has high affinity (2-7nm) for the brain BZ receptor but antagonizes certain in vivo effects of BZs and lowers the pentylenetetrazol (PTZ) seizure threshold.[60] It shows the same brain regional binding specificity in vivo as in vitro (more potent in the cerebellum than the hippocampus), and may exert its in vivo effects via binding to the BZ receptor[61-63] or a BZ receptor subpopulation (e.g. BZ_1).[64,65] Unlike ^{3}H-DZ, ^{3}H-βCCE binding to the BZ receptor is reportedly not enhanced by GABA.[66] β-CCE does, however, antagonize certain inhibitory actions of GABA, in addition to antagonizing certain behavioral effects of BZs. It has been suggested that β-carboline ligands may be agonists and BZs may be antagonists at the BZ receptor.[67] The methyl ester 1b (β-CCM) is also a potent inhibitor of ^{3}H-DZ binding; however, ^{3}H-β CCM binding is reportedly reduced by GABA.[65] Conflicting reports on the binding of the propyl ester, ^{3}H-CCP (1c) have appeared.[65] Thus, whether actual differences exist with regard to the effect of GABA on the binding of these three esters remains to be confirmed. ^{3}H-CCP has been used as a probe for investigating BZ receptor heterogeneity in support of BZ receptor subtypes, and as a selective radioligand for the BZ_1 receptor subclass.[65]



1a, R^1 = H, R^2 = $CO_2C_2H_5$
1b, R^1 = H, R^2 = CO_2CH_3
1c, R^1 = H, R^2 = $CO_2nC_3H_7$
1d, R^1 = CH_3, R^2 = H
1e, R^1 = H, R^2 = H
1f, R^1 = H, R^2 = CH_2OH

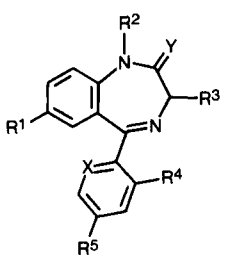
Other β-carbolines and related compounds under investigation as possible endogenous ligands include the convulsants harmane (1d) and norharmane (1e)[68,69] and related dihydro, tetrahydro and ester derivatives,[70] tryptophan derivatives,[71-73] and compound 1f which has been shown to antagonize some pharmacologic actions of DZ.[74]

Animal Models - The effects of anti-conflict drugs have been compared in three experimental animal models of anxiety: conditioned suppression of drinking, modified Geller-Seifter, and modified Estes-Skinner conditioned emotional response.[75] DZ antagonized the discriminative stimuli of PTZ, cocaine,[76] and yohimbine.

Yohimbine discrimination in rats has therefore been suggested as a model for detecting anxiolytics.[77] The conflict situation arising from combining foot shock punishment behavioral suppression with intracranial self-stimulation reward has also been proposed as a method for the evaluation of anxiolytics.[78] The neuropharmacological mechanisms of behavioral inhibition animal models of anxiety have been discussed.[58,79] The pharmacologic specificity of a recently described simple animal model, in which BZs increase mouse exploratory activity between a brightly lit open field and a small dark compartment, supports the use of this paradigm as a model for the behavioral effects of BZs.[80] Muscimol-induced myoclonus in mice has been proposed as a valid animal model to detect drugs effective in the treatment of clinical myoclonus (Lance-Adams Syndrome).[81,82] Rat models using kainic acid induced seizures,[83] kindled seizures[84,85] and flash-evoked after discharge[86] have been used to evaluate agents effective against seizures and epilepsy. A new model of insomnia in rats[87] and a rat gastric fistula model[88] have been used to evaluate sedative-hypnotics.

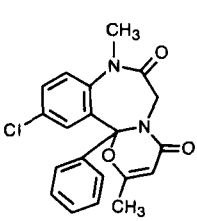
Benzodiazepines and Related Compounds

Anxiolytic Agents - The pharmacological and biochemical properties of BZs,[89-91] and the clinical use of BZs and other anxiolytics have been the subject of several



	R^1	R^2	R^3	R^4	R^5	X	Y
2a	Cl	CH_2 ◁	H	H	H	CH	O
2b	Cl	H	OH	Cl	H	CH	O
2c	Br	H	H	H	H	N	O
2d	Cl	H	OH	H	H	CH	O
2e	Cl	H	$CO_2C_2H_5$	Cl	H	CH	O
2f	NO_2	H	H	Cl	H	CH	O
2g	Cl	CH_3	H	H	Cl	CH	O
2h	Cl	CH_3	OH	H	H	CH	O
2i	Cl	CH_2CF_3	H	F	H	CH	S
2j	Cl	CH_3	OH	Cl	H	CH	O
2k	Cl	$(CH_2)_2N(C_2H_5)_2$	H	F	H	CH	O
2l	Cl	$(CH_2)_2NCS$	H	F	H	CH	O
2m	Cl	$(CH_2)_2NHCOCH_2Br$	H	F	H	CH	O

reviews.[92-94] Results of clinical studies on the treatment of various anxiety states with ketazolam (3),[95-97] prazepam (2a),[98,99] lorazepam (2b),[96,98,99] bromazepam (2c),[100] GP55,129 (4a),[101] alprazolam (4b),[102] oxazepam (2d)[103] and ethyl dirazepate (2e)[104] have been reported, as well as clinical studies[105,106] and a review of the pharmacological properties and therapeutic use in anxiety of clobazam (5).[107] The clinical pharmacokinetics of oxazepam and lorazepam have also been reviewed.[108] The use of clonazepam (2f) in the treatment of tardive dyskinesia[109] and the effect of DZ on memory and performance have been studied.[110] Ro21-8384 (6) was more potent than DZ in anti-conflict tests in rats and squirrel monkeys, with a favorable separation between anxiolytic and sedative properties.[111] The 1,5-BZ



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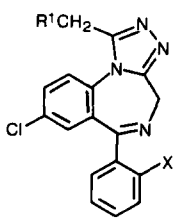
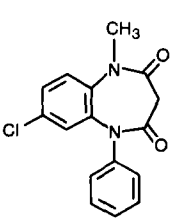


4a, R^1 = OH, X = H
4b, R^1 = H, X = H
4c, R^1 =, H, X = Cl



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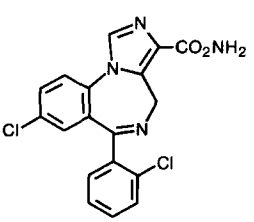

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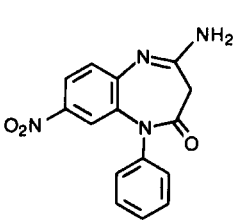
CP1414S (7) has a classical BZ pharmacological profile but is about ten times less potent than DZ in PTZ (ED_{50}=11.4 mg/kg ip), conflict, and rotorod (ED_{50}= 42 mg/kg ip) tests.[112] The 3,4-BZ tofisopam (8a) does not bind to BZ receptors but does enhance ^{3}H-flunitrazepam (FNM) binding.[113] GYKI 51,189 (8b), an analog of tofisopam, reportedly has tranquilizing effects comparable to chlordiazepoxide[114] but without anticonvulsant or muscle relaxant activity.[115] Ro5-4864 (2g) has been shown to bind with high affinity to BZ binding sites in membrane preparations of both rat kidney and cerebral cortex. The pharmacological specificity of the ^{3}H-Ro5-4864 binding site, with respect to binding of other drugs, appears to vary from that of the normal BZ binding site.[116] The convulsant BZ Ro5-3663 (9) binds to dihydropicrotoxinin (DHP) binding sites and has anxiogenic properties antagonized by DZ.[117] The imidazo-1,4-BZ Ro15-1788 (10) is a selective BZ antagonist in vitro and in vivo, acting at the level of the BZ receptor in the CNS. It is relatively non-toxic and produces none of the characteristic effects of BZs. It is being evaluated clinically as a BZ antagonist[118] and is used as a research tool for studying BZ receptors and possible endogenous ligands and to distinguish between BZ receptor agonists and antagonists in vitro.[119] Ro15-1788 has also been shown to be an antagonist of β-CCE.[60] An in vitro binding assay which differentiates BZ agonists and antagonists has been reported.[120]



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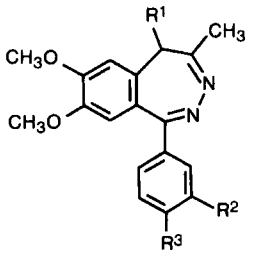
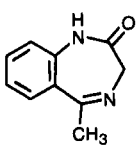


8a, $R^1 = C_2H_5$; $R^2 = OCH_3$; $R^3 = OCH_3$ 8b, $R^1 = H$; $R^2 = Cl$; $R^3 = H$



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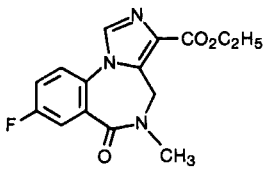
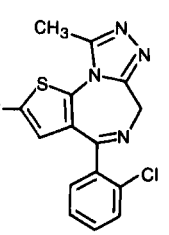


10

Sedative-Hypnotics - The pharmacological and therapeutic efficacy of the hypnotics temazepam (2h)[121] and triazolam (4c)[122] have been comprehensively reviewed. The clinical effectiveness of quazepam (2i) as a hypnotic for insomnia continues to be documented,[123-126] as has the effectiveness of lormetazepam (2j)[127,128] and brotizolam[129] (WE-941) (11). Other hypnotics studied in various clinical situations include ketazolam (3),[130] flurazepam (2k),[131,132] and midazolam (12).[133]



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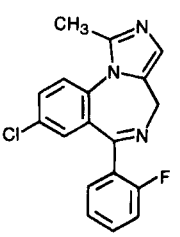
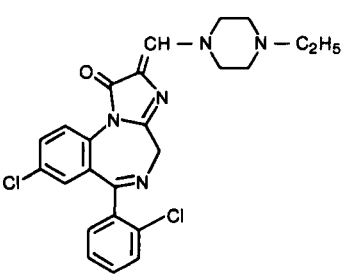


12

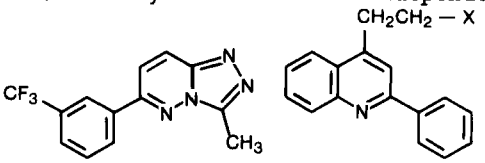
Anticonvulsants - The alkylating benzodiazepines irazepine (2l) and kenazepine (2m) inhibited ^{3}H-DZ binding and afforded a long-lasting protection of mice against PTZ-induced seizures when administered intracerebroventricularly but were inactive when administered parenterally.[134] In rodents and in baboons, RU31124 (13) demonstrated greater anticonvulsant potency than clonazepam but with less sedative and muscle relaxant side effects.[135]



13

Non-Benzodiazepines

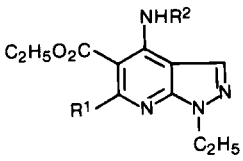
Anxiolytic Agents - Synthesis, pharmacology and SAR data have been reported for the series of 1,2,4-triazolo[4,3-b]pyridazines exemplified by CL218,872 (14).[136] Although clinical trials on this compound have been discontinued,[137] CL218,872 has been used to discriminate between BZ receptor subtypes.[138] The quinoline derivatives PK8165 (15a) and PK9084 (15b) appear to be "pure anti-conflict drugs," in that they increased punished responding in the rat lick shock conflict procedure (MED 6.5 and 1.8 mg/kg ip, respectively), but did not produce ataxia or sedation at doses 5-20 times higher than those effective in the conflict test, and did not demonstrate any anticonvulsant activity in standard animal tests. They selectively inhibit ^{3}H-DZ binding, showing greater potency for brain than kidney BZ binding sites. Binding is enhanced in the presence of halides, suggesting that PK8165 and PK9084 may act on a GABA-independent BZ receptor associated with a chloride



14

15a, X = NH

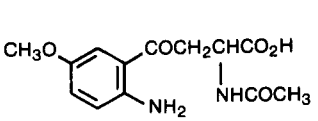
15b, X = N H



16a, R^1 = H, R^2 = n − C_4H_9

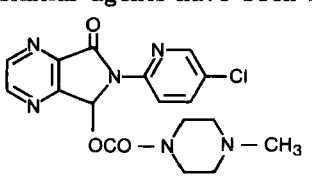
16b, R^1 = H, R^2 = N = $C(CH_3)_2$

16c, R^1 = CH_3, R^2 = n − C_4H_9



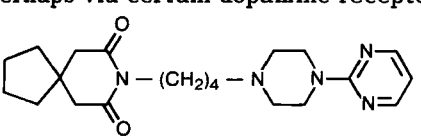
17

ionophore.[13] The anxiolytic pyrazolopyridines cartazolate (16a) and etazolate (16b), like pentobarbital, enhance BZ binding, possibly by acting at the picrotoxinin-sensitive chloride ion channel site.[139,140] Tracazolate (16c) enhances both BZ and GABA binding in vitro.[141] Details of its pharmacological properties and anxiolytic profile have been reported.[142] Melatonin (N-acetyl-5-methoxytryptamine) and its brain metabolite N-acetyl-5-methoxykynurenamine (17) were found to inhibit ^{3}H-DZ binding (IC_{50}=545 and 65 μM, respectively). These or similar agents have been suggested as possible endogenous modulators of the BZ receptor.[71] Although zopiclone (18) binds only weakly to the BZ receptor, it is reportedly twice as active as DZ in the Geller conflict test and suppresses aggressive behavior with essentially no muscle relaxation and no inhibition of electroshock-induced convulsions.[143]



18

Buspirone (19) has been shown to be active in the Geller conflict test in rats and monkeys[144] and to be a clinically effective anti-anxiety agent without anticonvulsant, sedative or muscle relaxing side effects. Since buspirone does not bind to either BZ or GABA receptors, its anti-anxiety effects may be mediated differently, perhaps via certain dopamine receptors.[145,146]



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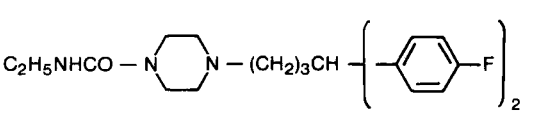
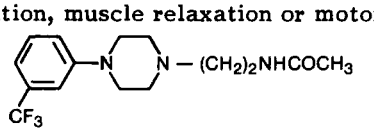


20

The piperazine derivatives amperozide (FG5606) (20)[147] and DU27,725 (21)[148] have shown pronounced selective anti-aggressive activity in animals with little or no sedation, muscle relaxation or motor impairment.



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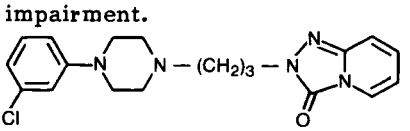

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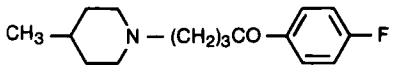
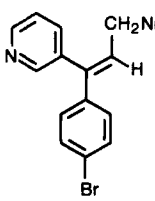


23

The use of the antidepressant trazodone (22) in the treatment of anxiety has been reviewed.[149] Clinical trials have indicated melperone (23)[150] and zimelidine (24)[151] to also be effective in certain anxiety conditions. The anxiolytic effect of zimelidine may involve 5-HT.[57] Several reports on the anxiolytic activity of clonidine have appeared.[152-155] The use of β-blockers in the management of stress and anxiety has been discussed. Contrary to BZs, β-blockers most likely exert their anti-anxiety effects mainly through peripheral influences.[156,157] The clinical use of prochlorperazine[158] and nabilone[159] in the treatment of special anxiety conditions has been studied. The pyrazolo [4,3-c] quinoline CGS 8216 (25a) is a potent BZ antagonist; interestingly, however, 25b is an agonist with anticonvulsant and anxiolytic activity, and 25c is a partial agonist.[160,161] PTZ and related tetrazoles competitively inhibit ^{3}H-DZ binding, and may elicit their convulsant effects by interaction with the BZ receptor.[162]



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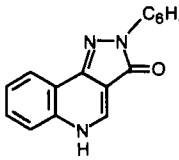
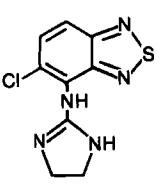


25a, X = H
25b, X = Cl
25c, X = OCH_3

Sedative-Hypnotics - The mechanism of action of barbiturates has been reviewed[163] and the effect of various barbiturates on specific neurotransmitter functions examined.[164-166] The amino acid lysine has been shown to enhance hexobarbital-induced sleep in the rat, and may act as a modulator of sleep behavior of animals.[167] The adenosine analogs cyclohexyladenosine and 2-chloroadenosine displayed sedative and anticonvulsant properties and reversed DZ-induced increase in exploratory behavior in mice.[168,169]



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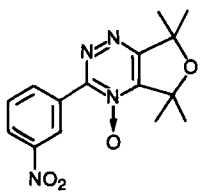
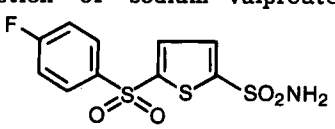


27

The skeletal muscle relaxant DS103-282 (26) was compared to DZ in the clinical treatment of muscle spasms of local origin, and was found to be a more potent and faster acting myoclonolytic agent than DZ, well tolerated with no notable side effects.[170]

From a series of novel furo[3,4-e] -as-triazines, the 4-oxide 27 was found to be a potent sleep inducer. In addition, there was some indication of potential anxiolytic activity from Geller conflict results.[171]

Anticonvulsants - Numerous articles and reviews concerning the mechanism of action of sodium valproate[172-174] and anticonvulsants in general have



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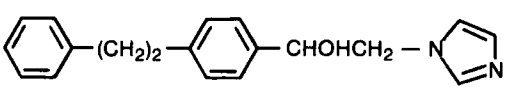
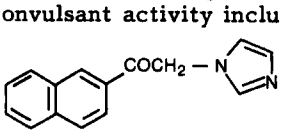


29

appeared.[175-178] Compounds found active in various animal models indicative of anticonvulsant activity include the cerebral vasodilator 28,[179] the imidazoles 29[180]



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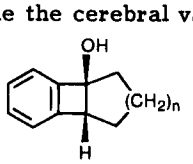
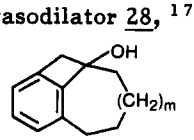
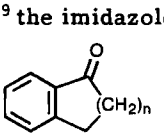


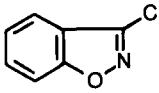
31, n = 1-4



32, m = 1-4

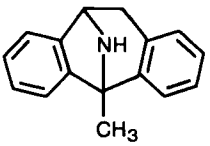


33, n = 1-6



34

and 30,[181] the benzocyclobutanols 31 and 32, the benzocyclanones 33,[182] and the benzisoxazole AD-810 (34).[183] MK-801 (35) is a potent anticonvulsant with central sympathomimetic and apparent anxiolytic properties. Details of its pharmacological and biochemical properties, mechanism of action and therapeutic potential have been discussed.[184]



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Chapter 3. ANALGESICS (PERIPHERAL AND CENTRAL), ENDOGENOUS OPIOIDS AND THEIR RECEPTORS

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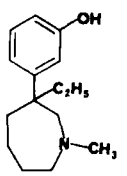
Introduction - Research related to the development of new and improved analgesics has proceeded forward on several different fronts. The acceptance of the non-steroidal anti-inflammatory drugs (NSAIDs) as effective analgesics has accelerated the search for more specific peripherally acting analgesics. The search for superior, orally effective, centrally acting analgesics has intensified and several potential candidates are under clinical evaluation. The opioid peptides and their receptors continue to be widely studied. There is now overwhelming evidence for the presence of two distinct populations of opioid receptors, the μ-(morphine) receptor and the δ-(enkephalin) receptor and there is good evidence for the existence of a third receptor, the κ-receptor. The physiological functions of these receptors are unknown but are actively being explored. Structure activity relationship (SAR) studies have focused on the discovery of receptor-specific agents as new analgesics.

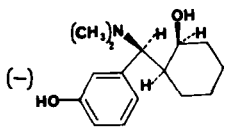
Clinical Studies

Centrally Acting Analgesics - The results of several clinical studies were published during the last year. Additional oral efficacy studies with butorphanol[1,2] (Stadol®) and nalbuphine[3] (Nubain®) were reported. Orally, both butorphanol and nalbuphine appear to be approximately one-fourth to one-fifth as potent as by the intramuscular route. Propiram continues to be clinically evaluated for oral analgesic activity. At 50 mg, propiram compared favorably to 60 mg codeine in episiotomy[4] and postsurgical[5] pain.

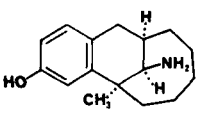
Clinical studies of meptazinol[6,7] and ciramadol[8] were reported. There was no difference in effectiveness between meptazinol (200 mg, p.o.) and pentazocine (50 mg, p.o.) in patients with chronic back pain,[7] whereas ciramadol (60 mg, p.o.) provided significantly better pain relief than pentazocine (50 mg, p.o.).[8] Reports on the effectiveness of parenteral dezocine (WY16225) have been issued.[9-11] In one study, dezocine (10 mg, i.m.) appeared equipotent with morphine (10 mg, i.m.). Doxpicomine, a structurally unique partial opiate agonist, at 400 mg, i.m., provided pain relief at least equivalent to morphine (8 mg, i.m.).[12] Side effects with doxpicomine were reported to be infrequent and minor.



MEPTAZINOL



CIRAMADOL



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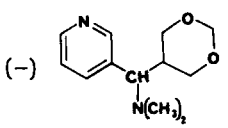

DOXPICOMINE

Preliminary clinical data with the enkephalin analog metkephamid (LY127623) indicate that it has analgesic activity.[13] A comparison of

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the clinical pharmacology of metkephamid and FK33-824, another synthetic enkephalin analog, indicates that metkephamid does not share many of the disturbing and limiting side effects reported for FK33-824.[14] Whereas, FK33-824 shows a selectivity for the μ-receptor,[15] metkephamid has been reported to have a higher preference for the δ-receptor relative to standard opiates.[13] SAR studies of metkephamid analogs have been reported.[16] Recent clinical reports have described the effects of FK33-824 on gastrointestinal motility[17] and endocrine effects on the pituitary.[18,19] Clinical results of a third enkephalin analog (FW34-569) have been published. Although FW34-569 (0.5 mg or 1.0 mg, i.m.) increased tolerance to experimentally induced pain, the pain threshold was unaffected. Other effects such as heaviness of body and nasal stuffiness were observed. It was concluded that FW34-569 has no therapeutic potential as an analgesic agent by systemic administration.[20]

H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2

Metkephamid

H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol

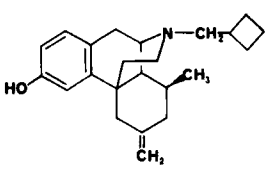
FK33-824

MeTyr-D-Ala-Gly-MePhe-Met(O)-ol

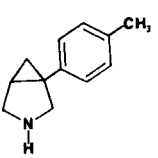
FW34-569

Picenadol (LY150720) and TR5379 are two new narcotic agonist-antagonist analgesics reported to be under clinical evaluation. TR5379 has been described as an analgesic with anti-antagonist properties.[21] SAR studies of TR5379 and related compounds have been published.[22,23] Picenadol, an unusual new analgesic, is a racemic mixture whose partial agonist properties are a consequence of the *d*-optical isomer being a potent opiate agonist and the *l*-isomer being a narcotic antagonist. The preclinical pharmacology[24] and SAR considerations[25] of picenadol have been published. Bicifadine is an analgesic thought to act centrally but not at opioid receptors.[26,27] In a clinical study in postoperative pain, only limited analgesic activity was demonstrated (100 and 150 mg, p.o.).[28] The GABA-mimetic THIP was reported to be undergoing clinical analgesic trials. In rodents THIP produced analgesia, with a potency comparable to that of morphine, that was not reversed with naloxone.[29]

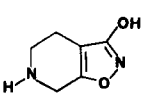
PICENADOL



TR 5379



BICIFADINE

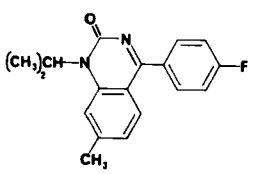


THIP

Levonantradol, a cannabinoid derivative, at intramuscular doses of 1.5, 2.0, 2.5 or 3 mg, was found to produce analgesic activity in acute postoperative pain; however, no significant dose-response curve was observed.[30] Alfentanil, a very short-acting narcotic analgesic structurally related to fentanyl, is undergoing clinical evaluation as an anesthetic agent.[31,32]

Peripherally Acting Analgesics - The four orally effective NSAIDs that were approved in 1980 for the treatment of pain (fenoprofen, Nalfon®; ibuprofen, Motrin®; naproxen, Anaprox®; and zomepirac, Zomax®) appear to have been well accepted. Additional clinical studies supporting the effectiveness of fenoprofen,[33] naproxen,[34] and zomepirac[35] were published.

There were reports of other NSAIDs under clinical evaluation as oral analgesics. In a supplement to Arzneimittel-Forschung/Drug Research,[36] detailed information of the animal pharmacology and clinical evaluation of fluproquazone (Tormosyl®) was published. In a multiple-dose study in postoperative pain patients, fluproquazone (100 mg) produced relief at least comparable to paracetamol (500 mg). Gastrointestinal side effects were observed in both groups.[37] Comparative efficacy studies with suprofen[38] demonstrated that at 200 mg it provided pain relief at least equivalent to the combination of aspirin (650 mg) and codeine (60 mg).[39,40] Similarly, indoprofen (200 mg) was found to be superior to acetaminophen (650 mg) with codeine (60 mg).[41] A review of clinical studies on indoprofen has been published.[42] Other NSAIDs that were reported to have demonstrated clinical analgesic effectiveness include flurbiprofen,[43,44] fentiazac,[45] and BPPC.[46]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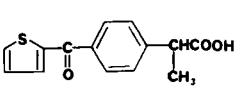
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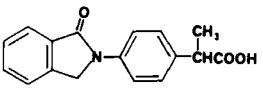
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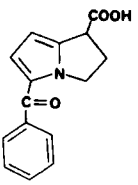
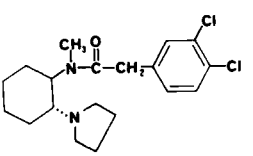
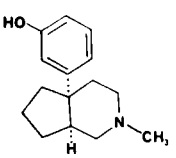

BPPC

SAR Studies

U-50,488 was reported to be a prototype of a new class of opioid analgesics with reduced physical dependence liability.[47] Its analgesic potency (approximately one-half that of morphine in the mouse writhing test) varies according to the intensity of the nociceptive stimulus. Resolution of compound 1 showed that the *d*-isomer was a potent morphine-like agonist while the *l*-isomer had properties of a partial opiate agonist.[24] The phenylpiperidine 2, a known pure antagonist, was changed by the addition of a 2-methyl group to the pure agonist (3) which is at least as potent as morphine. This reversal of effects was rationalized as a change in the conformational binding mode from an equatorial to an axial phenyl.[25]


U-50,488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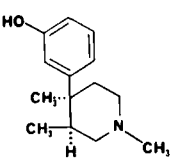
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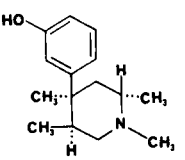
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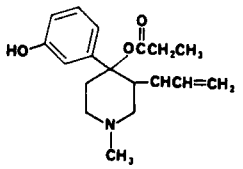
The m-hydroxy analogs of allylprodine (4) were found to be without appreciable narcotic agonist or antagonist activity.[48] This observation led to a proposal that different aromatic recognition sites exist on the μ-receptor and that these may correspond to the aromatic rings of the tyrosine or phenylalanine residues in enkephalins. The N-arginyl derivative 5 was found to be equally potent to methionine enkephalin (Met-enk) in its ability to inhibit tritiated naloxone binding to opioid receptors.[49] The (-)-4-methoxy-N-methylmorphinan-6-one 6 showed opioid receptor affinity one-third that of morphine,[50] and its analgesic potency (3X morphine) was at least equivalent to that of its 4-hydroxy

analog.[51,52] Compound 7 was found to have analgesic activity equivalent to that of etorphine, proving that the C-6 methoxy group present in etorphine was not necessary for potent analgesic activity.[53]



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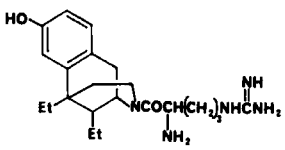

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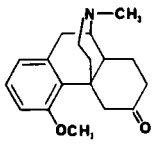

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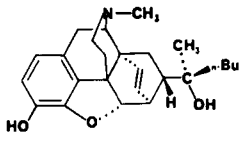
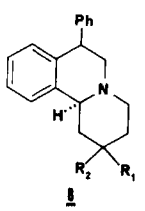


7

Four diastereomeric conformationally restricted analogs of fentanyl (8) were found to have only weak analgesic activity.[54] The opioid receptor affinities of other analogs of fentanyl were reported.[55] Narcotic antagonist activity was found with a number of derivatives of m-hydroxy substituted 4-aryl-4-aminocyclohexanone, 9.[56] Compound 10 proved to be a pure narcotic antagonist equal in potency to naloxone.[57] The N-allyl and N-cyclopropylmethyl analogs of (-)-α-acetylmethadol and (-)-α-methadol possess only weak opiate agonist activity.[58]

Practical total syntheses of the morphine alkaloids and the thebaine-based derivatives appear to have been achieved. The total synthesis of (-)-dihydrocodeinone was reported, and conversion to morphine and codeine by known processes should give overall yields in the range of 10-30%.[59,60] Conversion of codeine to noroxycodone (from which naloxone and nalbuphine could be synthesized) was also reported.[61] Efficient synthesis of the two morphine fragments 11[62] and 12[63] have been accomplished.



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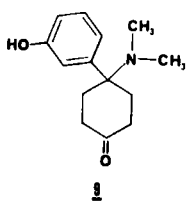

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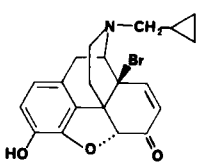

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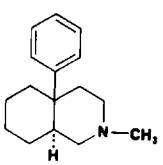

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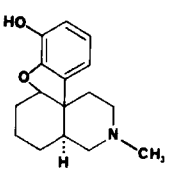
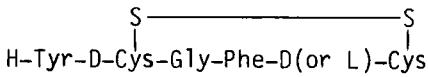


12

Opioid Peptides

An extensive review, "Endogenous Opiates:1980", was published during 1981.[64] Other review articles and lead references to topics of interest discuss the spinal action of opiates,[65,66] the epidural[67-69] and intrathecal[70] administration of opiates for obstetric analgesia, studies of enkephalins and the blood-brain barrier,[71] the problems of peptide transport,[72] the interaction of opiates and opioid peptides with the endocrine system,[73] the role of peptides as neurotransmitters,[74] and the effects of opiate-like agonists and antagonists on respiratory depression[75,76] and monoamine neurotransmitter release.[77-79]

Enkephalin Analogs - Several approaches to conformationally restricted enkephalin analogs were described during 1981. Some cyclic analogs (notably 13 and 14) possess strong in vitro and in vivo opiate-like effects, as well as increased resistance to enzymatic degradation.[80,81] Analogs with restricted rotation about the amide bond between residues 1, 2, 3, or 4, however, possess reduced opiate-like activities.[82-84]



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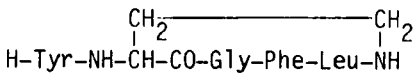

(14)

Extremely potent tetrapeptide enkephalin analogs which appear to act primarily at the μ-receptor have been reported.[85,86] Search for the minimal enkephalin fragment required for analgesia has led to development of substituted tripeptides with potent activity.[87,88] The dipeptide H-Tyr-D-Ala-NH-$(CH_2)_3$-C_6H_5 produces analgesia (i.c.v.), comparable to that of a standard (H-Tyr-D-Ala-Gly-Phe-Met-NH_2), as measured by the mouse tail-flick assay.[89]

Other Opioid Peptides - The complete 17 amino acid sequence of porcine dynorphin (DYN) has been published.[90] Although DYN is one of the most potent naturally occurring opioid peptides in the guinea pig ileum (GPI) assay, it does not produce appreciable analgesia. It has been suggested that DYN may be involved in the development of morphine tolerance and physical dependence.[91] Evidence has been presented that DYN(1-8) is the major DYN-related peptide in the rat pituitary.[92]

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-
-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH Dynorphin

The structure of α-neo-endorphin, a proposed leucine enkephalin (Leu-enk) precursor, has been revised. This decapeptide has potent in vitro (GPI) opiate-like activity which suggests a possible physiological function.[93]

H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-OH α-Neo-endorphin

Two new opioid peptides, dermorphin (DERM) and [hydroxyproline^6]-DERM, which have been isolated from methanol extracts of the skin of South American frogs, represent the first reported cases of the presence of a D-amino acid residue in natural peptides isolated from a vertebrate.[94,95] The DERM peptides possess potent opiate-like activities in vitro and in vivo (i.v. or i.c.v.). Studies have indicated that the minimum structure required for full DERM-like activity is H-Tyr-D-Ala-Phe-Gly-NH_2.[96]

H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 Dermorphin

In in vitro assay systems, β-casomorphin and related fragments (e.g., H-Tyr-Pro-Phe-Pro-NH_2, morphiceptin), although of limited potency, are selective for the μ-receptor, rather than the δ-receptor.[97,98]

Kyotorphin, H-Tyr-Arg-OH, has been reported to produce naloxone-reversible analgesia when administered intracisternally (ED_{50} = 35 nmol/mouse-tail pinch and 16 nmol/mouse-hot plate).[99] While it does not bind to opioid receptors, kyotorphin may produce analgesia by depolarization of enkephalinergic neurons to release Met-enk.[100]

The opiate-like materials humoral endorphin,[101] pancreatic β-endorphin-like polypeptide,[102] rat mesentery factor,[103] and a non-peptide morphine-like compound,[104] have been isolated but not fully characterized.

Substance P - Substance P (SP) has been described as a neurotransmitter for primary nociceptor afferents,[66] therefore, an SP antagonist was

predicted to be an analgesic agent with a novel mechanism of action. Two synthetic SP antagonists (15,16) have now been synthesized.[105,106] The SP antagonist 15 inhibited the inflammatory response promoted by application of SP or infrared radiation on the rabbit eye.[107] Although 16 depressed the scratching and biting behavior elicited by either topical application of nociceptive substances or by intrathecal injection of SP, it was without effect in the standard tail-flick and hot-plate analgesic assays.[108] Evidence continues to accumulate that SP itself can produce analgesia when given i.c.v.[109] or intrathecally.[110]

H-Arg-D-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH_2 (15)

H-Arg-D-Pro-Lys-Pro-Gln-Gln-D-Phe-Phe-D-Trp-Leu-Met-NH_2 (16)

Enkephalin Metabolism - An extensive review of enkephalin metabolism has been published.[111] Two inhibitors of enkephalinase activity, thiorphan [(N-(R,S)-3-mercapto-2-benzylpropionyl)glycine] and phosphoryl-Leu-Phe-OH, have been shown to protect selectively Met-enk released from brain slices[112] and to enhance the analgesia elicited (i.c.v.) by [D-Ala^2]-Met-enk amide,[113] respectively. The microbial aminopeptidase inhibitor bestatin and related analogs were shown to be effective inhibitors (K_i = 80-500 nM) of rat brain enkephalin aminopeptidase.[114] A novel method for the in vivo analysis of the stability of opioid peptides toward aminopeptidase degradation has been reported.[115,116]

Opioid Receptors

A plethora of reviews on the concept of multiple opioid receptors was published during 1981.[117-124] New efforts directed toward the isolation, purification, and characterization of opioid receptors have been described.[125-128] The relationship between opioid receptors and dopamine receptors has been studied.[129]

μ-Receptors and δ-Receptors - The regional distribution of μ- and δ-receptors has been determined in rat[130,131] and bovine[132] brain. In rat brain, regions which possess more μ- than δ-receptors (hippocampus and thalamus) also possess more Met- than Leu-enk neurons, whereas the central amygdala, which possesses more δ- than μ-receptors, has more Leu- than Met-enk neurons.[130] This correlation was not observed in bovine brain.[132] Binding studies in rat brain, however, have led several groups to postulate that opioid receptors do not exist as distinctly separate entities, but rather that μ- and δ-receptors are allosterically coupled[133] and they interconvert through a sulfhydryl-disulfide interchange mechanism.[134] Evidence continues to be presented indicating that μ-receptors are responsible for analgesia, while δ-receptors may be involved in other behavioral effects.[135-138] However, experiments in rats in which selective development of tolerance was produced with either the δ-receptor agonist [D-Ala^2,D-Leu^5]-enk or the μ-receptor agonist sufentanyl, have indicated that both μ- and δ-receptors are able to mediate analgesia and catatonia.[139] The structural requirements of enkephalin related peptides for specific recognition of μ- or δ-receptors have been explored.[140]

κ-Receptors - Binding studies with a radiolabelled benzomorphan, ([^{3}H]-ethylketocyclazocine), have resulted in the characterization of κ-receptors in the guinea pig brain,[141] rabbit vas deferens,[142] and the human placenta.[143] A specific DYN receptor has been demonstrated in the GPI-myenteric plexus,[144] and DYN has been postulated to be the endogenous κ-receptor ligand.[145,146] Selective tolerance experiments utilizing the GPI-myenteric plexus resulted in the demonstration of μ-, κ-[147] and

σ-receptors.[148] Novel binding sites for benzomorphans have been characterized in the rat brain.[149,150]

σ-Receptors - Studies of the binding of the putative σ opiate, N-allyl-N-normetazocine ([^{3}H]-SKF 10,047), in rat brain membranes have led to the characterization of a low affinity binding site which has tentatively been attributed to the σ-receptor.[151] The psychotomimetic effects of the benzomorphan σ opiates and the dissociative anesthetic phencyclidine (PCP) may be mediated through a common receptor.[152]

ε-Receptors - Further efforts have been directed toward the characterization of the ε-receptor, which has been identified as the binding site for β-endorphin in the rat vas deferens.[153] The N-terminal 21 amino acid residues of β-endorphin are required for ε-receptor activation.[154]

Antagonists - The use of irreversible narcotic agonists and antagonists is proving to be a useful technique in the investigation of opioid receptors. The fumarate methyl ester derivative of naltrexone (B-FNA) was found to be a selective antagonist for pure agonists but had little effect on the activities of mixed agonist-antagonists.[155] Investigations with the irreversible antagonist naloxazone have led to the proposal that opiate and opioid peptide analgesia are mediated through a single subpopulation of receptors.[138] The first enkephalin peptides (e.g., 17) which have been reported to possess *in vitro* characteristics of an opiate antagonist, appear to be selective toward the μ-receptor.[156]

H-Tyr-D-Ala-Gly-MePhe-N(CH_2-CH_2-C_6H_5)-CH_2-CH_2-CH(CH_3)$_2$ (17)

Specific antagonists for the δ-receptor have not yet been developed; however, aliphatic alcohols can selectively inhibit the binding of enkephalins to δ-receptors.[157,158]

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Chapter 4. Peptides in the Central Nervous System: Focus on Thyrotropin Releasing Hormone and Neurotensin

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Although substance P was isolated in the central nervous system (CNS) in 1931,[1] interest in neuropeptides languished for four decades, until Guillemin and his associates[2] and Schally and his associates[3] announced the amino acid sequence of thyrotropin releasing hormone (TRH). Interest in peptides was further kindled by the discovery and sequencing of β-endorphin and met- and leu-enkephalin (see Gesellchen and Zimmerman, this volume). In the few years since then, many peptides have been discovered in the CNS and other tissues. To review comprehensively the data generated by these discoveries would require volumes; indeed, books have been devoted to single peptides. The goal, then, of this brief chapter must be truncated. Only an inkling of data pertaining to humans can be provided: TRH appears to be a useful tool in psychoendocrine research;[4] neurotensin (NT) appears to be diminished in the cerebrospinal fluid (CSF) of some schizophrenic patients;[5] somatostatin (somatotropin release inhibiting factor, SRIF) appears to be lowered in certain brain regions of patients with Alzheimer's disease.[6] We will emphasize findings in animals and focus on pharmaco-behavioral effects at the expense of peripheral and endocrine effects. We will comment succinctly about peptides selected for current interest and provide references to recent reviews. We will then discuss TRH and NT more extensively for two reasons: TRH and NT exert a broad range of effects, and they are the two peptides to which the efforts of our research group have been largely directed. A consideration of the actions and interactions of TRH and NT may provide the reader with at least a beginning sense of an aspect of current CNS peptide research.

I. A PEPTIDE POTPOURRI

Hypothalamic Hypophysiotropic Hormones - These substances are released from nerve terminals in the median eminence of the hypothalamus into the hypothalamo-hypophyseal portal system, in which they are transported to the anterior pituitary gland, where they stimulate or inhibit the secretion of adenohypophyseal hormones.[7] As mentioned above, the tripeptide TRH was the first hypothalamic releasing hormone to be identified and sequenced. Four other putative hypothalamic hypophysiotropic hormones -- luteinizing hormone-releasing hormone (LHRH, GnRH), melanocyte stimulating hormone-release-inhibiting hormone (MIF), SRIF, and corticotropin-releasing hormone (CRF, CRH) -- have been identified. The decapeptide LHRH was discovered in 1971 by Guillemin[8] and Schally.[9] Immunoreactive LHRH is distributed heterogeneously in the mammalian CNS, though it is not as widespread as TRH or SRIF.[10,11] When administered peripherally, LHRH stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. An impressive demonstration of an extrapituitary effect of a hypothalamic peptide is the induction of lordosis behavior in hypophysectomized or ovariectomized, steroid-primed female rats by low doses of LHRH.[12,13] Direct gonadal effects of LHRH have also recently been described.[14]

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The tripeptide MIF (Pro-Leu-Gly-NH_2) has been reported to inhibit the release of MSH from the pituitary.[15] Whether this substance, which is identical to the side chain of oxytocin, indeed exerts physiological control over MSH secretion, is unclear. Recently Manberg and his colleagues developed a radioimmunoassay which recognizes the sequence of MIF (and also therefore oxytocin). Using several chromatography techniques, they found that all MIF immunoreactivity in rat brain was, in fact, oxytocin.[16] Nevertheless, the synthetic tripeptide produces a variety of pharmacobehavioral effects in laboratory animals.[17]

Somatostatin, a tetradecapeptide, was discovered in mammalian hypothalamus by Brazeau, Guillemin and their associates.[18] Like other hypothalamic hypophysiotropic hormones, it is widely distributed in mammalian CNS and in the gastrointestinal tract.[10,11] Somatostatin inhibits the secretion of growth hormone (GH), thyrotropin (TSH) and a variety of other hormones.[19] The peptide produces several behavioral effects in rodents, including a reduction in spontaneous locomotor activity.[19] Corticotropin-releasing hormone is a peptide composed of 41 amino acids.[20] It causes the release of corticotropin (ACTH) and β-endorphin from the anterior pituitary gland.

Anterior Pituitary Peptides - Prolactin (PRL), GH, LH, FSH, TSH and ACTH are all localized in pituicytes of the adenohypophysis. In addition, all are reported to be present in CNS tissue and/or CSF. The functional significance of these findings is unclear.[21] Pharmacobehavioral effects have been observed after administration of most of these peptides.[19]

DeWied and his associates[22] have comprehensively examined the effects of ACTH, MSH and their fragments on learning and memory. Prolactin has been reported to induce parental behavior in a variety of avian species.[19] Growth hormone has been reported to alter neurotransmitter metabolism, to increase REM sleep and to protect against restraint-stress-induced gastric ulcers.[19] Luteinizing hormone has been reported to produce behavioral alterations in male starlings, and to exert potent electrophysiological effects on hypothalamic neurons.[19] Follicle-stimulating hormone has been reported to enhance brain norepinephrine turnover.[19] We are not aware of any pharmacobehavioral effects of TSH that cannot be attributed to activation of the thyroid axis.

Posterior Pituitary Peptides - The two octapeptides vasopressin (VP) and oxytocin (OXY) are synthesized in the paraventricular and supraoptic nuclei of the hypothalamus and transported, bound to neurophysins, down the axons of the neurohypophyseal tract to terminate in the posterior pituitary. These peptides are also found in extrahypothalamic brain regions. The former conserves body water by an action on the kidney, while the latter induces lactation and uterine muscle contraction. DeWied and his colleagues[23] have shown that a single injection of lysine VP (1 μg) inhibits the extinction of a conditioned avoidance response for as long as three weeks. Recently Pedersen and Prange[24] have reported that intracerebroventricular (i.c.v.) injection of oxytocin induces complete maternal behavior in virgin female rats.

Other Neuropeptides - As noted, substance P was first discovered in 1931 by von Euler and Gaddum.[1] Almost 40 years later the undecapaptide was sequenced by Leeman and her associates.[25] The peptide has been reported to alter nociceptive threshold. It produces a variety of other behavioral effects after CNS administration, including grooming and alterations in locomotor activity.[26]

Cholecystokinin (CCK), composed of 33 amino acids, is found in both gastrointestinal tissue and the CNS. It is one of only a few peptides that are found in high concentration in cerebral cortex.[27] CCK may be a physiological satiety hormone.[28,29] Reduction in food consumption by the C-terminal octapeptide of CCK, which possess the complete biological activity of the parent compound, occurs without alterations in levels of brain or plasma aromatic and neutral amino acids.[30] Immunohistochemical and radioimmunoassay data indicate that CCK and dopamine co-exist in certain midbrain neurons.[31] The peptide has been reported to be diminished in brains of genetically obese mice,[32] though others have disputed this finding.[33]

Considerable evidence supports the hypothesis that the renin-angiotensin system plays a physiological role in water balance, and there is evidence that the brain contains an endogenous renin-angiotensin system.[34] Angiotensin, whether injected peripherally or into the CNS, increases water intake in the non-water deprived rat. The subfornical organ is one neuroanatomical locus where angiotensin II is believed to act to produce this effect.[35,36]

Bombesin, a tetradecapeptide first isolated from amphibian skin and heterogeneously distributed in mammalian brain,[37] exerts many peripheral and CNS effects.[38] Brown and his colleages[39] have conducted extensive studies on the effects of bombesin after administration into CSF of rodents. The peptide produces marked hypothermia in cold-exposed rodents but unlike NT (vide infra), it produces hyperthermia in rodents exposed to warm environmental temperatures. Thus, it is considered a poikilothermic substance.[40,41] The peptide, when administered centrally, produces hyperglycemia, increases plasma epinephrine levels, reduces gastric acid secretion and is cytoprotective against stress-induced gastric ulcers.[42] When injected intracisternally, bombesin potentiates ethanol-induced sedation and hypothermia and reduces ethanol-induced withdrawal signs.[43,44]

Vasoactive-intestinal peptide (VIP), originally isolated from gastrointestinal tissue, is composed of 28 amino acids.[45] One of the few peptides found in high concentrations in cerebral cortex, it fulfills many neurotransmitter criteria. Recently, VIP has been shown to stimulate the enzymatic breakdown of glycogen in mouse cerebral cortex in vitro, a finding that supports the view that the peptide may regulate local CNS energy metabolism.[46] Space limitations do not allow discussion of other neuropeptides that have been reported to be present in mammalian brain: calcitonin, delta-sleep-inducing peptide, gastrin, insulin, glucagon, carnosine, secretin, the endogenous opioids (β-endorphin, met- and leu-enkephalin, dynorphin), pancreatic polypeptide and bradykinin.

II. THYROTROPIN RELEASING HORMONE (TRH)

Isolation - TRH was isolated by the groups of Guillemin[2] and Schally.[3] Comprehensive reviews of the isolation, distribution, metabolism, endocrine actions and CNS effects of TRH (pyroglutamyl-histidyl-prolineamide) have been published by our group[19,47,48] and others.[49,50,51] In addition, the previous volume in this series contained a review of hypothalamic peptides which included a brief discussion of TRH.[52]

Localization - After controversy concerning the nature of immunoreactive TRH found in one or another tissue, a consensus has emerged that TRH-like immunoreactivity in the CNS is indeed the authentic tripeptide.[53] It is found in highest concentrations in the hypothalamus but is present in other brain regions as well.[54,55] The peptide had previously been thought

to be absent from cerebellum, but recently Parker[56] and Pacheco et al.[57] reported that TRH is present in human fetal and rat cerebellum. Whether the TRH-like immunoreactivity reported to be present in peripheral tissues and fluids such as pancreas, heart, kidney, placenta, urine and blood is identical to the native pGlu-His-Pro-NH_2 is still not resolved.[53] Koivusalo et al.[58] have recently obtained data from RIA and reverse phase HPLC techniques that support the view that the TRH-like immunoreactivity is indeed the tripeptide, pGlu-His-Pro-NH_2. Jackson[59] has recently reported the presence of large quantities of immunoreactive TRH in the alfalfa plant.

Synthesis - Little is known about the biosynthesis of TRH. Early work indicated that the peptide might be synthesized enzymatically, but this does not appear to be the case. Recently Rupnow et al.[60] reported that frog brain contains a macromolecule that can be converted by enzymatic and chemical modifications to TRH. These data are consistent with the view that TRH, like many other peptides (e.g., ACTH), is formed by cleavage of a large prohormone.

Receptor Binding - High affinity, saturable, reversible binding sites for [^{3}H]-TRH have been found to be heterogeneously distributed in rat brain.[61] In a recent study,[62] Burt and Taylor reported that the binding of radiolabelled TRH to receptors in the nucleus accumbens-septal area of sheep brain resembles the binding of the peptide to the pituitary. Similar findings have been obtained in the retina.[63]

Central Nervous System Effects - The pharmaco-behavioral effects of TRH have been previously reviewed.[47-51] In early studies peripherally administered TRH was found to potentiate the stimulant effects of L-DOPA[64] and 5-hydroxytryptophan[65] in mice, even in hypophysectomized and thyroidectomized animals. When administered i.c.v. or peripherally, TRH markedly antagonizes the sedation and hypothermia induced by a wide variety of CNS depressants, including barbiturates, ethanol, reserpine, chloral hydrate and chlorpromazine.[66,67] It is of interest to note that histidylproline diketopiperazine, a metabolite of TRH, is a potent antagonist of ethanol and ketamine[68] but not barbiturate-induced narcosis.[69] Direct i.c. injections of TRH have been performed to determine its CNS site of action to reverse pentobarbital-induced sedation; the septum is the most sensitive of all the brain regions studied.[70] Recently Nagawa et al.[71] reported that TRH blocks pentobarbital-induced reduction in the rate of glucose utilization throughout the brain.

Because of certain reported similarities between the pharmacological profiles of TRH and d-amphetamine (AMPH), we compared the effects of these two agents in rats.[72] We compared the arousal and hyperactivity produced by low and moderate doses of TRH (10-60 mg/kg i.p.) and AMPH (0.3-2 mg/kg i.p.) by measuring the occurrence of discrete behavioral items. Compared to controls, both TRH and AMPH produced arousal from sleep, but TRH, at all doses tested, produced less arousal than moderate AMPH and produced a pattern of behavioral responses different from those of both low and moderate AMPH. Moderate AMPH produced marked increases in forward locomotion and rearing, but low AMPH and TRH did not. Both TRH and low AMPH increased grooming (perhaps by increasing wakefulness), but TRH failed to increase sniffing, a cardinal feature of AMPH-induced excitement. Unlike AMPH, TRH produced wet-dog shakes, piloerection, tail elevation and teeth chattering. Both moderate AMPH and TRH produced increased activity when compared to controls, but the AMPH effect was greater. Recently Stanton et al.[73] have reported that injection of very low doses of TRH (0.1 ng) into the dorsal hippocampus of hibernating ground squirrels produced prompt arousal with marked increases in body temperature and heart rate.

The literature concerning effects of TRH on body temperature in laboratory animals is confusing.[48] The peptide administered i.c.v. is reported to produce hyperthermia in rabbits and hypothermia in cats. We have not observed any effect of i.c. TRH on colonic temperature of cold exposed (4°C) rats[74] or mice.[75] Yehuda and Youdim[76] confirmed our finding that TRH did not alter body temperature of rats at 4°C. Of particular interest is the report of Prasad et al.,[7] who injected rats i.c.v. with TRH antiserum. This treatment, designed to neutralize endogenous TRH, produced significant hypothermia. These data imply a role for TRH in thermoregulatory physiology.

Hedner et al.[78] reported that i.c.v. TRH produces an increase in respiratory frequency and minute volume in rats. Brown[79] has advanced the hypothesis that TRH may be a CNS regulator of the autonomic nervous system. When injected i.c.v., TRH produced significant hyperglycemia and increases in plasma levels of epinephrine, norepinephrine and glucagon.

A novel approach has been taken by Morier et al.,[80] who synthesized a new drug composed of TRH and amphetamine (pGlu-His-Pro-amphetamine). This compound as well as a related one (pGlu-His-amphetamine) produced marked CNS effects including alterations in thermoregulation and neurotransmitter levels. Finally, electrophysiological effects of TRH have been reported.[4,49] Recently Braitman et al.[81] studied the responses to TRH of 38 identified neurons in sensory-motor cortex of the cat applied iontophoretically. Both excitation and inhibition were observed after TRH, depending on the neurons studied. Kalivas et al.[82] reported that i.c.v. TRH produces a significant increase in both hippocampal and cortical EEG synchrony in pentobarbital pretreated rats. Finally, of great potential clinical interest is the report of Faden et al.[83] who reported that TRH, administered intravenously, resulted in dramatic recovery from spinal cord injury in cats.

III. NEUROTENSIN

Isolation - In 1973, Carraway and Leeman[84] discovered in bovine hypothalamus a substance which, when injected i.v. in rats, produced dose-dependent vasodilation. They identified the amino acid sequence of NT as pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH.[85] The peptide was later synthesized,[86] making possible the production of antiserum, which in turn led to the development of both radioimmunoassays (RIA) and immunohistochemical analysis.

Localization - Ninety percent of total body NT occurs outside brain and spinal cord, the majority in small intestine[87] (see also Brown and Miller, this volume). Hypothalamus and brainstem each contains 35% of total NT in brain; most of the remainder is found in cerebral cortex (17%) and thalamus (11%). Uhl and Snyder[88,89] assayed the subcellular distribution of NT in calf and rat brain. Immunoreactivity to NT was preferentially localized in synaptosomal and microsomal fractions. Using a microdissection procedure, Kobayashi et al.[90] studied the distribution of NT in brain nuclei of the rat. Highest concentrations were found in the nucleus accumbens, preoptic area and medial basal hypothalamus. Uhl and Snyder[91] have adduced evidence from lesion studies for the existence of a NT-containing pathway arising in the central amygdaloid nucleus and terminating, in part, in the stria terminalis. In the brains of normal humans, substantial quantitites of NT were found in the amygdala, nucleus accumbens, hypothalamus, substantia nigra, periventricular gray, anterior perforated substance and septum. Spinal fluid contained about 235 pg/ml of NT.[92]

Interactions with Barbiturates and Ethanol - We found that many peptides (e.g., TRH) antagonize the depressant actions of pentobarbital, but only NT, centrally administered, potentiates them.[3] However, given i.p., NT had no effect. Given centrally, NT caused a marked delay in the disappearance of pentobarbital from liver, brain, and plasma. Luttinger et al.[43] found that i.c. NT also augments ethanol-induced sedation and hypothermia. However, this effect is not associated with changes in ethanol levels. Our group has evaluated the effects in mice of i.c. administered NT on the ethanol-induced impairment of motor coordination. Although NT did not alter the aerial righting reflex in untreated mice, NT (30 μg i.c.) potentiated motor impairment in ethanol-treated mice.[44]

Effects on Thermoregulation - As little as 30 ng of NT administered i.c. to mice in a cold environment (4°C) produces hypothermia.[74,75] It also produces hypothermia at an ambient temperature of 23°C, but hypothermia does not occur after peripheral administration of even large doses.[110] Using structural analogs of NT, Rivier et al.[94] and Loosen et al.[95] found that the C-terminal region, particularly the two arginine moieties, is essential for hypothermic activity, a finding consistent with structure-activity studies in peripheral tissues. The (D-Tyr^{11})-NT congener is more potent than the parent compound. Martin et al.[96] measured rectal temperature of rats after injection of NT (2.5 μg) into 135 different brain sites. NT produced a hypothermic effect at 63 loci, NT-sensitive sites being concentrated in the medial preoptic area, anterior hypothalamus and the periaqueductal gray, all regions that contain substantial quantities of endogenous NT. Kalivas et al.[97] mapped the rat brain for sites at which 5 μg NT (2.5 μg/side) produces hypothermia.

With drugs we have manipulated the activity of specific brain pathways to determine effects on NT-induced hypothermia.[98] Pretreatment with the neuroleptic haloperidol produces a dose-related augmentation. Other agents, including a serotonin depletor, naloxone, phenoxybenzamine, propranolol, atropine and thyroid hormone are ineffective. Bissette et al.[99] found that the hypothermia induced by NT (10 μg i.c.) in mice is blocked by AMPH (1-2 mg/kg i.p.) and methylphenidate (10 mg/kg i.p.), two indirect DA agonists, while apomorphine (2.5 or 5.0 mg/kg i.p.), a direct DA agonist, did not alter it. TRH (20-80 μg i.c.) antagonized NT-induced hypothermia.[98] Thus, reduced CNS DA activity enhances NT-induced hypothermia; indirectly-enhanced CNS DA activity reduces NT-induced hypothermia.

Interactions with Dopaminergic Drugs - Motivated by findings of interactions of NT with DA systems in thermoregulation and by findings that centrally administered NT, like neuroleptics, potentiates barbiturate and ethanol effects, we set out systematically to compare effects of NT and neuroleptic drugs. Neuroleptic drugs produce a reduction in locomotor activity in rodents. We found NT (30 μg i.c.v.) reduced locomotor activity of adult male rats,[74] and Jolicoeur et al.[100] confirmed this effect. AMPH causes a variety of behavioral effects, many of which appear to be associated with DA release. Stereotypic behavior after high doses of AMPH appears to be mediated by the nigroneostriatal DA system; increased locomotor activity and rearing after low doses appear to be mediated by the mesolimbic DA system, with release of DA from nerve terminals in the nucleus accumbens. Intracisternal administration of NT blocks the enhanced locomotor activity induced by AMPH, methylphenidate and cocaine, all of which enhance DA activity indirectly. On the other hand, NT does not block the increase in locomotor activity observed after injection of direct DA agonists apomorphine and lergotrile.[101] To clarify site of action we studied the effects of injection of NT into the nucleus accumbens (a termination site of the mesolimbic DA system) or into the nucleus caudatus

(a termination site of the nigrostriatal DA system) on the behavioral responses to i.p. AMPH. Intra-accumbens injections of NT (0.3-5 μg), like haloperidol, significantly blocked AMPH-induced locomotion and rearing, but intra-caudate injection did not block stereotypic behavior.[102] These data suggest that NT preferentially modulates the mesolimbic DA system, which is thought to play a role in schizophrenia.[103] In a discrete-trial, conditioned-avoidance response paradigm, NT (0.6-17.9 nmol i.c.v.) produced a neuroleptic-like effect in the rat, i.e., a decrease in avoidance responding with no effect on escape responding.[104]

Neurochemical studies also have revealed interactions between NT and DA systems in the CNS. Garcia-Sevilla et al.[105] found that NT injected i.c.v. in the rat increases DA, 5-HT, and NE turnover in various brain areas, and Widerlöv et al.[106] reported that NT (1-100 μg i.c.) increases the content of major DA metabolites in nucleus accumbens, striatum, and olfactory tubercle. Similar effects are seen after treatment with neuroleptic drugs. Govoni et al.[107,108] reported that the concentration of immunoreactive NT in the nucleus accumbens is significantly increased after a single injection of haloperidol (2 mg/kg i.p.), while repeated daily injections produce a gradual increase in NT in both accumbens and striatum.

Kalivas et al.[109] have obtained data that further support an interaction of NT with the mesolimbic DA system. Rats were implanted bilaterally with cannulae in the VTA, the origin of the mesolimbic DA system. Injection of NT (2-5 μg) produced a significant increase in locomotor activity. Haloperidol i.c.v. abolished this NT effect. Intra-accumbens injection of NT also abolished it. Thus, intra-accumbens NT had the remarkable property of blocking the stimulant effects of intra-VTA NT.

Antinociceptive Effects - Clineschmidt and McGuffin[110] reported that low doses of NT in the mouse produce a dose-related increase in reaction time to a hot plate. In another nociceptive test (i.p. injection of acetic acid), i.c. NT also produced a significant reduction of writhes, with effective doses ranging from 0.025-0.25 ng. Naloxone did not alter this effect. Nemeroff et al.[111] compared the potency of NT (1 μg i.c.) with that of equimolar doses of 11 other endogenous peptides and morphine in a hot water tail flick model of nociception. Except for β-endorphin, NT on a molar basis was the most potent analgesic studied. Pretreatment with peripherally or centrally administered TRH significantly blocked NT-induced analgesia.

Martin et al.[112] performed direct i.c. injections to determine the loci at which NT elicits analgesia. Intrathecal NT failed to alter responses, while i.c. and i.c.v. NT produced significant antinociception. Kalivas et al.[97] studied the effects of bilateral NT (2.5 μg/side) microinjection at 22 brain sites on responses of rats to a heat stimulus.

Effects on Gastric Ulcers and Gastric Acid Secretion - Osumi et al.[113] found in rats that NT (1-10 μg i.c.v.) decreases basal gastric acid output and gastric mucosal blood flow. However, Tepperman and Evered[114] reported that lateral hypothalamic injection of NT (100 pmol) did not alter gastric acid secretion. We investigated the effect of i.c. NT on the development of cold-restraint-stress-induced gastric ulcers in rats.[115] In this paradigm NT (30 μg i.c.) completely prevented the development of gastric ulcers while NT (100 μg/kg i.v.) had no effect. The effect of i.c. NT did not appear to be mediated by effects on gastric acid secretion, for i.c. NT did not alter gastric pH. Moreover, neither a neuroleptic (haloperidol) nor a hypothermic drug (oxotremorine) afforded cytoprotection in this model.

IV. DISCUSSION

The first peptide that attracted the interest of our research group was TRH. After we had demonstrated the analeptic properties of this substance, we examined a series of substances for activity in the pentobarbital-sedated mouse. Several substances, like TRH, shortened barbiturate-induced sleep, but only one reliably extended it--NT. Dualism of central action between TRH and NT has been extended to a variety of parameters, and this dualism, along with the quality of effects exerted by the two peptides, allows the broad hypothesis that, in the terms of Hess,[116] TRH subserves the ergotropic functions of the organism, NT its trophotropic functions. The notion of the former includes the concepts of preparation for activity, engagement with the environment and catabolism. The concept of the latter connotes the converse set of the organism. The dualism between the central effects of TRH and NT, whatever its best interpretation, appears unusual among peptides.

Although differences can be noted, NT exerts many behavioral effects that characterize neuroleptic drugs. Moreover, it occurs in brain in loci that subserve the functions at fault in schizophrenia, notably the limbic system. Furthermore, the peptide interacts with DA systems, which may play a pathophysiologic role in schizophrenia. At present it must be regarded as unique among peptides in its ability both to stimulate and inhibit the activity of a neurochemical pathway, the mesolimbic DA system. Clearly, TRH and NT and their interactions deserve further study.

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Chapter 5. Antidepressants

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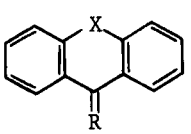
Introduction - Maprotiline was marketed early in 1981. It may have a more rapid onset of action than its eight tricyclic antidepressant (TCA) predecessors in the United States.[1] Alprazolam, a benzodiazepine derivative with combined antianxiety-antidepressant actions, was also marketed.[2] Trazodone, a "second generation" antidepressant, likewise received approval by the FDA; however, it did not appear on the market before the end of the year.[3] The intense interest in this field is reflected by the nearly 25 additional antidepressants currently in the development phase in this country. Primary objectives are to develop new antidepressant drugs with fewer side effects and a more rapid onset of action. Following our review last year,[4] many reviews concerning clinical,[5-7] biologic[8] and medicinal chemical[9] aspects, as well as cardiovascular[10,11] and anticholinergic[12] side effects and overall perspectives[13] of these agents, have appeared.

Tricyclic Compounds with Antidepressant Activity - TCAs,[14] their therapeutic utility,[15,16] biology, metabolism and side effects[17] were the subject of numerous reviews. Some established TCAs, e.g., amoxapine,[18] amineptine, clomipramine,[19] oxaprotiline,[20] desipramine (DMI)(and its 2-OH metabolite),[21] 2-cyanoimipramine (DAC, Ro-11-2465),[22] mianserin[23] and butriptyline,[24] as well as many other TCAs, were extensively studied. Combined TCA-antipsychotic therapy was also investigated.[25] The influence of the seven-membered ring conformation of "6-7-6" TCAs was examined.[26]

Several new aminoalkylated derivatives are chemically related to known TCAs. 2-Nitroimipramine selectively and irreversibly inhibited serotonin (5-HT) uptake and imipramine binding in human platelets.[27] At low doses in rodents prooxen (1) was a more potent antidepressant than amitriptyline, whereas neuroleptic effects were seen at higher doses.[28] A series of imine analogs of TCAs was studied. The cyclopropa(c)cycloheptene derivatives were most potent. The most effective member was 2; it was about six times more potent than amitriptyline in causing exploratory loss and as an antagonist of tetrabenazine-induced ptosis in mice.[29] Antidepressant actions were also shown by related oximino ethers, e.g., mariptiline (3).[30]



1, X=O; R=$CHCH_2CH_2N(CH_3)_2$
2, X=Δ; R=$NCH_2CH_2N(CH_3)_2$
3, X=Δ; R=$NOCH_2CH_2NHC_2H_5$

In a structure-activity relationship (SAR) study of mianserin analogs, presynaptic α-adrenergic blockade and inhibition of norepinephrine (NE) and 5-HT reuptake into rat brain synaptosomes were measured. Heteroatom replacement, hydroxylation or methylation of the methylene bridge between rings A and C, CH_3 or Cl substitution of rings A and C, N-substitution and modification of the D ring, including analogs with an exocyclic amino group, were investigated. Presynaptic α-blockade was noted only in molecules with an overall bent shape. Flat molecules, whether rigid or flexible, were not active. Six-membered, chair form D rings (containing an

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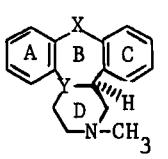
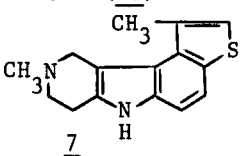
NCH_3 grouping) were more potent than five or seven membered ring homologs.[31] α-Adrenolytic activity resided in the (-)R enantiomer of mianserin; however, the (+)S isomer (4) was more potent (300 X) as an inhibitor of NE uptake into rat hypothalamic synaptosomes and in animal tests for antidepressant activity.[32] Single crystal X-ray analysis of the more potent uptake inhibitory (+) antipode of the 10-oxa analog 5 of mianserin showed that it also had S stereochemistry.[33] The C isostere (6, Mo-8282) of mianserin also had antidepressant properties.[34] Other new TCAs included tienocarbine (7),[35] tianeptine (8)[36] (an analog of amineptine that inhibits 5-HT reuptake), the lactam LM 1404 (9) and its more potent demethylated derivative LM 1580 (10).[37]



4, X=CH_2; Y=N
5, X=O; Y=N
6, X=CH_2; Y=CH



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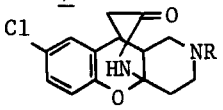
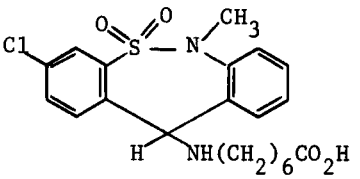


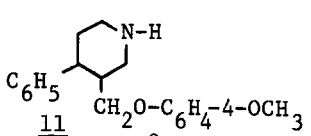
9, R=CH_3
10, R=H



8

Non-tricyclic Compounds with Antidepressant Activity - Many non-TCAs, particularly trazodone,[38] zimelidine,[39] bupropion,[40] nomifensine,[41] clovoxamine,[42] binodaline,[43] and alprazolam,[2] have been the subject of intense clinical study and review. General research and clinical implications of "second generation" antidepressants were reviewed.[13]

Several non-TCAs are substituted aminoalkylated aryl and diaryl derivatives. 2-$C_6H_5CH_2C_6H_4O(CH_2)_4NHCH_3$ (MCI-2016)[44] was one of the most potent members (0.78 X amitriptyline) of a series of aminoalkoxyaryl compounds in a test for prevention of reserpine-induced hypothermia in mice. It and several relatives were comparatively free of the antihistaminic and anticholinergic side effects of many TCAs.[45] Other aminoalkoxyaryl derivatives with antidepressant-like activity included 2-$CH_3OC_6H_4OCH_2CH(CH_3)$-$NHCH_3$, an antagonist of reserpine-induced ptosis in mice[46] and norfemoxetine (11, FG 4996), a femoxetine metabolite with similar 5-HT uptake inhibiting activity.[47] GBR 13069 (12) and GBR 13098 (13) inhibited dopamine (DA) uptake into brain homogenates.[48] A related compound, amperozide (14, FG-5606), had antidepressant activity in a behavioral despair test in rats.[49] LY-125180, 4-$CH_3C_6H_4OCH_2CH_2CH(C_6H_5)N(CH_3)_2$, was a 5-HT and DA uptake inhibitor in vitro, but caused only 5-HT inhibition in rats.[50] Antidepressant activity was also cited for clobamine (15);[51] and 16, one of a series, had imipramine-like activity in various tests in mice.[52] Within a series of alaproclate relatives the keto analog, 4-$ClC_6H_4CH_2C$-$(CH_3)_2COCH(CH_3)NH_2$, was the most potent although it was less effective than the parent.[53] SAR of a series of zimelidine analogs indicated that norzimelidine (17) was the most potent inhibitor of 5-HT reuptake. Ortho-substitution of the phenyl ring of Z isomers enhanced NE uptake inhibitory potency.[54]



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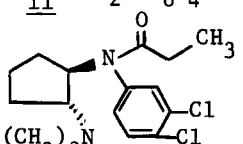

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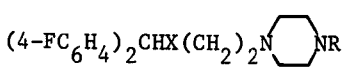
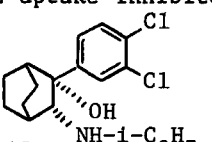


12, X=O; R=$CH{=}CHCH_2C_6H_5$

13, X=O; R=$(CH_2)_3C_6H_5$

14, X=CH_2; R=$CONHC_2H_5$



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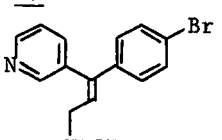
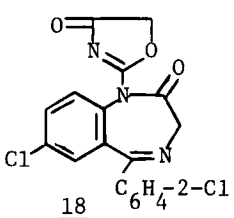


17

Antidepressant actions were also produced by many bicyclic compounds via a variety of mechanisms. Foremost among these were 1,4-benzodiazepines and related structures that combine antianxiety and antidepressant actions, e.g., SC-33963 (18).[55] SAR of a series of pyrazolodiazepines, showed that zometapine (19), currently in clinical trial, was more potent (4X) than imipramine in a methamphetamine-potentiation test in rats.[56] Several naphthalene derivatives, e.g., napactadine (20, DL-588),[57] the thioacetamidoxime 21,[58] and Wy 26002 (22), had antidepressant actions in vitro and in vivo.[59]



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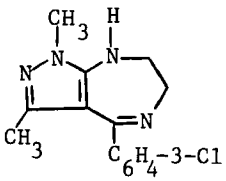

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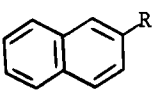
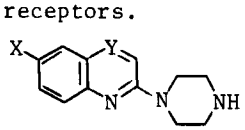


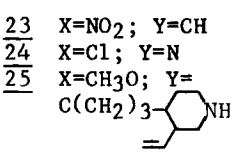
20, R=$CH_2C(=NCH_3)NHCH_3$

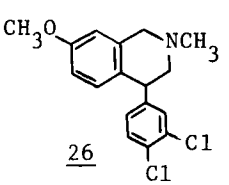
21, R=$SCH_2C(=NOH)NH_2$

22, R=CH_2N  $NHCONHCOC_6H_5$

A number of quinolines and related structures have antidepressant properties. The piperazinylquinoline DU 24565 (23), a quipazine analog and 5-HT uptake inhibitor, is being studied as a potential antidepressant.[60] In a series of related quinoxalines, 24 was 4 times more potent than clomipramine as an inhibitor of 5-HT uptake in rats.[61] These effects are closely associated with the arylpiperazine grouping. Thus, 3-trifluoromethylphenylpiperazine (TFMPP), is a potent 5-HT agonist.[62] It may have antidepressant activity as is suggested by the utility of 5-HT precursors in the management of depression.[63] Another quinoline, PK 5078 (25), was more than 20 times more potent than clomipramine as an inhibitor of 5-HT uptake in vitro and induced some 5-HT release. Its epimer, PK 7059, was 100 times less potent in inhibiting 5-HT uptake, but was a potent 5-HT releaser.[64] 1-Phenyl-3-piperazinylisoquinoline (perafensine, HR-459) is a potent antagonist of reserpine-induced effects and inhibits NE uptake into rat hypothalamic synaptosomes (IC_{50}=21 nM).[65] The tetrahydroisoquinoline diclofensine (26, Ro-8-4650) is a clinically effective antidepressant that inhibits NE, DA and 5-HT uptake.[66] Animal tests indicated that 1-(3-dimethylaminopropyl)-2-phenylindole (L 22005) had antidepressant activity similar to that of iprindole; however, it caused hepatotoxicity.[67] Other bicyclic compounds having antidepressant activity include 5-chloro-1-(4-piperazinyl)-2-benzimidazolone (RP 29676), an inhibitor of 5-HT uptake into rat blood platelets,[68] pirenperone (27, R 47465), a 5-HT_2 antagonist,[69] trans-1-methylamino-4-phenyltetralin (tametraline, CP-24,411),[70] and a series of spiroaminocyclohexaneisobenzofurans, e.g. 28, with antitetrabenazine activity approximating that of amitriptyline in mice.[71] Several benzodioxanylimidazolines, e.g. 29,[72] and -imidazoles, e.g. 30,[73] apparently owe their antidepressant activity to blockade of central α_2-adrenergic receptors.



23 X=NO_2; Y=CH
24 X=Cl; Y=N
25 X=CH_3O; Y= 



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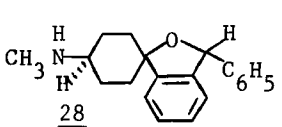

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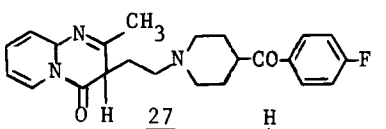

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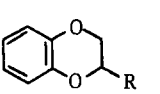
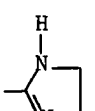
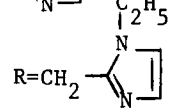
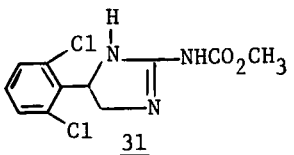


29, R= 

30, R=CH_2 

Various other imidazoles and aryl derivatives have antidepressant properties. Extensive pharmacological study suggests that RS-51324 (31) acts via inhibition of NE reuptake;[74] M44K58 (32) inhibits central DA reuptake.[75] Pyrazolepropanamine I (33) inhibits NE uptake into rat brain synaptosomes.[76] 1-Ethyl-1-[(2-methylphenyl)methyl]aziridinium iodide (AZI) is an irreversible inhibitor of NE uptake into various tissues.[77]



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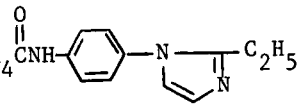

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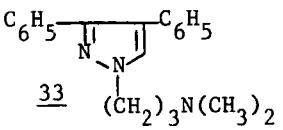
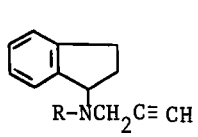


33

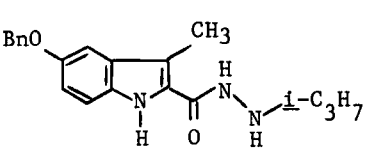
Antidepressant effects have been associated with thyrotropin releasing hormone (TRH), pyroGlu-His-ProNH$_2$, a peptide which potentiates the effects of imipramine on brain 5-HT systems.[78] TRH relatives, e.g., pyroGlu-His-(3-Me)ProNH$_2$ (RX74355), pyroGlu-His-(3,3-Me$_2$)ProNH$_2$ (RX 77368),[79] an isostere in which the NH of the pyroGlu residue is replaced by O (DN 1417),[80] an orotyl congener, 6-orotyl-His-ProNH$_2$ (CG 3509),[81] and L-pyro-2-aminoadipyl-His-thiazolidine-4-CONH$_2$ (MK—771),[82] produce antidepressant-like actions in animals. Another peptide, taftsin (Thr-Lys-Pro-Arg), caused central stimulation in rats.[83]

Monoamine Oxidase Inhibitors (MAOIs) - MAOIs were reviewed.[84] Studies have focused on newer MAOIs, e.g., caroxazone[85] and pirlindole (pyrazidol),[86] and on the benefit of combined therapy of MAOIs with various psychotropic agents.[87] In contrast to earlier studies, recent investigations indicate greater antidepressant efficacy for (+)-tranylcypromine, the more potent MAOI, than for the (-) isomer, a more effective uptake inhibitor.[88]

Study of the A and B subtypes of MAO has continued. They appear structurally to be different; the two forms were separated by immunoaffinity chromatography of human liver extracts.[89] However, species differences in DA oxidation were observed. DA was deaminated by type A-MAO in rat brain, but by type B in humans.[90] Similarity of MAO-A and B was indicated by the identity of primary structures near the pargyline binding sites of both types.[91] The possible correlation between the failure of (-)-deprenyl, a type B selective MAOI, to alleviate depression and its freedom from the "cheese effect" was probed.[92] Selective acetylenic suicide and reversible type A and B MAOIs were studied. AGN 1135 (34) and AGN 1133 (35, J-508) were most selective for type B-MAO.[93,94] Interestingly, (+)-FLA 336 [(+)-2-CH_3-4-$(CH_3)_2NC_6H_3CH_2CH(CH_3)NH_2$], a selective type A MAOI, seems to lack the "cheese effect."[95] Cimoxatone (M770515),[4] a long acting reversible type A MAOI,[96] was studied clinically. Its N-demethyl metabolite was also effective.[97] Other new MAOIs included LY 51641 (2-$ClC_6H_4OCH_2CH_2$ NH-c-C_3H_5)[98] and IH 3 (36),[99] which appeared selective for MAO-A, as well as methylaplysinopsin (37).[100]



34, R=H
35, R=CH_3



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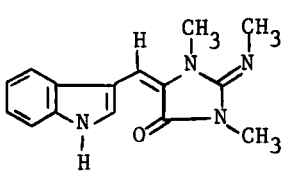

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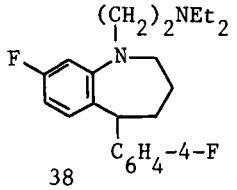
Central Nervous System (CNS) Stimulants - Many new CNS stimulants and nootropic agents (cognitive activators) were reported in 1981. Only a few of these are cited. CNS stimulants, e.g., amphetamine, are of doubtful value in treating depression.[101] The CNS stimulant effects of caffeine[102] and related purines were recently correlated with their ability to bind to central adenosine (A_1 and A_2) receptors, thus preventing the depressant action of endogenous adenosine on nerve firing.[103] More than 100 purine bases and related heterocycles were studied for their ability to inhibit the adenosine-induced increase in c-AMP in a VA13 fibroblast line. Three families of adenosine antagonists were found: i.e., xanthines, 8-(4-bromophenyl)xanthine (A 40), which was 96 times more potent than theophylline; 9-methyladenine (ca. 0.1 X theophylline); and benzo[g]pteridines, e.g., alloxazine (ca. 4 X theophylline).[104] CNS stimulation was also produced by some 1-benzazepines, e.g., VUFB 14043 (38)[105] and by a prostaglandin analog BW245C (39).[106] L-Cysteinesulfinic acid (CSA) was identified as an endogenous CNS stimulant.[107] CNS stimulant effects were produced by CERM 3726 (40).[108] 3-Phenoxypyridine (CI-844) was the most potent of a series in enhancing retention of passive avoidance learning in mice; it is a potential cognitive activator.[109]



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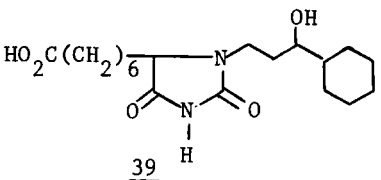

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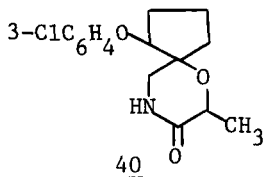

40

Miscellaneous Antidepressants, Diagnosis and Therapy - Clinical evidence suggests that 5-HT function may be altered in patients with affective disorders.[110] A variety of therapeutic agents, such as β-adrenoreceptor agonists and antagonists, e.g., salbutamol[111] and propranolol,[112] neuroleptics,[113] and antihistaminics,[114] as well as 5-HT precursors[63] and ergoline derivatives, e.g., bromocriptine,[115] were reported to have antidepressant activity.

Other antidepressant treatments, namely electroconvulsive shock (ECS) therapy[116] and the prophylactic and therapeutic utility of lithium[117] continue to receive documentation. ECS therapy, for which memory impairment is a major side effect, may find increased application in the future because new technologies are enhancing its precision, efficacy and safety.[118] Side effects of renal and liver toxicity accompanied Li therapy.[119]

Perhaps the most significant advance in the treatment of depression was the derivation of methodologies for diagnosis. Current thoughts about neurochemical and neuroendocrinological changes in affective disorders were the subject of a symposium .[120] Concentrations of NE may provide biochemical markers for identification of mental states.[121] The dexamethasone suppression test (DST) offers another diagnostic possibility. In general, dexamethasone administration lowers blood cortisol; however this appears not to be the case in 30-80% of endogenously depressed persons.[118] Other possible biochemical markers of depression are endorphin concentrations in spinal fluid,[122] suppression of cortisol levels upon i.v. dextroamphetamine administration[123] and various peptide challenges.[124]

Screening Methods - Behavioral models of depression for antidepressant screening were reviewed.[125,126] Chronic TCAs, MAOIs and ECS restored the ability of chronically stressed rats to respond to acute stress (noise and

light) with increased open field activity.[126] Behavioral despair was reviewed.[127] Various antidepressants reduced the duration of immobility by activating DA and/or NE mechanisms but the test was insensitive to drugs activating or reducing 5-HT mechanisms;[128] antidepressants could be distinguished from methamphetamine, caffeine, scopolamine and diphenhydramine by extending the period of observation and by chronic administration.[129] Inhibition of muricide in olfactory bulbectomized (OB) rats was potentiated by chronic ECS and antagonized by pretreatment with phenoxybenzamine but not sotalol.[130] Chronic TCAs, bupropion and mianserin, but not MAOIs, attenuated the passive avoidance deficit in OB rats two and three days after drug withdrawal;[131] the passive avoidance deficit in OB rats may reflect functional 5-HT deficiency.[131,132] Chronic nortriptyline counteracted escape deficits produced in rats by inescapable shock.[133] Increased locomotor activity in rats following ten months of social deprivation was blocked by acute mianserin, iprindole, nomifensine, salbutamol, cyproheptadine and apomorphine.[134] The ability of TCAs and maprotiline to inhibit aggressiveness in socially deprived mice was potentiated by chronic administration.[135] Chronic TCAs, mianserin or iprindole increased shock- or isolation-induced fighting in rats.[136,137]

TCAs, MAOIs and atypical antidepressants, but not ECS, potentiated yohimbine-induced toxicity in mice; several types of false positives, e.g., antihistamines, anticholinergics and stimulants were also detected. The yohimbine potentiation test in dogs did not detect stimulants as false positives.[138] The decrease in β-receptor density after only one dose of antidepressant plus phenoxybenzamine could serve as a rapid screening procedure for antidepressant drugs.[139] Hypothermia induced by a high dose of apomorphine was antagonized by TCAs, amineptine, amoxapine, viloxazine, nomifensine and β-agonists but not by neuroleptics.[140] Potentiation of TRH-induced hyperthermia in mice may be used to select antidepressants that activate α-adrenergic systems.[141]

Mechanism(s) of Action - Research on the mechanism(s) of action of antidepressants continued to focus on changes in receptor sensitivities induced by their chronic administration; reviews and commentaries have appeared.[142-146] Behavioral and electrophysiological evidence accumulated for enhanced postsynaptic α-receptor sensitivity following TCAs, iprindole, maprotiline or zimelidine, but not fluoxetine, in mice and rats.[147,148] However, TCAs or ECS did not alter α_1-receptor binding in rats.[149,150] Evidence for presynaptic α_2-receptor subsensitivity in depressed patients following TCAs or clorgyline[151-153] and in mice and rats following TCAs, ECS or rapid-eye-movement sleep deprivation (REMD), but not mianserin, iprindole or nisoxetine, was reported.[154-157] Mianserin caused supersensitivity.[158] However, while amitriptyline and MAOIs decreased α_2-receptor binding,[159,160] TCAs, iprindole, mianserin, ECS and REMD had no effect.[149,150,157,160] Decreased β-receptor binding or c-AMP response following DMI or mianserin, but not after Li or iprindole, was indicated by electrophysiological experiments.[161,162] Decreased β-receptor binding induced by DMI or MAOIs was potentiated by phenoxybenzamine,[139] while the DMI- or ECS- induced decrease was abolished by ganglionectomy or pretreatment with 6-hydroxydopamine,[150,163] suggesting a role for noradrenergic innervation in the down-regulation of the β-receptor. Selective changes in 5-HT availability did not affect β-receptor binding or the c-AMP response in rats.[164] Zimelidine reduced β-receptor binding.[165] Chronic TCAs decreased DA receptor binding,[166] while zimelidine or ECS had no effect;[150,165,167] DMI, ECS and REMD caused subsensitivity or supersensitivity of pre- and postsynaptic DA receptors.[143,167-169] The effects of antidepressants on cholinergic and

histaminergic receptors have been reviewed.[146] TCAs and ECS, but not zimelidine, increased neuronal responses to 5-HT,[170] while clorgyline, but not TCAs or deprenyl, reduced the response.[171] 5-HT_2 receptor binding was increased by ECS [172] and decreased by amitriptyline, iprindole, MAOIs or Li.[172,173] Coadministration of trazodone and phenoxybenzamine decreased 5-HT_2 receptor binding, while trazodone alone had no effect.[174] Zimelidine induced low affinity 5-HT binding and attenuated 5-HTP-induced behavior [175] or had no effect on low affinity binding;[165] fluoxetine and Li decreased binding.[173,176]

High affinity [^{3}H]-mianserin binding was associated with postsynaptic 5-HT and histamine receptors;[177] high affinity [^{3}H]-imipramine and [^{3}H]-DMI binding are associated with 5-HT and NE uptake sites, respectively.[144,178] The affinities of different structural types of antidepressants for various neurotransmitter receptors have been determined.[179]

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Section II - Pharmacodynamic Agents

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Chapter 6. Pulmonary and Antiallergy Agents

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Introduction - Highlights of 1981 in the area of allergy and asthma research include the first International Symposium devoted entirely to the leukotrienes and other lipoxygenase products and the introduction of albuterol (1) to the United States market.

Important review articles dealt with adrenergic receptors and their role in atopic diseases,[1] the molecular mechanism of action of salicylates,[2] an historical account of glucocorticoids as they affect phospholipase A_2,[3] and the biochemistry of allergic reactions.[4]

Biochemistry and Pharmacology of Asthma - Recent studies dealing with the biochemistry and pharmacology of asthma emphasize the systemic nature of this disease and the existence of subpopulations of asthmatics. The response of leukocytes from asthmatic and normal subjects to various histamine releasing agents has been studied.[5] Cells from asthmatics were hyporesponsive to C5-peptide and N-formyl-met-leu-phe and hyperresponsive to deuterium oxide when compared with the cells from normal individuals. The H_1-antagonist hydroxyzine decreased the response to subcutaneous histamine, compound 48/80, or antigen in normal and asthmatic subjects, and there was no difference in the responses of the two populations.[6] The H_2-antagonist cimetidine had no effect on the response to antigen in either population of subjects. A group of asthmatic children divided into two populations with respect to the ability of theophylline to inhibit anti-IgE induced histamine release from their leukocytes.[7] In another study involving leukocytes from children, it was found that tylophorine (an alkaloid from Tylophora-asthmaticus) increased cyclic AMP levels in the cells of asthmatic donors and failed to affect the cells from normal subjects.[8] Two studies have dealt with arachidonic acid metabolites. Platelets from asthmatics converted added arachidonic acid to lipoxygenase products to a greater extent than did platelets from normal individuals.[9] Pulmonary responses to antigen in atopic subjects with and without asthma were compared.[10] Indomethacin increased the non-asthmatics sensitivity to antigen while having no effect on the already sensitive asthmatic population.

β-Adrenergic Agonists - Albuterol (1) was approved for inhalation therapy,[11] and approval of related drugs is expected. Reviews of the pharmacology and toxicology,[12] clinical use as an aerosol,[13] worldwide experience,[14] and use in chronic airway disease[15] of albuterol have appeared.

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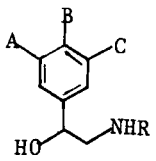

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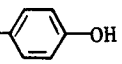
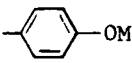
3	OH	H	OH	$CHMeCH_2$–  –OH
4	HNCOH	OH	H	$CHMeCH_2$–  –OMe
5	OH	H	OH	CMe_2CH_2OH
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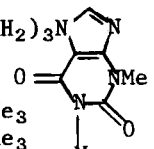
7	OH	H	OH	$(CH_2)_3N$– 
8	$HNCONH_2$	OH	H	CMe_3
9	Cl	NH_2	Cl	CMe_3
10	OH	OH	H	$CHMe_2$
11	$HNSO_2$	NH	H	$CHMe_2$
12	H	$HNSO_2CF_3$	H	$CHMe_2$

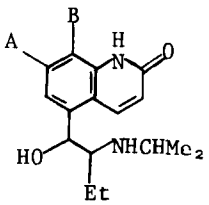
Oral fenoterol (3) produced significant bronchodilation in asthmatic children over a six month period.[16] Inhaled[17] but not oral fenoterol protected against histamine and methacholine challenge in 12 asthmatics.[18] Formoterol (BD-40A, 4) had a greater antiallergy effect than 2 in rat, mouse, and guinea pig PCA.[19]

Several analogs of terbutaline (2) were reported to have bronchodilating activity. KWD 2131 (5) provided a protective effect against allergen-induced wheals[20] but had no protective effect at subbronchodilating doses against allergen bronchoprovocation in asthmatics.[21,22] Wy-14319 (6) showed diminished bronchodilating and cardiostimulating activity compared to 1 by i.v. and aerosol routes and was less bronchoselective at higher doses.[23] Reproterol (7) (30 mg) blocked antigen-induced bronchospasm[24,25] better than 1 (5 mg) and attenuated methacholine-induced bronchospasm in asthmatics.[25,26] Inhalation of 1 mg of 7 gave better protection against exercise-induced bronchospasm (EIB) than 0.2 mg of 1.[27]

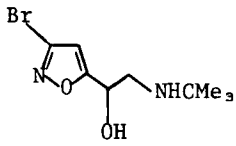
Significant bronchodilation occurred with 2 or 3 mg of oral carbuterol (SK&F 40383, 8) tid accompanied by a fall in pulse rate and blood pressure in asthmatic patients.[28] Single, oral doses of clenbuterol (NAB 365, 9) had similar cardiac, pulmonary, and tremorigenic effects as 1, but there was a higher incidence of transient headaches and nervousness at the high (80 μg) dose in asthmatics.[29]

Procaterol (13) given i.v. to rhesus monkeys inhibited allergen-induced bronchospasm,[30] while another analog (14) was 42 times less potent as a bronchodilator and 87 times less potent as a chronotropic agent than isoproterenol (10) in anesthetized dogs.[31]

The heterocyclic compound, pirbuterol, significantly increased FEV_1 and MMF at 10 and 15 mg in asthmatics.[32] However, when given to patients with chronic congestive heart failure, tolerance developed to its hemodynamic effects, and β-adrenergic receptor density decreased in



13 A=H, B=OH
14 A=OH, B=H



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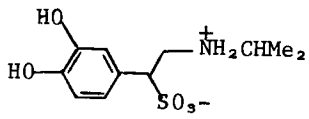

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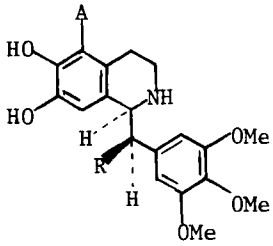


	A	R
17	H	H
18	H	Me
19	Me	H

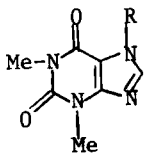
lymphocytes.[33] A novel heterocyclic β-adrenergic agonist, Z 1170 (15), was ten times less active than isoproterenol in the guinea pig atria[34] and 100 times less active in the cat soleus muscle suggesting relatively less tremorigenic potential.[35]

Of several derivatives of tretoquinol (17) reported, 18 was more potent than 17 with enhanced selectivity for guinea pig trachea.[36] Methylation of 17 yielded 19 which was more selective and less potent than the parent compound,[37] while rearrangement of the hydroxy substituents of 17 generally abolished or reduced activity.[38] Some modifications of isoproterenol led to loss of adrenergic activity. The sulfonic acid derivative (16) lost α- and β-adrenoceptor binding affinity.[39] Replacement of the catechol moiety with the more acidic 2,1,3-benzothiadiazole 2,2-dioxide ring (11) or triflanilide (12) destroyed bronchodilator activity and adrenoceptor affinity.[40]

α-Adrenergic Blockers - The hypothesis that α-adrenergic blockers might be of value in clinical asthma has been tested and found wanting. Moxisylyte (thymoxamine) produced no change in PEFR in asthmatics.[41] The selective α_1-adrenergic blocker prazosin, was found to be ineffective in asthmatic subjects.[42,43]

Other Bronchodilators - ABC 12/3 (21) was a more potent bronchodilator than theophylline (20) on the guinea pig trachea in vitro and in the Konzett-Rossler guinea pig.[44] Another 7-substituted xanthine (22) showed oral bronchodilator activity in the guinea pig.[45] Verofylline (23) was effective in blocking EIB,[46] but side effects were dose-limiting.[47,48]

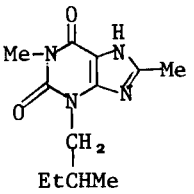
BB-1502 (24) (id) had 5x the bronchodilating activity of theophylline in dogs and guinea pigs, and relaxed guinea pig trachea at a concentration 600x lower than theophylline. In an ascaris-sensitive dog model, 24 gave complete protection at an oral dose of 1 mg/kg.[49] LA 2851 (25) eliminated histamine-induced bronchospasm in the dog and guinea pig at 10 mg/kg i.v.[50] Compound 25 also showed modest anti-allergy effects in rat models (PCA and mast cells). Dipyridamole, a



20 R=H

21 R=CH_2CH(O)(O)

22 R=$CH_2N(CH_2CH_2OH)_2$



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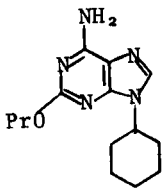

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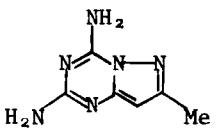

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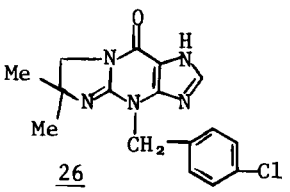


26

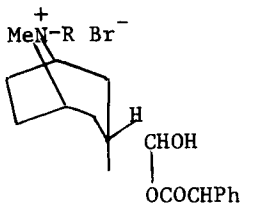
potent phosphodiesterase inhibitor, had no significant bronchodilator effect in nine asthmatics.[51] The structure of fenprinast (MJ 13401, 26), a bronchodilator and antiallergy agent, has been firmly established by chemical[52] and X-ray[53] methods.

Both positive and negative clinical results have been reported for ipratropium (27).[54-57] Ipratropium had a generalized action throughout the airways, was synergistic with albuterol (1)[58] and was also effective in controlling rhinorrhea associated with the common cold.[59] Oxitropium (Ba 253, 28) was less effective than fenoterol (3) in antigen-challenged allergic subjects.[60] Animal bronchodilator investigations have also been reported for diphemanil (29)[61] and several xanthene-9-carboxylic esters (30).[62]

DHET-PGE_2 (31) produced bronchodilation in guinea pigs and dogs, but produced bronchoconstriction or bronchodilation in asthmatic patients. Both 31 and PGE_2 caused contraction of isolated human bronchus, whereas PGE_1 produced relaxation.[63] Human bronchus is now recommended for testing prostaglandin analogs. TR4979 (32) was 0.8, 0.06, and 0.02x as active as PGE_1 on guinea pig trachea, human bronchial muscle, and cat trachea, respectively. Thus it is selective for the putative 'Ψ' prostanoid receptor in airways.[64]

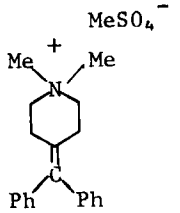
Calcium Antagonists - The role of calcium ions in the pathogenesis of asthma has been reviewed.[65,66] The cardioprotective agents nifedipine and verapamil have recently been evaluated as bronchodilators and antiallergy agents.[67-69] Although histamine-induced[70] and exercise-induced[71-73] bronchoconstriction can be modified, the effect is small[70,74] and probably not therapeutically useful.[75]

Corticosteroids - Beclomethasone dipropionate and flunisolide (33) have recently been introduced in the U.S. for intranasal treatment of seasonal and perennial rhinitis.[76] In seasonal rhinitis 33 was superior to DSCG[77] and did not suppress the pituitary-adrenal-axis.[78] Budesonide (34) showed significant activity in patients with seasonal rhinitis.[79] Long-term follow-up studies on the use of beclamethasone dipropionate in asthma have been reported.[80,81] Both beclamethasone dipropionate and 34 inhibited antigen-induced release of SRS but not histamine from guinea pig chopped lung tissue.[82] Histamine release from human basophils was inhibited after incubation 24 hr with corticosteroids.[83]



27 R=CHMe$_2$

28 R=Et



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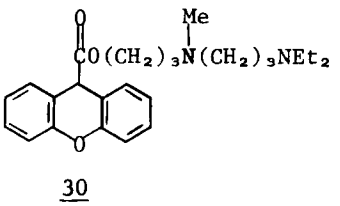

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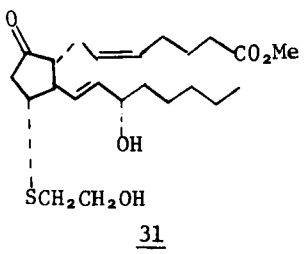

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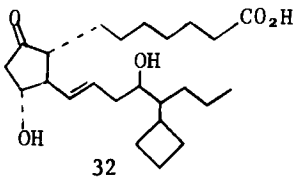

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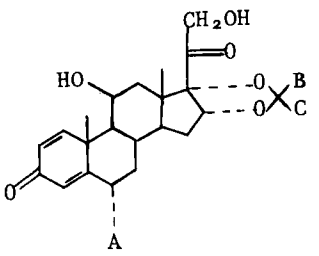
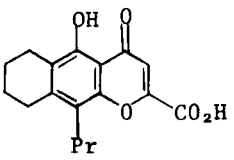


	A	B	C
33	F	Me	Me
34	H	Pr	H

Inhibitors of Mediator Release - Perhaps the most recent advance in the area of mediator release was the description of a method for the purification of human basophils.[84] Advances have also been made in the dispersion and purification of human lung mast cells. The release of several proteolytic enzymes from partially purified dispersed human mast cells has been described.[85] Preliminary pharmacology of human mast cells[86] indicates that these cells will be pharmacologically similar to the human basophil and dissimilar to the rat peritoneal mast cell. Three new releasing agents have been described. A prostaglandin releasing factor of anaphylaxis defined as an acidic polypeptide has been found in human lung fragments.[87] Tetradecanoylphorbol acetate (TPA),[88,89] an agent best known for its ability to promote tumors, and hyperosmolar solutions, e.g. mannitol,[90] have been found to release histamine from human leukocytes. Studies with a series of rat basophilic leukemia cell lines have elegantly demonstrated the need for a series of phospholipid methylation reactions for histamine release in this cell type.[91] These reactions may also be necessary in human basophils.[92]

A review of the use of cromolyn sodium (DSCG) in the treatment of asthma has appeared.[93] While its mechanism of action is unknown, growing evidence indicates that it is not all mast cell related.[94] Because DSCG attenuates the bronchospasm caused by cold air in normal subjects,[95] it may have a nonimmunological mode of action, perhaps acting directly on the smooth muscle[96] or a bronchial irritant receptor.[97] Because its therapeutic action may not be entirely related to mast cell stabilization,[98] questions concerning the relevance of the rat PCA and tests involving mediator release from animal mast cells[99] and human lung fragments[100] have arisen.

The development of ulcers and renal tumors in two year rat



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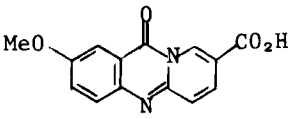

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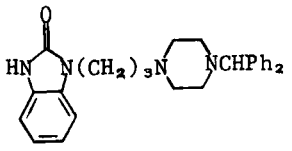

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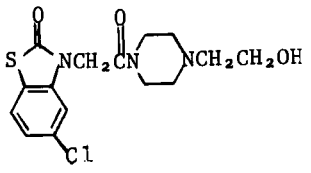

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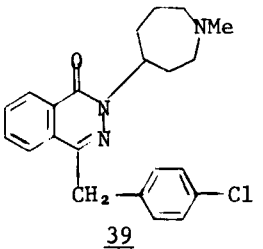

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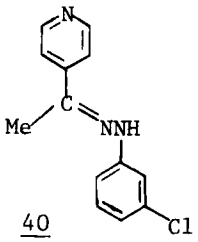
toxicity studies[101] have halted further work on proxicromil (35). Ro 21-7634 (36) had good oral activity in rat PCA and in the anesthetized rat against antigen-induced bronchospasm (ID_{50}=0.2 mg/kg).[102] It inhibited the release of histamine from rat mast cells[103] and both histamine and SRS release from guinea pig lung fragments.[104]

Several clinical trials[105] of ketotifen in atopic asthmatics have not shown a beneficial effect, but significant improvement of allergic rhinitis was observed.[106-110] However, on long term treatment (3-6 mo.) a reduction of asthmatic symptoms was noted.[111] A review of pharmacological studies on oxatomide (37) has appeared.[112] In clinical asthma, 37 had little effect,[113] but it did produce a protective effect in EIB.[114] Oxatomide was also effective in treating allergic rhinoconjunctivitis[115,116] and chronic urticaria.[117,118] Tiaramide (38) has both bronchodilating and antiallergy activity in rats and guinea pigs[119] and is active in clinical and experimental asthma.[120-123] Azelastine (39) inhibited histamine release from guinea pig mast cells[124] and rat mast cells and was orally active in the 48 hr guinea pig homologous PCA test.[125] It also prevented histamine-induced bronchospasm in anesthetized guinea pigs.[126]

Slow Reacting Substance (SRS) - SRS was identified two years ago as a mixture of leukotrienes C and D (LTC_4 and LTD_4). Since then no additional compounds have been defined which have comparable SRS activity. Several review articles have appeared recently.[127-131] See also chapters 26 and 32.

Phospholipase Inhibitors - Anti-inflammatory steroids indirectly inhibit phospholipase A_2 activity in some cell types by inducing the synthesis of a polypeptide(s)/protein(s) capable of inhibiting the enzyme. Several studies of these inhibitors (lipomodulin and macrocortin) have appeared in the last year.[132-134]



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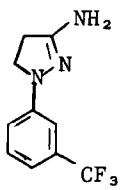

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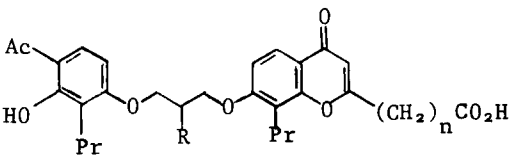

42 R=OH, n=0

43 R=H, n=1

Lipoxygenase Inhibitors - The largest number of inhibitors of the overall conversion of phospholipid to leukotrienes appear to act at the level of the lipoxygenase enzyme that converts arachidonic acid to 5-HPETE. Two studies[135,136] have described the inhibition of the 5-lipoxygenase by 15-HETE, the final product of the 15-lipoxygenase. In the more recent of these studies[136] using human T-lymphocytes, it was noted that 11-HETE and prostaglandin E_2 production was unaffected. Numerous acetylenic fatty acids have now been synthesized and their ability to inhibit lipoxygenases and other related enzymatic conversions has been assessed. In a study utilizing human platelets,[137] the majority of the compounds tested inhibited 12-lipoxygenase and cyclo-oxygenase with the 4,7,10,13-ETYA being both the most potent and selective inhibitor of the 12-lipoxygenase. In another study,[138] these compounds were found to be poor inhibitors of the 5-lipoxygenase compared with their ability to inhibit production of SRS activity from either arachidonic acid or LTA_4. The inhibition of the 12-lipoxygenase from human platelets by a series of ferric iron chelators has been described.[139] The lipoxygenase of human platelets has been studied in an acetone-pentane powder preparation.[140] A series of phenylhydrazones, e.g. 40, inhibit this enzyme as well as soybean lipoxygenase. The 15-lipoxygenase in rabbit peritoneal polymorphonuclear leukocytes has been partially purified and studied.[141] The enzyme is sensitive to sulfhydryl blocking agents and is inhibited by ETYA and BW 755C (41). Colchicine, cytochalasin B, and isoproterenol (10) have no significant effect on HETE formation in rabbit polymorphonuclear leukocytes.[142]

Diethylcarbamazine has been shown to inhibit SRS, histamine, and prostaglandin F_2 production or release from sensitized guinea pig chopped lung challenged with antigen.[143] Inhibition of the SRS activity occurred at lower concentrations than did the other two effects. These findings are of interest in light of early clinical studies claiming therapeutic effectiveness of diethylcarbamazine in the treatment of asthma.[144]

LTA_4 Synthesis Inhibitors - 5,8,11,14-Eicosatetraynoic acid (ETYA) inhibits the formation of LTA_4 from 5-HPETE at lower concentrations than causes other effects.[145]

SRS Antagonists - No compounds, other than FPL 55712 (42) and its newer analog FPL 59257 (43), have been described which specifically antagonize SRS effects. FPL 55712 has been studied in a variety of systems, including bronchoconstriction produced by LTC_4 and LTD_4 in man where it inhibits the bronchoconstriction in some patients.[146,147]

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Chapter 7. Antihypertensive Agents

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Introduction - Hypertension remains a major risk factor in cardiovascular disease. Studies continue to indicate that lowering of blood pressure reduces complications and death rate;[1] however, the aggressive management of borderline hypertension remains controversial.[2-5]

Although the pathogenesis of hypertension remains to be elucidated, much of the effort to understand the basis of the disease remains focused on the kidney. The regulatory factors impinging on renal function and their relationship to essential hypertension have been reviewed.[6] In essential hypertension there is a reduction of renal plasma flow. This reduction is limited to the cortical nephrons while juxtamedullary nephrons maintain normal post-glomerular flow. A genetically determined defect in the cortical nephron autoregulatory control mechanism may be responsible.[7] This inherited defect diminishes the ability of the kidney to eliminate sodium and may cause an increase in the concentration of a circulating sodium transport inhibitor. This results in an increase in arteriolar intracellular sodium which is reflected in an increase in vascular reactivity.[8] Certain factors such as vasopressin appear less likely to be causative.[9] The importance of others, such as a circulating inhibitor of Na^+,K^+ ATPase, remains to be more completely elucidated.[10]

Agents opening new directions for improved therapy have been reviewed.[11] These include dopaminergic agonists, calcium antagonists, gamma aminobutyric acid agonists, renin inhibitors, phenethylamine-N-methyltransferase (PNMT) inhibitors, morphinomimetic peptides, prostaglandins, vasopressin antagonists, tonin inhibitors, bradykinin, α_2-adrenoceptor antagonists and medullary neutral lipids.[12]

Renin-Angiotensin System - Novel antihypertensive agents that operate through inhibition of the renin-angiotensin system remain a focal point for research, and the various approaches directed toward interfering with this system have been reviewed.[13,14]

Renin - Prostaglandins (PG) have been shown to mediate renin release secondary to their stimulation of the renal baroreceptors and the macula densa. Activation of α-adrenergic receptors involves a PG mediated renin release while stimulation of β_1-adrenergic receptors[15,16] involves a renin release that is independent of the PG system.[17,18] Calcium influx through voltage sensitive calcium channels has an inhibitory coupling role on the control of renin secretion.[19] Since aprotinin suppresses active renin without affecting inactive renin, it has been suggested that a kallikrein-like enzyme is responsible for the activation of prorenin.[20]

Converting Enzyme - Mechanisms other than inhibition of plasma converting enzyme may be involved in the antihypertensive action of captopril in man and animals. In spontaneously hypertensive rats captopril alters the permeability of sodium in the vascular smooth muscle membrane.[21] In man

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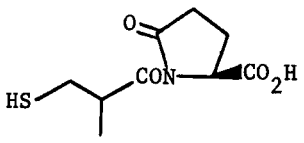
after long term administration of captopril, serum converting enzyme returns toward control levels. In addition, indomethacin modifies the antihypertensive effect suggesting involvement of local kinin-PG system.[22]

Clinical and preclinical reports have appeared describing the higher potency and longer duration of action of the non-sulfhydryl angiotensin converting enzyme inhibitor enalapril (MK-421).[23,24] Other novel inhibitors, both with[25,26] (1,2) and without[27] (3) sulfhydryl functions, have been described.



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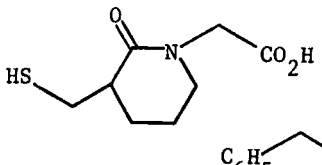

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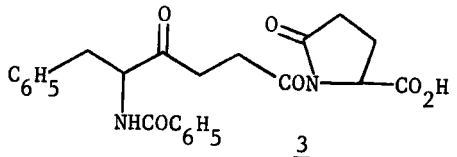


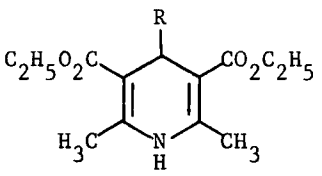
3

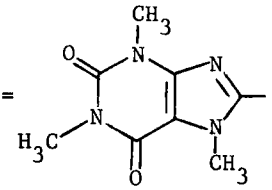
Angiotensin II - The incorporation of hexafluorovaline (Hfv) into angiotensin II (AII) yields a derivative [AcAsn[1], DL-Hfv[8]] AII having potent in vitro and in vivo AII inhibitory activity.[28]

Calcium Channel Inhibitors - Calcium channel blocking agents continue to constitute an area of broad interest for the treatment of angina, arrhythmias and hypertension. Complete reviews of the voltage dependent calcium channel[29] and cellular calcium modulation and control[30,31] have been published. The mode of action and the comparative pharmacology of the three principal classes of calcium channel blockers, i.e., verapamil, diltiazem and the dihydropyridines, have been described.[32-34] Several methods for the reliable estimate of relative potency of the different calcium channel blockers have been developed.[35] A method which depends on the glucose stimulated release of insulin from isolated rat islets[36] may provide a method of comparison for the tendency of calcium channel blockers to induce hyperglycemia.

Structure-activity overviews on the dihydropyridine class have appeared.[37,38] The antihypertensive activity within the nifedipine series is retained when a 2-alkyl moiety is replaced by an amino group.[39] Examples have been prepared involving substitution of the nitrophenyl substituent in nifedipine by a 1,3-dimethylxanthine[40] (4) and a benzoxadiazole (5) group, PY-108-068.[41] The latter example is highly potent in vitro and is not competitive in its blockade. An evaluation of nifedipine analogs indicates that those containing mixed ester functionalities, illustrated by nitrendipine, are comparatively superior to examples having identical ester groups.[42] The influence of ring substituents on the activity of verapamil has been investigated by means of qualitative and quantitative analyses.[43] Structural types other than verapamil, diltiazem and nifedipine have been shown to possess calcium channel blocking activity. These include cyproheptadine[44] and L9394 (6).[45]

The processes involved in activation and inhibition of the calcium channel are being clarified. In the rabbit, histamine stimulates the slow calcium channel via activation of H_1 receptors,[46] and in rats the influx of extracellular calcium necessary for vasoconstriction is initiated by stimulation of vascular postsynaptic α_2 adrenoceptors.[47] A fraction of the sites labeled by the α_1 ligand [^{3}H]WB-4101 in membrane





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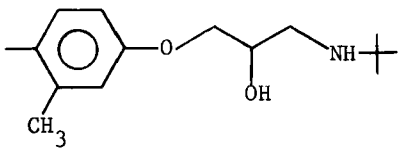

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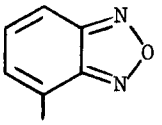

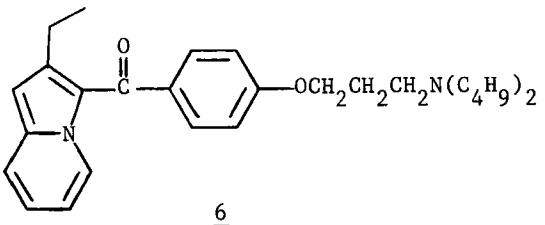

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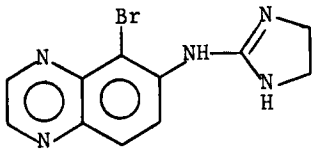
preparations from brain and NG-108-15 cells may be accounted for by binding to calcium channels.[48] At least one member of the dihydropyridine class, felodipine, interacts with intracellular calcium binding proteins such as calmodulin,[49] while diltiazem appears to have effects on intracellular stores of calcium.[50] The exact mechanism through which nifedipine acts may also be more complex than anticipated. This calcium channel blocker was found to normalize the high blood pressure of salt-loaded Dahl salt-sensitive rats. Since a decrease in peripheral resistance would not be expected to induce this effect, other mechanisms must be responsible.[51]

Additional reports have appeared on the combination of nifedipine with β-adrenergic blocking agents for the treatment of severe hypertension.[52] Structural hybrids of the dihydropyridine class having a functionality capable of inducing β-adrenergic blockage have been prepared (7); however, vasodilation did not appear to be the mechanism for the antihypertensive activity.[53]

Centrally Acting Antihypertensive Agents - The role of the central nervous system in cardiovascular control has been reviewed.[54-56] The major thrust in the area of centrally acting antihypertensives continues to be directed toward selective α_2 adrenergic agonists. Hypotensive activity has been correlated with hypertensive activity in pithed rats and lipophilicity for a series of structurally diverse clonidine analogs.[57] Additional evidence indicates that the bradycardiac effects of clonidine and related structures are due to activation of peripheral presynaptic α_2 adrenoceptors.[58] The in vitro effects of UK 14,304 (8) have been compared to those of clonidine; the new compound exhibits higher selectivity for and greater potency at the α_2-adrenoceptor.[59] The 6-aryltetrahydropyrroloimidazole ICI 106270 (9), although less potent than clonidine as an antihypertensive, appears to be relatively less sedating.[60] The pharmacological profile of M6434 (10) has been described and its mechanism of action appears to be due to direct α-adrenergic stimulation.[61] Azepexole (BHT-933), which has been classified as a "clonidine type" hypotensive

agent, may be acting primarily through a peripheral mechanism involving presynaptic α_2 receptors.[62]



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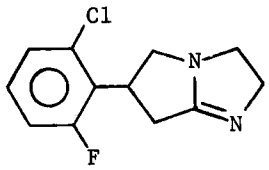

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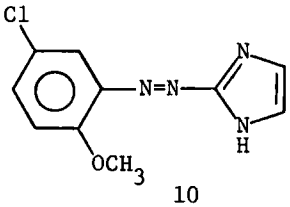
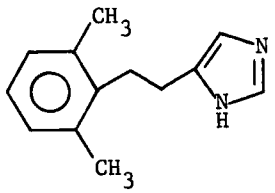


10

A class of centrally acting agents, represented by MPV 295 (11), has been reported. Although the exact mechanism has not been determined, a decrease in norepinephrine turnover does not appear to be involved.[63,64]



11

The possible involvement of serotonin (5-HT), epinephrine and glutamate in the central regulation of blood pressure is receiving attention. The enhancement of 5-HT function, either directly by 1-(m-trifluoromethylphenyl)piperazine or indirectly by fenfluramine, lowers blood pressure (bp) in spontaneously hypertensive rats.[65] Improved mapping of the central catecholamine pathways has strengthened the regulatory role of adrenaline neurons.[66] Evidence has been presented that L-glutamic acid, not substance P, is the primary neurotransmitter of baroreceptor afferents terminating in the nucleus tractus solitarius.[67]

Further studies in the control of bp via inhibition of phenylethanolamine-N-methyltransferase (PNMT) have been reported. The antihypertensive properties of SKF 29661 in the DOC-salt model suggest an important role for central epinephrine in regulating peripheral sympathoadrenomedullary and baroreceptor reflex activity.[68] An approach to antihypertensive therapy through inhibition of adrenal medullary PNMT has also been advanced.[69] The role of PNMT in the formation of an active metabolite of α-methyldopa has been suggested. Although α-methylepinephrine, the putative metabolite, has lower affinity for the α_2-adrenergic receptor than α-methylnorepinephrine, it nevertheless has the potential for pharmacological relevance.[70]

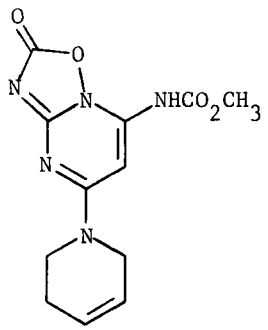
Vasodilators - The approach to antihypertensive therapy via a reduction in elevated total or regional peripheral vascular resistance remains an attractive target. The concept, the neurohumoral and local regulatory mechanisms, and the established vasodilating agents have been reviewed.[71] The advances have been modest and most efforts have centered around modification of standards such as hydralazine, minoxidil and adenosine.

Both animal[72] and human studies[73] have been reported with the minoxidil derivative carpazadil, RO 12-4713, (12). Although this vasodilator did not produce tachycardia and fluid retention in animals, a doubling of plasma renin activity and increases in heart rate were seen in man. Intensified diuretic therapy was needed to control weight gain or edema.

Pharmacokinetic and metabolism studies of the vasodilator tolmesoxide have revealed high bioavailability and rapid conversion to the sulfone Rx 71,112.[74] Mechanistic studies with tolmesoxide and its

metabolite suggest that inhibition of transmembrane flux is not an essential part of the vasodilating action.[75]

Studies into the mode of action of hydralazine and propildazine suggest interaction with a smooth muscle receptor sensitive to endogenous ATP and adenosine.[76] Possible mechanisms for hydralazine-induced lupus-erythematosus have been proposed.[77-78] The hydralazine analogs, budralazine,[79] cadralazine[80] and a 6-methylimidazolyl derivative[81] (13) may offer advantages in potency and/or toxicity. A central mode of action has been postulated for the hydrazinopyridazine GYRI 11679 (14); however, an additional peripheral component was not excluded.[82]



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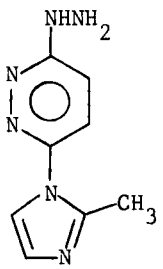

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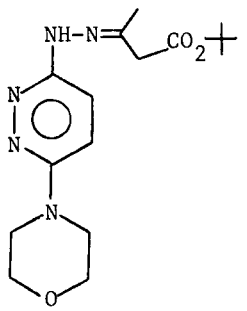
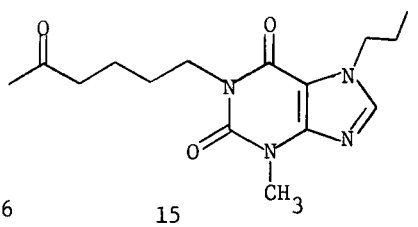


14

In man pinacidil (P1134), a compound structurally related to guancydine, lowers blood pressure apparently by relaxation of precapillary resistance vessels.[83,84] As with other vasodilators, fluid retention was observed in man.

Within the purinergic class, analogs of the P_2 agonist doridosine have been described; none were superior to the parent.[85] The xanthine derivative HWA 285 (15) was found to decrease blood pressure and total peripheral resistance in dogs by preferential dilation of small arterioles.[86]



15

β-Adrenoceptor Antagonists - In independent, double-blind, randomized studies, timolol,[87] metoprolol[88a] and propranolol[88b] have been found to reduce mortality rate 44.6%, 36% and 26%, respectively, in patients surviving acute myocardial infarction. In animal studies infarct size was also significantly reduced by the administration of atenolol, nadolol or propranolol. These latter studies imply that in the dog the limitation of infarct size by β-adrenoceptor antagonists is not influenced by relative cardiac selectivity or membrane depressant activity.[89] Although the precise mechanism of this protective effect is not understood, it is possible that the protection of platelets against aggregatory stimuli may be involved.[90] In addition to limiting infarct size, nadolol has been reported to counteract the reduction in cortical nephron plasma flow observed in essential hypertension.[91]

Reports continue to appear on structurally novel agents having unique properties in their pharmacological profile, such as the long duration of action observed with FM24[92] (16) and N696[93] (17). Unsuccessful attempts have been made to combine diuretic activity and β-adrenergic antagonism into a single molecular entity.[94] Efforts to shift selectivity

toward the β_2-adrenoceptor by the introduction of a γ-methyl substituent into the aminopropanol side chain generally have been unrewarding.[95,96]

β_1 Selective Adrenoceptor Antagonists - Based on results with atrial preparations from rat, guinea pig and cat, it has been suggested that β_1 defines innervated receptors that respond to neuronally released norepinephrine whereas β_2 defines hormonal receptors mediating responses to circulating epinephrine.[97] In support of the concept that the β-receptor subtypes are indeed discrete entities, a difference in the thermal sensitivity has been observed between solubilized β_1 and β_2 adrenoceptors from rabbit lung.[98]

The use of β_1 selective agents in the treatment of hypertension and cardiovascular disease should reduce the propensity for side effects such as bronchospasm, associated with inhibition of the β_2-receptors. To assist in the clinical evaluation of β_1-selective antagonists, a new method for quantitative measure of bronchial β-adrenoceptor blockade has been developed.[99] However, the possibility of locally enhanced levels of mediators such as histamine causing a bronchospasm has not been completely eliminated.[100]

In a comparison of the cardioselective agent atenolol with pindolol, it has been suggested that pindolol by β_2-mediated inhibition of lipolytic, glycogenolytic and growth hormone releasing effects may favorably influence oxygen consumption and make myocardial performance more economical.[101]

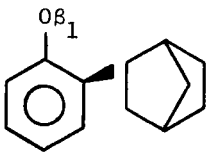
. The optical isomers of the β_1 selective agent bometolol have been prepared; the (S) isomer is both more potent and β_1 selective than the (R) enantiomer.[102]

Vasodilator/β-Adrenoceptor Antagonist - The search continues for the ideal symbiotic agent possessing the two complementary features of vasodilation and β-adrenoceptor blockade. The four chiral forms of the α,β-adrenergic receptor antagonist labetalol have been prepared: the R,R isomer, Sch 19927, is four times as potent as labetalol in blocking β-receptors but only one-third as potent in blocking α-receptors. The antihypertensive effect observed in animals with Sch 19927 is suggested as being the result of direct vasodilation rather than α blockade.[103] The N-phenylbutyl analog of the vasodilator/β-adrenoceptor antagonist MK-761 exhibits both α/β-adrenergic receptor blocking activity. Chirality at each of the two asymmetric centers independently influences binding characteristics with the S,R isomer having the highest receptor affinities.[104]

The vasodilator/β-adrenergic antagonist prizidilol (SKF 92657) reduces blood pressure in a variety of animal models with no indication of tolerance in the rat. It is equipotent to propranolol as a β-adrenoceptor antagonist with partial agonist properties and no selectivity in its blockade. Prizidilol reduces blood pressure in human subjects and its bioavailability is influenced by the rate of metabolic acetylation. Like hydralazine, it appears to be a substrate for polymorphic N-acetyltransferase. Clinically, the dose used to reduce blood pressure induces slight tachycardia.[105]

The effect of bucindolol in rats has been described. The compound has potent β-adrenoceptor blocking properties and exhibits an apparent direct relaxant effect on vascular smooth muscle.[106] The introduction of

a 2-isoxazolylethenyl substituent into the phenoxypropanolamine moiety (18) introduces α-adrenergic blockade, yielding a more potent α,β-adrenoceptor antagonist and antihypertensive agent than labetalol.[107]



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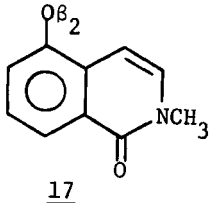

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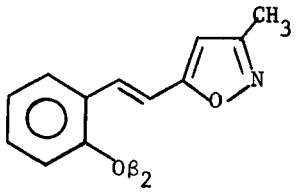
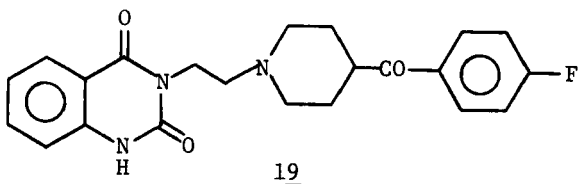


18

β_1 = $-CH_2CHOHCH_2NHCH(CH_3)_2$ β_2 = $-CH_2CHOHCH_2NHC(CH_3)_3$

Miscellaneous - Both N,N-di-n-propyldopamine[108] and pergolide[109] lower arterial blood pressure in rats by activation of presynaptic dopamine receptors located on vascular sympathetic neurons. Stimulation of these receptors inhibits the neurogenic release of norepinephrine, thereby inducing a passive relaxation of vascular beds.

An antihypertensive effect has been observed in man with the serotonin antagonist ketanserin, R41468 (19). An inhibition of the direct vasopressor properties of serotonin is suggested.[110]



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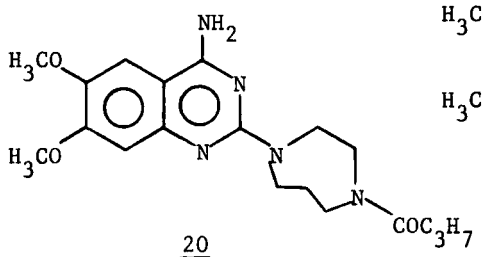

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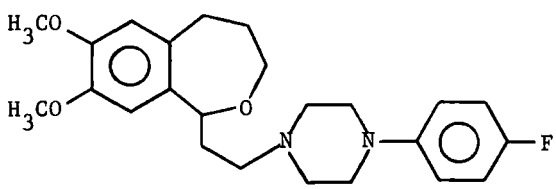

21

The antihypertensive activity of α-fluoromethyldopa depends on a decrease in sympathetic function due to depletion of peripheral transmitter stores. This depletion results from the irreversible inhibition of L-aromatic amino acid decarboxylase.[111]

Human pharmacokinetic studies of the α_1-adrenergic antagonist prazosin indicate substantial binding to albumin and α_1-acid glycoprotein, with wide variations in plasma concentrations between patients and between days in the same patient.[112] Specific blockade of α_1-adrenoceptors is also the basis for the antihypertensive action of E-643[113] (20) and the tetrahydrobenzoxepine (21).[114]

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Chapter 8. Antianginal and Anti-ischemic Agents

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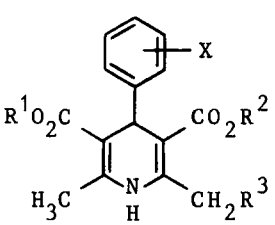
Introduction - Angina pectoris is one of the most frequent clinical syndromes associated with ischemic heart disease. The main progress in research and development of antianginal and anti-ischemic agents since the last review in Annual Reports[1] has clearly been among the calcium antagonists. No fundamentally new mechanistic approaches to anti-ischemic therapy have emerged.

Calcium Antagonists - The definition and mechanism of action of calcium antagonists have been treated in reviews.[2,3] Calcium antagonists offer an alternative to ß-blockers and nitrates for the treatment of ischemic heart disease. The three agents most extensively studied for this indication are verapamil, diltiazem and nifedipine (1), the latter recently obtaining NDA approval.

Numerous recent clinical reviews show the wide applicability of these drugs in the treatment of cardiac oxygen-deficiency diseases, particularly angina pectoris in its various forms.[4-12] The high rate of therapeutic success compared to standard treatment is accompanied by only a slight incidence of side-effects. These new drugs have been compared with one another in two clinical trials,[13,14] nifedipine emerging as the most potent. This result is supported by comparative pharmacological studies.[15,16] Primary research interest has therefore been directed particularly towards the variable structure of the highly active calcium-antagonistic 4-aryl-1,4-dihydropyridine-3,5-dicarboxylates. The current status of drugs under development from this group[17] and the chemistry of the dihydropyridines have been surveyed.[18] Qualitative structure/activity studies have been concerned with the nature of the ester groups,[19,20] the replacement of the methyl group in the 2-position with an amino function,[21] and the introduction of "ß-blocker sidechains" in the phenyl ring.[22]



	X	R^1	R^2	R^3
1	2-NO_2	CH_3	CH_3	H
2	3-NO_2	$nPrOCH_2CH_2$	$nPrOCH_2CH_2$	H
3	3-NO_2	C_2H_5	CH_3	H
4	2-NO_2	CH_3	$(CH_3)_2CHCH_2$	H
5	3-NO_2	C_2H_5	C_2H_5	OH
6	3-NO_2	CH_3	$C_6H_5CH_2N(CH_3)CH_2CH_2$	H
7	3-NO_2	CH_3	$(CH_3)_2CH$	H
8	2,3-Cl_2	C_2H_5	CH_3	H

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The results from these qualitative studies have been confirmed in the first analyses of quantitative structure/activity relationships (Hansch approach,[23] torsion angle at C_4 of the dihydropyridine nucleus[24]).

Dihydropyridines with non-identical ester functions ($R^1 \neq R^2$) are usually superior to the corresponding symmetrically substituted derivatives in terms of vasodilating action.[25] Such compounds possess a chiral center at C_4. The dextrorotatory enantiomer of nicardipine (6) has three times the vasodilating action of (-)-nicardipine in the dog.[26] Even larger differences have been found between the enantiomers of (7). Synthesis *via* diastereomeric intermediates of known absolute configuration has enabled conclusions to be drawn regarding the preferred configuration at C_4. The (-)(4S)-enantiomer of (7) was found to be 10x more active than the (+)(4R)-antipode in the barium-stimulated guinea pig ileum test.[27] These results show that the dihydropyridines, like verapamil and diltiazem, exhibit stereoselectivity in their calcium-antagonistic activity, which makes the existence of a stereospecific binding site probable: ^{3}H-Nitrendipine (3) binds reversibly to saturable structures of cardiac muscle cell membranes. The high affinity binding site has a dissociation constant of ca. 0.1 nmol/L and a density of 300 fmol/mg protein.[28-30] The binding sites for ^{3}H-nitrendipine are stereospecific as they differentiate between the enantiomers. Interestingly, verapamil and diltiazem show no affinity for the 1,4-dihydropyridine binding site.

Niludipine (2) has a similar pharmacological profile to nifedipine but is characterized by less cardiodepression in the dog.[31] Its anti-anginal activity and good tolerance have been confirmed in clinical trials.[32]

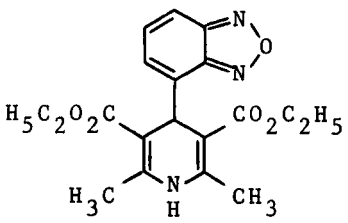
Nicardipine (6) is a highly active calcium antagonist, as has been shown on potassium-depolarized aortic strips,[33] so its weak inhibitory action upon phosphodiesterase can hardly be thought to contribute to vasodilation. Its pharmacokinetics are characterized by the rapid achievement of steady state with low plasma concentrations following prolonged administration to various animal species and to humans.[34] The metabolites have been synthesized and found to have no significant vasodilatory activity themselves.[35]

Nisoldipine (BAY k 5552, 4) is a new dihydropyridine derivative which is 4-10x more active than nifedipine in *in vitro* and *in vivo* experiments. This substance exhibits a selective peripheral vasodilatory effect.[36,37] Pharmacological investigations in comparison to verapamil and diltiazem confirmed its potent effect on blood flow in the ischemic myocardium.[38] In a preliminary clinical study, a dose of 1.5 µg/kg i.v. caused a 28% reduction in total peripheral resistance.[39]

FR-7534 (5) has a similar pharmacological profile to nifedipine, albeit at higher doses, on the basis of studies on the isolated guinea pig heart,[40] on potassium-depolarised coronary arteries[41] and collateral coronary flow in the dog.[42]

Felodipine (H 154/82, 8) is comparable to nifedipine in its potency. Animal studies have shown it to be a selective peripheral vasodilator.[43,44] Highly significant increases in coronary sinus flow and reductions in systemic and coronary vascular resistance were found at single doses of 10 mg p.o. in a clinical trial in patients with coronary heart disease.[45]

The proof of a strong binding of ^{14}C-felodipine to calmodulin is of great significance with respect to the molecular mechanism of action of 1,4-dihydropyridines.[46] As calmodulin is also capable of causing membrane activation,[47] an intracellular mechanism of action involving this Ca^{2+}-binding protein is not to be ruled out. It has been shown that neither verapamil, diltiazem nor nifedipine influences the uptake of $^{45}Ca^{2+}$ at concentrations which inhibit vascular contraction.[48] This is contradicted by a more complete study of differences between calcium channels which showed that gallopamil (D 600, 10) does inhibit unidirectional $^{45}Ca^{2+}$-uptake in such concentrations.[49] Interactions of verapamil and nifedipine with calmodulin have also been reported.[50]



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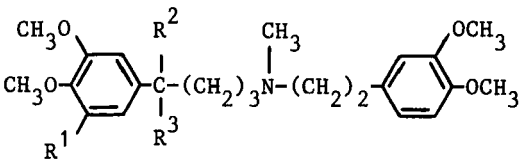


10 $R^1 = OCH_3$ $R^2 = iPr$ $R^3 = CN$

11 $R^1 = H$ $R^2R^3 = SO_2(CH_2)_3SO_2$

PY-108-068 (9) is the first dihydropyridine derivative under development which has a condensed aromatic system in the 4-position. Calcium-antagonistic properties have been demonstrated in vitro on the coronary artery of the dog. At similar doses to nifedipine, PY-108-068 increases coronary blood flow and reduces blood pressure in the anaesthetised cat. In this animal model a drop in heart rate was observed with PY-108-068, in contrast to nifedipine. A marked increase in renal perfusion was measured using the microsphere technique.[51]

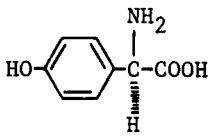
Qualitative and quantitative structure/activity relationships have been established in the verapamil series with respect to the negative inotropic action on cat papillary muscle. The substitution in the phenyl ring neighboring the chiral center has the greatest influence on activity. The 3,4-Cl_2-, 3-CF_3- and 3,4,5-$(OCH_3)_3$-substituted derivatives are more active than verapamil for otherwise identical functionality.[52] The enantiomers of the latter (gallopamil, D 600, 10) exhibit marked differences in the blockade of the contractions of various vascular strips following potassium-depolarization or activation of muscarinic or α-adrenergic receptors. The (-)-enantiomer was 6-180 x more active depending on the vessel and stimulation method.[53] In a double-blind crossover study against pindolol, verapamil in combination with a thiazide diuretic showed a comparable antihypertensive activity.[54]

Tiapamil (Ro 11-1781, 11), a verapamil analog, had a similar profile to verapamil and gallopamil in clinicopharmacological and electrophysiological studies, but at a tenfold i.v. dosage.[55,56] Its therapeutic efficacy in the treatment of supraventricular and ventricular cardiac

rhythm disturbances, coronary heart disease and Wolff-Parkinson-White syndrome has been demonstrated in several trials.[57-59]

New pharmacological results with calcium-antagonists, which could broaden their clinical application, include the reduction in blood pressure and in mortality of saltsensitive Dahl rats (following prolonged administration of nifedipine[60]), and an anti-atherosclerotic effect in rabbits fed on cholesterol (8-week administration of ca. 18 mg/kg p.o. nifedipine per day).[61] In a clinical study, nifedipine showed therapeutic activity against stress-induced asthma.[62] The strong inhibition by nicardipine (10^{-6} g/ml) of potassium-induced contraction of isolated guinea pig trachea must be considered in this context.[63]

Miscellaneous Antianginal Agents



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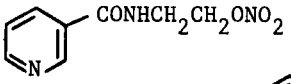

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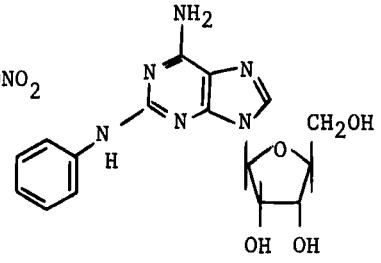


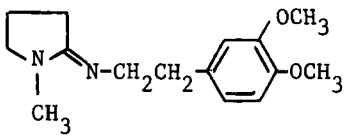
14

Although the major proportion of myocardial energy supply comes from oxidation of fats, carbohydrates require less oxygen for the same energy yield. Oxfenicine (UK 25842, L-4-hydroxyphenylglycine, 12) reduces the uptake of free fatty acids in myocardial cells.[64] The basic mechanism is activation of pyruvate-dehydrogenase,[65,66] which then results in an improved glucose utilization[67] and hence an enhanced myocardial efficiency. Oxfenicine is actually a prodrug of the active entity, 4-hydroxyphenylglyoxylic acid, which, in comparison with other para-substituted phenylglyoxylic acids, caused the strongest enzyme activation in a qualitative structure/activity study.[68] In healthy volunteers, oxfenicine prevented the initial drop in glucose levels under exercise tests.[69] Clinical pharmacological studies on heart-catheterized patients suffering from coronary artery disease gave no indications of haemodynamic changes. The activation of carbohydrate metabolism revealed itself in an enhanced lactate and pyruvate extraction and increased arterial concentration of free fatty acids.[70] Angina pectoris patients responded to higher doses (3-12 mg/kg i.v.) with a substantially increased time of onset of an angina attack induced by pacing.[71] In contrast, the same parameter for exercise-induced angina (single dose 420 mg i.v.) was not influenced significantly compared to placebo.[72]

Nicorandil (SG-75, 13) is a long-acting nitrate currently undergoing Phase III studies in Japan as an antianginal drug. A hardly detectable development of tolerance, compared with other nitrates, is its major advantage.[73] I.v. nicorandil has a relatively high selectivity of action on canine coronary vessels, the vasodilating effect on renal and femoral vessels being less marked. This selectivity distinguishes it from nitroglycerine and the calcium-antagonist diltiazem.[74] The primary step in its

metabolism is the rapid cleavage of the nitrate group yielding the hydroxyethyl-amide which is then partly oxidized to nicotinylglycine.[75] Clinical studies to date in Japan indicate good therapeutic successes in the treatment of angina pectoris.

CV 1808 (14), currently undergoing Phase I testing in Japan, is a new C_2-substituted adenosine derivative which, like adenosine itself, is a strong coronary vasodilator.[76] In contrast to the parent compound it is potentially orally active in man (active dose 10-25 µg/kg i.d. in the cat) and characterized by a longer duration of action. Doses of 1.1 µg/kg i.v. caused a doubling of coronary blood flow in the dog. In this model, CV 1808 is thus more active than nifedipine (12 µg/kg i.v.), nitroglycerine (38 µg/kg i.v.) and dipyridamol (95 µg/kg i.v.).[77]



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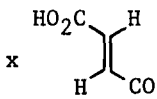

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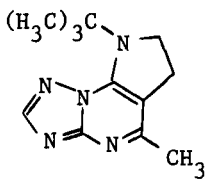

16

The majority of drugs which lower heart rate also reduce myocardial contractility. Mixidine fumarate (15)[78] causes a dose-dependent reduction in heart rate of long duration in the anaesthetized cat. A reduction in rate without a concomitant decrease of contractility is observed in the spontaneously beating guinea pig atrium (from 3 µM/L). In contrast, under electric pacing, a positive inotropic effect was observed at 10 µM/L, which could not be antagonised by a β-blocker. Oral doses of 15 mg/kg and greater significantly weakened canine sinus tachycardia induced by treadmill exercise. This activity profile makes mixidine fumarate an interesting candidate for antianginal therapy in man.[79,80]

The pharmacological profile of bumepidil (CS-611, 16) is similar to that of calcium-antagonists of the verapamil type. In the dog, doses of 0.1-1 mg/kg i.v. cause a marked, dose-related rise in coronary blood flow, without a significant effect on heart rate or AV conduction. The anti-arrhythmic activity of bumepidil is comparable to that of lidocaine in arrhythmias induced by coronary ligature. In-depth studies on the effect of bumepidil on sinus rhythm and AV conduction in the dog showed that i.v. doses of 1 mg and more lead to a loss of sinus rhythm and to a temporary AV block.[81,82]

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Chapter 9. Antithrombotic Agents

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Introduction - The impact of thrombosis on mortality and morbidity in western civilisation is considerable. Arterial thrombosis plays a role in acute myocardial infarction, stroke, and in the growth and terminal occlusion of the atherosclerotic lesion. Pulmonary embolism, resulting from venous thrombosis, is a major threat to life in the post-operative state and is a frequent cause of death among hospitalised adult patients. Furthermore, two or more episodes of non-fatal pulmonary embolism are estimated for every fatal episode. Thromboembolism induced by contact of blood with foreign surfaces is a serious problem in the use of prosthetic devices implanted within the heart and blood vessels or for extracorporeal circulation. Enhanced formation of platelet aggregates in the small peripheral vessels may be a cause of increased vascular resistance, thereby inducing pathology in a variety of diseases among which are hypertension, stroke, diabetes and cancer.[1]

Medical treatment of thrombosis may be divided into three distinct areas: platelet suppression, anti-coagulant and fibrinolytic therapy. Drugs used in these areas were last reviewed in this series three years ago.[2] This review reports on the progress made since that time.

Platelets and Platelet Aggregation Inhibitors

Platelets are useful as markers of thromboembolic disease; normal platelet physiology and platelet malfunction in disease has been reviewed.[3-5] The use of ^{111}indium-labelled platelets to allow external imaging of localised clots has been described.[4-6] When activated in response to vascular injury or through participation in blood coagulation, platelets release several proteins not normally found in the plasma. Elevated levels of these proteins may therefore indicate the presence of vascular disease. In some patients with considerable coronary artery disease platelet factor 4 (PF-4) levels were found to increase during exercise-induced myocardial ischaemia.[7] Similarly, β-thromboglobulin and PF-4 levels were significantly elevated in blood samples taken from patients experiencing unstable angina pectoris.[8] Abnormal platelet behaviour has been detected in patients with transient ischaemic attacks and cerebral infarction.[9] New evidence has accumulated indicating possible interactions of cancer cells with the haemostatic system, and the clinical relevance of such interactions has been discussed.[10]

In recent years the discovery of a vasoconstrictor, pro-aggregatory factor, thromboxane A_2 (TxA_2), formed in blood platelets, and a vasodilator and anti-aggregatory factor, prostacyclin (PGI_2), formed in the endothelial layer of the vasculature, has had a major impact on our understanding of vascular homeostasis. These two prostanoids, together with PGD_2, PGE_2 and $PGF_{2\alpha}$, are part of the arachidonic acid cascade and are generated from a common precursor, PGH_2 endoperoxide. In the normal situation, the prostacyclin and thromboxane biosynthetic pathways are in balance. However, should vascular prostacyclin synthesis be impaired

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and/or platelet activation be enhanced, then thrombosis and vasoconstriction will be favored. Abnormalities of platelet behavior and an alteration in the prostacyclin/thromboxane balance have been reported in patients with diabetes mellitus[11] and unstable angina pectoris.[12] Increases in TxA_2 levels have also been correlated with the severity of early post-infarction arrhythmias induced in the dog following acute coronary artery ligation.[13] Comprehensive reviews on the role of the prostanoids in haemostasis and thrombosis have been published.[14,15]

Calcium ion is a pre-requisite for the formation of a vascular plug, and the mechanisms by which calcium-dependent signals are translated into biological responses have been the subject of intense scrutiny. An important finding in this area was the discovery that the calcium-binding regulatory protein calmodulin is present in platelets.[16] When fully activated by Ca^{++}, calmodulin stimulates the activity of phospholipase A_2, and thus is able to control the formation of endoperoxides and TxA_2 in the platelet. In addition, the contraction of platelet actomyosin after the formation of TxA_2 is under calmodulin control.[17]

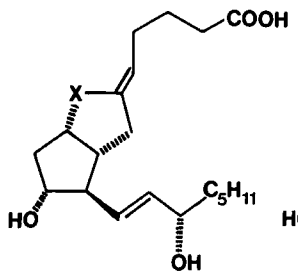
The crucial role played by platelets in haemostasis and thrombosis is evident. Thus, in recent years, therapeutic approaches to the problem of thrombosis, particularly arterial thrombosis, have increasingly focused on the inhibition of platelet aggregation. The compounds that have been reported to influence platelet aggregation will be discussed as follows: 1) prostacyclin and analogues, 2) thromboxane synthetase inhibitors and thromboxane antagonists, 3) aspirin, sulfinpyrazone and non-steroidal anti-inflammatory agents and 4) miscellaneous agents.

Prostacyclin and Analogues - Prostacyclin (1) inhibits platelet aggregation by stimulating adenylate cyclase, leading to an increase in cAMP levels in the platelets. The generation of prostacyclin by the endothelial cells protects the vessel walls against deposition of platelet aggregates.[18,19] In neutral or acidic solution, prostacyclin is unstable due to hydrolysis and, as it is orally inactive, continuous intravenous infusions are necessary.[20] When administered to man, prostacyclin inhibited platelet aggregation and, at higher doses, dispersed circulating platelet aggregates.[20,21] Vasodilator side effects were also observed, and a systematic approach to dose-response relationships has now been carried out.[22] It was found that short (30-60 minute) infusions were generally well tolerated up to a maximum of 10ng/kg/min. Because of significant complaints of headache, nausea, anxiety and vomiting, along with marked facial flushing and reduction in diastolic blood pressure, higher doses were considered inadvisable even for short periods of time.

In dogs, infusion of prostacyclin prevented blockage of partially obstructed coronary arteries.[23] In coronary-ligated animals, prostacyclin was found to be both anti-arrhythmic and arrhythmogenic, depending on the dosage.[24-26] Prostacyclin is a powerful anti-metastatic agent against B16 amelanotic melanoma cells.[27,28] This effect, which appears to result from the platelet anti-aggregatory action of prostacyclin, is potentiated by theophylline. Inhibition of prostacyclin synthesis apparently increases metastasis, and prostacyclin synthesis *in vivo* may play a role in preventing the spread of metastatic disease.[27]

Patients with severe peripheral vascular disease of the lower extremities have been given prostacyclin infusions for periods up to 72 hours.[29-33] The results ranged from good to excellent, with alleviation of rest pain, eventual reversal of necrosis and healing of ischaemic ulcers. Despite the fact that the pharmacological actions of prostacyclin

are short-lasting, the clinical improvement often persisted for several months. Prostacyclin has also been successfully employed in patients with pulmonary hypertension,[34] Raynaud's syndrome,[35] thrombotic thrombocytopenia purpura,[36] and chronic renal disease.[37] It has also been successfully used in patients undergoing charcoal haemoperfusion.[38]



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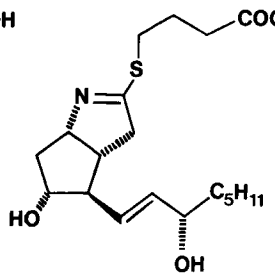

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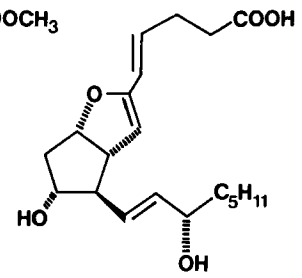

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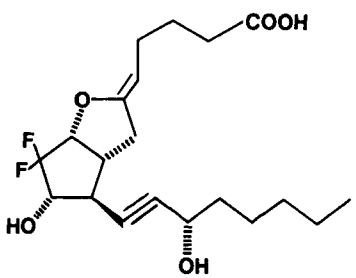
The presence of an acid-labile enol ether group in prostacyclin limits the therapeutic usage of this substance. As a result, efforts have been made to obtain more stable analogs by replacement of the ether oxygen by sulphur (2),[39] methylene (3),[40-42] nitrogen (4)[43] or by insertion of a trans diene system as in 5.[44] Although prostacyclin-like activity was observed with these analogs, potency was reduced. Carbacyclin (3) for example was 0.1 times as active as prostacyclin in inhibiting ex vivo platelet aggregation in both rabbit and dog.[40,41] Although chemically stable, carbacyclin was considered to be metabolically unstable because of its short duration of action in vivo.[41]



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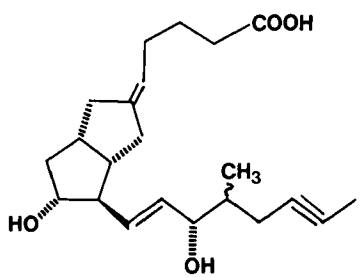

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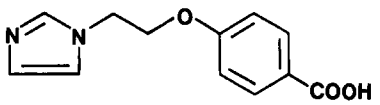
10,10-Difluoro-13-dehydroprostacyclin (6)[45] was resistant to both hydrolytic cleavage and oxidation by 15-hydroxy prostaglandin dehydrogenase. The compound was 3-4 times more potent than prostacyclin in some tests and has a half-life of 24 hours in solution.[46] However, after intravenous administration to dogs, the haemodynamic effects were only of the same duration as those of prostacyclin. ZK-36,374 (7) is a carbacyclin derivative that is equipotent to prostacyclin.[47,48]

Other prostacyclin analogues modified in the ring[49] and the side chain[50] have been synthesized and evaluated.

Thromboxane Synthetase Inhibitors and Thromboxane Receptor Antagonists - Selective inhibition of the thromboxane synthetase enzyme is a potentially attractive approach to antithrombotic therapy. Because the platelet cyclo-oxygenase enzyme will be unaffected by this approach a build-up of endoperoxides will be promoted. Although the endoperoxides are mildly

pro-aggregatory, they should be metabolized by vascular endothelium and white cells into anti-aggregatory prostacyclin.



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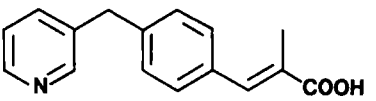

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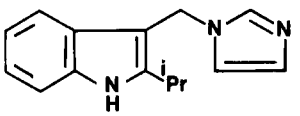

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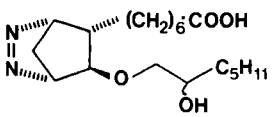
Dazoxiben (UK-37,248, 8) is a selective TxA_2-synthetase inhibitor which, when administered to rabbits, prevented arachidonic acid-induced mortality and reduced the associated thrombosis, vasospasm and elevation of TxB_2.[51] Dazoxiben is orally active, and was found to have a biological half-life of 5-6 hours after administration to man.[52] In a double-blind placebo controlled study in normal humans, the reduction of serum TxB_2 levels by dazoxiben was accompanied by a rise in the levels of 6-keto $PGF_{1\alpha}$, the main metabolite of prostacyclin.[53] Similar findings were made in patients with severe atherosclerotic heart disease[54] and stable and unstable angina.[55] In addition to its potent TxA_2-synthetase inhibitory activity, dazoxiben antagonizes the effects of carbocyclic thromboxane A_2 (CTA_2) on rabbit pulmonary arteries.[56] Various papers on OKY-1555 (9) and its sodium salt OKY-1581 have been published.[57-60] Intravenous injection of OKY-1581 prevented blockage of a partially obstructed coronary artery in the dog.[61] The effect was independent of the degree of partial obstruction and resembled that observed with i.v. prostacyclin. The SAR of 9 and its imidazole analogs has been described.[62,63] 1-Carboxyheptyl imidazole was found to be the most potent of a series of substituted alkyl imidazoles,[64,65] the degree of inhibition of TxA_2-synthetase being dependent on the length of the alkyl chain. 1-Carboxyheptyl imidazole is reported to inhibit tumor growth.[28] UK-34,787 (10) is one of a series of 3-(imidazolylmethyl) indoles whose SAR is described.[66]

When hypertensive patients were treated with dl-propranolol, thromboxane synthesis by their platelets was significantly inhibited, as was platelet aggregation induced by thrombin or arachidonic acid.[67] Similar effects were found with d-propranolol, which has very little beta-blocking activity. Timolol and metoprolol, along with propranolol, were claimed to inhibit TxA_2-synthetase.

The azaprostanoic acid (11) inhibits TxA_2-synthetase and blocks PGH_2/TxA_2 receptors in human platelets.[68,69] Although inactive against the synthetase enzyme 12 functioned as a PGH_2/TxA_2 receptor antagonist in platelets.[70,71] An SAR analysis of various azaprostanoids indicates that the TxA_2 receptor in platelets may be different from the TxA_2 receptor in blood vessels.[72] Pinane thromboxane A_2 (PTA_2, 13) was found to both inhibit the synthesis of TxA_2 and to function as a receptor antagonist. The close analogue CTA_2 (14) had however a rather different profile, the compound inhibited TxA_2 synthetase in platelets but when evaluated *in vivo* it functioned as a very potent vasoconstrictor agent.[76,77] Dual antagonist and synthetase inhibitory actions have been reported for (15)[78] whereas SQ-26,536 (16),[79] EP 045 (17)[80] and AH-19,437 (18)[81-83] were purely thromboxane receptor antagonists.



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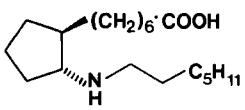

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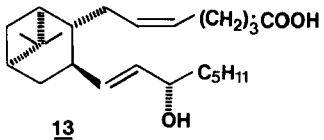

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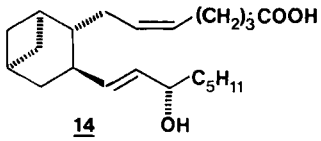

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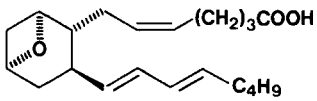

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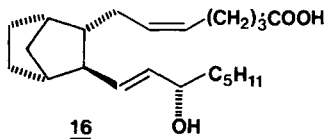

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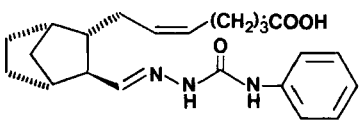

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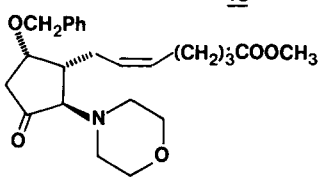

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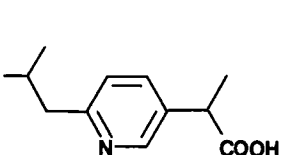
Aspirin, Sulfinpyrazone and Non-steroidal Anti-inflammatory Agents - The results of three major clinical trials concerned with the treatment of patients who have survived an acute myocardial infarction (MI) have been published. The Aspirin Myocardial Infarction study (AMIS) was a randomised, double blind, placebo-controlled trial designed to test whether the regular administration of aspirin for an average of 3 years would result in a reduction in total mortality.[84] The results were inconclusive, as were the results of a similar trial carried out over a 1 year period.[85] The Persantine-Aspirin Reinfarction Study (PARIS) was a 3 year study designed to assess the effects of a combination of persantine plus aspirin compared with the effect of aspirin alone and with placebo on mortality after MI.[86] Again, the results were not statistically significant. However, it did appear that treatment was more likely to influence mortality if started early after MI (i.e. within 6 months). Accordingly, another study (PARIS II) has been designed.[87] The Anturane Reinfarction Trial (ART)[88,89] evaluated the effect of sulfinpyrazone against placebo over an average of 16 months in patients who had survived an acute MI. All the patients were entered for the trial within one month of the infarction. Sulfinpyrazone caused a remarkable 74% reduction in sudden deaths during the critical period of 2-7 months after the MI. However, marketing approval of sulfinpyrazone for MI was withheld because it was claimed that the cause-of-death classification and all conclusions based on it were unreliable.[90] Sulfinpyrazone has been reviewed[91] and the effect of the compound and its sulfide metabolite on platelet function reported.[92]

Most non-steroidal anti-inflammatory (NSAI) compounds inhibit the enzymatic conversion of arachidonic acid to PGG_2 and PGH_2 endoperoxides. This may be undesirable in an anti-thrombotic drug because, although TxA_2 formation is inhibited, the formation of the anti-aggregatory prostacyclin is inhibited also. However, aspirin given in very low doses preferentially inhibits the platelet cyclo-oxygenase enzyme,[14] and may possibly offer advantages over conventional aspirin doses.[93,94]



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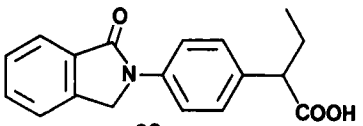

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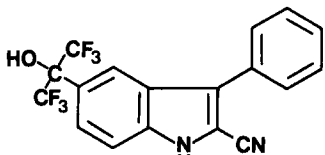

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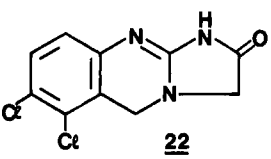
Ibuprofen[95] and its 3-pyridyl analogue (19)[96] both possess moderate platelet aggregation inhibitory activity. The effect of indobufen (K-3920, 20)[97] on platelet function has been investigated in patients with venous thrombosis.[98] Like aspirin, indobufen acts on the platelet release reaction and therefore suppresses the second wave of aggregation. Potent inhibition of the cyclo-oxygenase enzyme, with consequent inhibition of platelet aggregation was reported for (21).[99]

Miscellaneous Agents - Anagrelide (22), a potent inhibitor of platelet PDE, effectively inhibits experimental thrombosis and platelet aggregatability in vivo;[100] details of the metabolism in man of anagrelide have been published.[101] SAR studies on analogs of DH-6471 (23)[102] have indicated the essential contribution of the lactam structure and the lipophilic substituents on the thiophene ring. The anti-aggregant activity of plafibrate (24) results from its effects on platelet PDE.[103] Cilostamide (25), a highly selective cAMP PDE inhibitor, inhibited platelet aggregation induced by collagen, ADP and arachidonic acid.[104]



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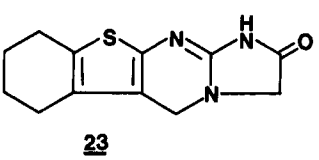

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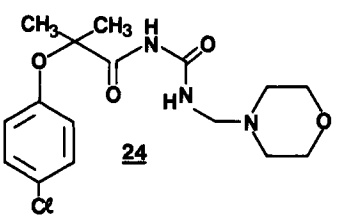

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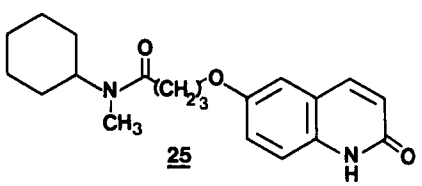

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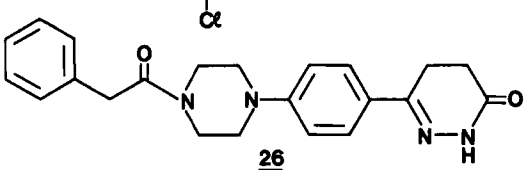

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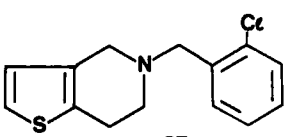

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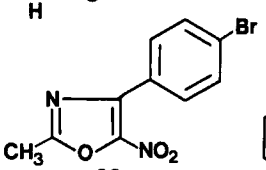

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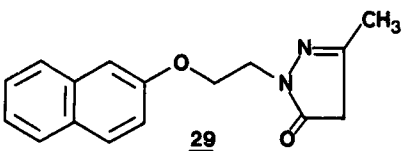

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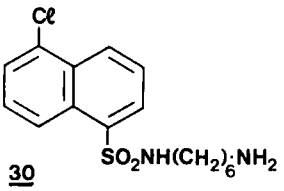
Aggregation of human platelets by collagen, ADP, thrombin and arachidonic acid was inhibited by CCI-17,810 (26).[105] In rats, 28 inhibited thrombus formation.[106] Ticlopidine (27) is said to inhibit platelet aggregation by both activating platelet adenylate cyclase and preventing PGE_2-induced depression of the cyclase activity, thereby raising platelet cAMP levels.[107] General pharmacological[108] and cardiovascular[109] effects of ticlopidine as well as the results of various studies in man have been published.[110-113] The anti-thrombotic activity of a nitro-oxazole S-20,344 (28) is reported.[114] Nafazatrom (Bay g 6575, 29)[115] had no effect on blood coagulation, fibrinolysis or platelet aggregation in vitro, but it inhibited platelet aggregation in vivo. It is thought that the anti-thrombotic properties of nafazatrom are due to

stimulation of prostacyclin release from the vascular endothelium. Administration of nafazatrom to man resulted in prolonged inhibition of ADP-induced platelet aggregation.[116] The calmodulin inhibitors trifluoperazine[17] and W-7 (30)[117,118] completely inhibited platelet aggregation induced by a variety of stimuli. W-7 combines inhibition of platelet aggregation with vascular relaxant activity. The calcium entry blockers nifedipine and verapamil inhibited platelet aggregation in man at therapeutic doses,[119,120] and verapamil was shown to potentiate the anti-aggregatory effects of prostacyclin. Favorable results have been reported for nifedipine[121] and diltiazem[122] in the treatment of Raynaud's phenomenon.



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Anticoagulants

Heparin has been reviewed,[123] and evidence suggesting that heparin would be useful in the prophylaxis and therapy of atherosclerosis has been discussed.[124] Low-dose heparin has been advocated for prophylaxis against pulmonary embolism.[125] A prospective double blind study on the incidence of thrombocytopenia in man with different heparin preparations has been carried out.[126] The results showed that there was a higher (3-4 times) incidence of thrombocytopenia associated with bovine-lung heparin than with heparin from intestinal mucosa. In an attempt to explain this finding, the biological and chemical properties of lung and mucosal heparin have been discussed.[126] The effect of various heparin fractions on platelet aggregation has been examined,[127] and it was concluded that heparin of low molecular weight and high anti-thrombin affinity may provide safer anticoagulant therapy. A new heparin preparation has been characterised and found to be twice as potent as commercial heparin after intravenous administration to man.[128]

A randomised double-blind 2-year clinical trial has been undertaken with the objective of evaluating long-term efficacy and side-effects of oral anticoagulant therapy after MI.[129] The patients were all over 60 years of age, and the anticoagulants employed were acenocoumarin and phenprocoumon. In this selected group of patients continuation of therapy substantially reduced the risk of recurrent MI. In another clinical trial,[130] it was found that warfarin was more effective than low-dose heparin in preventing recurrent venous thromboembolism, but its use was accompanied by a significant risk of bleeding. The effects of the coumarin tioclomarol on coagulation factors in patients have been reported.[131]

Fibrinolytic Agents

The results of a controlled trial of streptokinase in patients who have had a myocardial infarction have been published.[132] It was concluded that a sub-group of "medium-risk" patients, admitted to a coronary-care unit within 12 hours of onset of symptoms, benefited from the fibrinolytic therapy. Although fever, chills and bleeding complications occurred frequently, streptokinase treatment was said to be generally well tolerated. This trial, along with two earlier studies, has been critically discussed,[133] and guidelines for the use of streptokinase and urokinase have been put forward.[134] Studies on human tissue plasminogen activator suggest that this is a more specific and effective fibrinolytic agent than human urokinase.[135]

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Chapter 10. Agents for the Treatment of Peptic Ulcer Disease

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Introduction - Advances in the treatment of peptic ulcer diseases depend not only on the identification of novel and efficacious medicinal agents, but also on the controlled clinical assessment of their therapeutic benefits. Although much of the interest has focused on the use of H_2-receptor antagonists,[1-4] a recent review of other antiulcer agents has also appeared.[5] In addition, proceedings[6] of a symposium on the etiology and treatment of peptic ulceration and reports regarding the adverse effects,[7] acute toxicity,[8] and safety[9] associated with antiulcer therapeutics have been published.

Investigation of the biochemical mechanisms involved in the gastric secretory process continued to receive considerable attention.[10-13] Although the therapeutic benefit of cytoprotection to the ulcer healing process remains to be established, examination of drugs that enhance mucosal defense, as well as those that concomitantly reduce gastric acidity, are of particular interest.[14,15]

Mechanisms of Gastric Acid Secretion - Cellular mechanisms by which gastric acid secretion is controlled are complex. The transport of hydrogen ion across a million-fold concentration gradient necessitates a significant energy requirement in addition to an adequate supply of metabolic substrate to sustain secretion of hydrochloric acid.[16] Models proposed initially for the transport mechanism were designed to account primarily for the energy requirements of the transport process. This approach led to the development of two distinct, but not mutually exclusive, mechanistic proposals for gastric acid formation. The redox hypothesis specifies that the energy from cellular oxidation-reduction reactions is that used to translocate hydrogen ions.[17] The second mechanism proposed is the ATP hydrolysis hypothesis.[18,19] This alternative proposal postulates that the energy released during hydrolysis of ATP serves to drive the hydrogen ion transport. Although these two mechanisms have been investigated vigorously, neither has been established unequivocally.

Gastric acid secretion is regulated via the autonomic nervous system and hormonal mechanisms, both endocrine and paracrine. There is considerable evidence to suggest that histamine is a common mediator in gastric parietal cell secretions.[20,21] The cellular mechanism by which prostaglandins (PGs) elicit their antisecretory effect is even less clear than the mechanism by which histamine stimulates the secretion of hydrochloric acid. It has been proposed that gastrin and acetylcholine receptors (vagal innervation) regulate histamine release from proximal histaminocytes.[20] Histamine can then directly interact with the acid secreting parietal cell mediated by the intracellular "second messenger" cyclic AMP.

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A dual regulatory mechanism with histamine and PGs controlling a system of balance between cytoprotective substances (mucopolysaccharides) and gastric acid has also been proposed.[25] Both the PG receptor on non-parietal cell gastric epithelia and histamine receptors located on the surface of parietal cells are mediated through the intracellular messenger cyclic AMP via PG-sensitive and histamine-sensitive adenyl cyclase, respectively. Blockade of the histamine receptor not only results in a decrease in gastric acid secretion, but also permits the cytoprotective component of the proposed mechanism to proceed unopposed.

Mechanisms of Cytoprotection - The term cytoprotection was employed initially to describe the ability of PGs to protect the cells of the gastric epithelium against potentially noxious agents. The cytoprotective effect has been observed with all PGs examined, whether or not they possess gastric antisecretory properties.[26,27]

The mechanism of cytoprotection by PGs or other agents is not known. Several theories have been proposed which include: (1) increased mucus synthesis and secretion;[28-31] (2) increased epithelial cell half-life;[32] (3) strengthening of the gastric mucosal barrier;[33] and (4) increased secretion by the non-acid producing cells of the gastrointestinal epithelium.[34] Another theory which relates directly to prostaglandins suggests that endogenous PGs are cytoprotective and their elimination by aspirin and other acidic non-steroidal antiinflammatory drugs results in necrosis of the epithelium. Introduction of exogenous PGs prevents the damage resulting from these agents.[35] In this regard, gastrointestinal ulceration could result from a deficiency of naturally occurring PGs.[36-38] Increase in mucosal blood flow,[30,39-44] stimulation of bicarbonate secretion,[45,46] stimulation of sodium ion transport,[47] stimulation of cyclic AMP formation,[30,48] and stimulation of chloride ion transport[49] are other proposed mechanisms of cytoprotection.

Histamine H_2-Receptor Antagonists - The major emphasis in peptic ulcer disease therapy continues to be H_2-receptor antagonism. The subject has been reviewed from several perspectives.[1,2,50] Cimetidine (1) has an established position in the medical management of peptic ulcer.[51,52] There are only rare occurrences of severe adverse reactions to cimetidine,[7] however the controversy concerning the formation of N-nitrosocimetidine (2)[53] and N-nitrosamines in gastric juice, and the resulting potential for gastric cancer, continues. Elevated N-nitrosamine levels and growth of nitrate-reductase-positive organisms were identified in cimetidine-treated patients.[54-55] In another study, N-nitrosocimetidine and N-methyl-N'-nitro-N-nitrosoguanidine, a potent laboratory animal carcinogen, were shown to similarly methylate DNA.[56] The importance of the nitrosamine level studies has been disputed,[57] and a preliminary report of no abnormal findings in dogs treated chronically with 144 mg/kg/day of cimetidine for 53 months was issued.[58]

Ranitidine (3) was marketed in the UK on October 5, 1981, under the tradename Zantac™,[59] and the Proceedings of 1st International Symposium on Ranitidine were published.[60] Several comparative studies of ranitidine and cimetidine were reported. In vitro, ranitidine has a pA_2 of 7.2 on the guinea-pig isolated right atrium, which is 4.5 times more potent than cimetidine.[61] Ranitidine was 4.2-9.6 times more potent than cimetidine as an inhibitor of histamine-stimulated gastric acid secretion

in the dog.[62] Clinical studies further substantiated the comparative potencies: ulcer healing rates were not significantly different for ranitidine, 150 mg bd (77%) and cimetidine, 200 mg tds and 400 mg at night (84%).[63,64] Both ranitidine and cimetidine increased rat gastric mucosal damage following ethanol treatment, and partially abolished cytoprotection by PGs.[65]

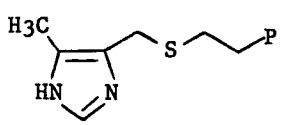
Two clinical studies of tiotidine (4), demonstrating the increased potency and duration of action of this agent relative to cimetidine, were reported.[66,67] These studies were apparently completed prior to the suspension of clinical trials due to a dose-related incidence of intestinal metaplasia in rats.[68]

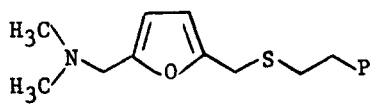
Oxmetidine (5) is four times as potent as cimetidine as an inhibitor of meal-stimulated gastric acid secretion in normal volunteers.[69]

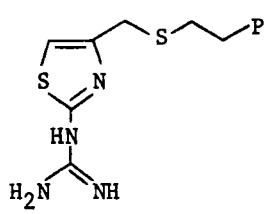
SK&F-93479 (6) is 20 times more potent than cimetidine as an inhibitor of meal-stimulated gastric acid secretion in normal volunteers.[70] The maximum antisecretory effect was maintained beyond three hours and there was a significant effect for longer than eight hours.

BL-6341A (7) is a newly reported and highly potent histamine H_2-receptor antagonist, wherein the cyanoguanidine functional group of tiotidine is replaced by a 1,2,5-thiadiazole-1-oxide ring.[71] In conscious Heidenhain pouch dogs, stimulated with histamine, orally administered BL-6341A is 17 and 2.5 times more potent than cimetidine and ranitidine, respectively.[72]

YM-11170 (8), which contains a sulfamoyl amidine functionality replacing the cyanoguanidine of tiotidine, is 10 times more potent than cimetidine on the guinea-pig isolated atrium and up to 200 times more potent than cimetidine against dimaprit-induced gastric acid secretion in Heidenhain pouch dogs.[73]



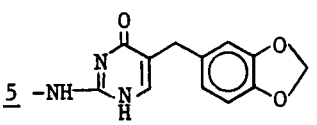




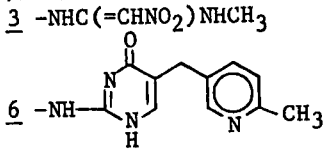
P

1 -NHC(=NCN)NHCH$_3$

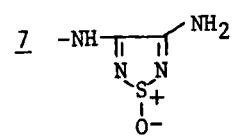
2 -NHC(=NCN)N(NO)CH$_3$



3 -NHC(=CHNO$_2$)NHCH$_3$



4 -NHC(=NCN)NHCH$_3$



8 -C(=NH)NHSO$_2$NH$_2$

Prostaglandins - The gastrointestinal effects of PGs and their clinical significance relative to peptic ulcer disease has been reviewed.[14] The proceedings of the International Workshop on Protective Actions of Prostaglandins on Gastrointestinal Mucosa, Santa Monica, California, 1981, were published.[74] The focus of PG research continues to be cytoprotection of the gastric mucosa.[75] It was demonstrated that the rat gastric mucosa can generate PGE_2, and that aspirin, administered either intravenously or intragastrically, reduces the generation of these PGs.[76]

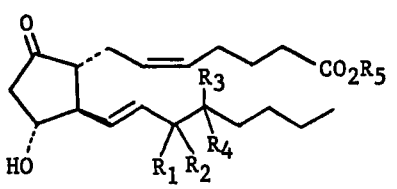
Arbaprostil, 9, at 10-40 μg orally, afforded cytoprotection against aspirin-induced mucosal damage in normal volunteers as determined by endoscopic study.[77] Rheumatoid patients receiving 200 μg/day were not consistently protected from the mucosal damaging effect of non-steroidal anti-inflammatory drugs.[78] 16,16-Dimethyl PGE_2 (10), 1 μg orally, inhibited gastric acid hypersecretion by 85% in six Zollinger-Ellison syndrome patients.[79] Interestingly, 10 prevented the aspirin-induced fall in gastric potential in humans,[80] but failed to prevent a reduction in gastric potential produced by ethanol,[81] suggesting a lack of cytoprotection against ethanol-induced gastric mucosal damage.

SC 29333, (±)-15-deoxy-16-hydroxy-16-methyl PGE, methyl ester, (11) is a more potent gastric mucosal vasodilator than 16,16-dimethyl PGE_2, suggesting that these two PGs may mediate gastric cytoprotection via different mechanisms.[82]

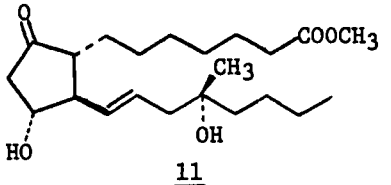
The stable prostacyclin analogs, 6β-PGI_2 (12) and 5,9-epoxy-16-phenoxy-ω-tetranor-PGF_1 (13) inhibit histamine-induced acid secretion from the lumen-perfused isolated whole stomach of the rat.[83] Further studies with these agents on isolated canine parietal cells demonstrate that their antisecretory action is mediated by a decrease in cyclic AMP formation in parietal cells, suggesting that inhibitory and stimulatory PG receptors linked to adenylate cyclase may be of therapeutic importance.[84]

A 16-phenoxy-ω-tetranor-PGE_2 analog, RS 84135 (14), is presumed to act topically on the gastric mucosa. Oral ED_{50} values for the prevention of histamine-, pentagastrin-, and carbacol-stimulated acid secretion were 17, 40, and < 2 μg/kg, respectively.[85]

Further studies indicate that thromboxane A_2 (TXA_2), a potent vasoconstrictor, may induce ulcer formation,[86] and that in duodenal ulcer patients, a disturbance of the PGI_2/TXA_2 balance may be involved in the pathogenesis of peptic ulcer disease.[87]



	R_1	R_2	R_3	R_4	R_5
9	CH_3	OH	H	H	H
10	H	OH	CH_3	CH_3	H



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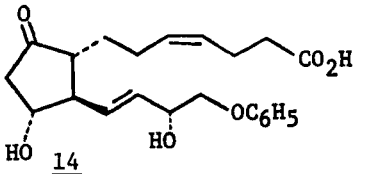

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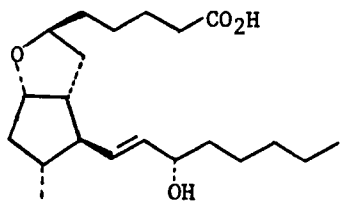

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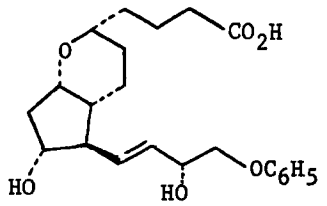
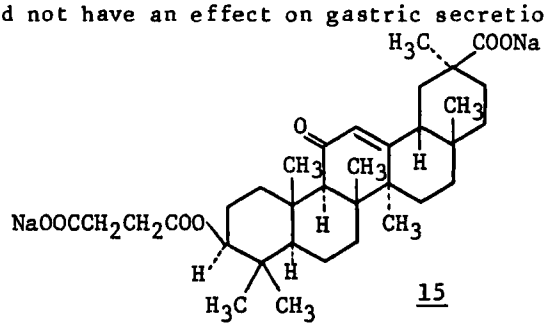


13

Non-prostaglandin Cytoprotectants - Carbenoxolone (15) is an antiulcer agent that stimulates the production of mucus secretion. In glycoprotein synthesis, the enhanced incorporation of ^{3}H -thymidine relative to control following carbenoxolone treatment has been described.[88] Carbenoxolone has also been reported to reduce the severity of ethanol-induced lesions in a dose-responsive manner in the rat when administered orally but not intravenously.[89]

Geranylgeranylacetone (E36U31, 16) is an acyclic polyisoprenoid antiulcer agent that may protect the stomach against its own secretions by enhancing the mucus and mucosal barrier.[90] In rats, prophylactic administration of 16 reduced gastric ulcer formation induced by exposure to cold-restraint stress and by administration of indomethacin, aspirin, prednisolone, or reserpine; it also reduced duodenal ulcers induced by cysteamine. However, it was ineffective against Shay's ulcer. Curative treatment with geranylgeranylacetone accelerated the healing process of gastric ulcers induced by topical application of acetic acid or thermocautery and by the administration of aspirin with exposure to cold-restraint stress. In the pyloric-ligated rat, geranylgeranylacetone did not have an effect on gastric secretion.



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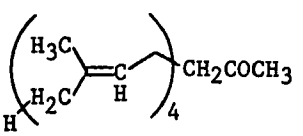
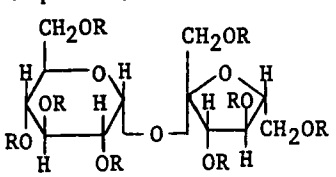


16

Sucralfate (17) is a basic aluminum salt of a sulfate disaccharide,[91] which is effective in preventing aspirin-induced gastric lesions.[92] The selective binding of sucralfate to gastric ulcers in man has been reported.[93]



$R=SO_3[Al_2(OH)_x(H_2O)_y]$

17

Sulglycotide is a polysulfated glycopeptide derived from pig duodenum. The cytoprotective effect of sulglycotide against necrosis of the rat mucosa produced by a number of adjuvants has been reported.[94]

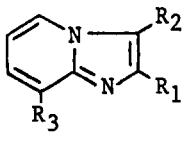
Zolimidine (18) healed prepyloric ulcers and ulcers of the lesser curvature when administered in doses of 1200 mg/day (divided). In

patients with ulcers of the lesser curvature, zolimidine decreased tyrosine equivalents in gastric juice which are markers for mucopolysaccharide content. Zolimidine did not effect hydrochloric acid or N-acetylneuraminic acid secretion.[95]

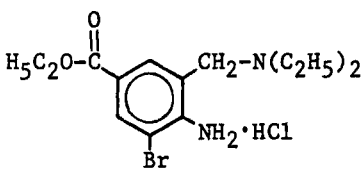
Sch 28080 (19) is an antiulcer agent with gastric antisecretory and cytoprotective properties. It was 4 and 10 times more potent than cimetidine in the pyloric-ligated rat when given orally one hour before ligation or intraperitoneally at the time of ligation, respectively. Gastric necrosis induced in rats by oral administration of ethanol was inhibited by Sch 28080.[96] There was a statistically significant dose-related inhibition of volume of gastric aspirate, acid output, acid concentration, and pepsin activity in both the unstimulated and pentagastrin-stimulated states observed in man following oral administration of Sch 28080.[97]

ABA-571 Cl (20) is a mucolytic agent with phosphodiesterase inhibitory properties which protects the gastric mucosa.[98] ABA-571 Cl afforded significant protection against aspirin damage of the mucosa in man when administered prior to the aspirin dose.

CF-19415 (21) is a member of a series exhibiting antisecretory[99] and cytoprotective properties.[100] CF-19415, administered orally at 100 mg/kg, increased alcian blue binding in fasted and in cold-restraint rats. CF-19415 also inhibited gastric damage and stimulated mucus secretion when administered at 100 mg/kg orally.



	R_1	R_2	R_3
18	4-$CH_3SO_2C_6H_4$-	H	H
19	CH_3	$-CH_2CN$	$C_6H_5CH_2O$-



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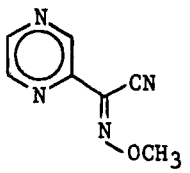
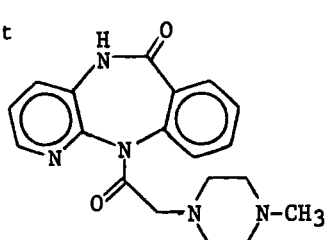


21

Other Antisecretory/Antiulcer Agents - An extensive clinical investigation of the tricyclic antiulcerant, pirenzepine (LS 519, 22), has been reported recently.[101] The degree of inhibition of basal and stimulated gastric secretion was comparable to that obtained using conventional anticholinergic agents at optimal effective doses, but without the usual associated side-effects.[102,103] Pirenzepine appears to act as a muscarinic drug; however a non-competitive inhibition of pentagastrin-stimulated gastric acid secretion has been observed. Pirenzepine is also a potent carbonic anhydrase inhibitor in vitro.[104]

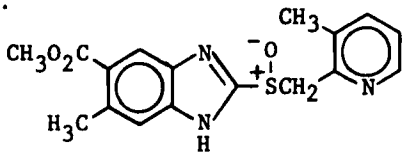
A double-blind study to assess the effect of maintenance pirenzepine therapy on duodenal ulcer relapse has been reported.[105] The combined application of ranitidine and pirenzepine inhibited meal-stimulated acid secretion more effectively and produced fewer side effects[106] than the combination of cimetidine and pirenzepine.[107] The absorption, distribution, and excretion of pirenzepine in rats has also been reported.[108]



22

H 149/94 (23) represents a new class of agents which inhibits gastric acid secretion by blocking $(H^+ + K^+)$ATPase.[109] This enzyme, which has been found only on the secretory surface of the parietal cell, catalyzes the exchange of H^+ for K^+ and is considered to be the "proton pump" responsible for acid secretion. Thus, inhibition at this step may afford a very selective antisecretory agent.

The mast-cell stabilizers, FPL-52694 (24) and sodium cromoglycate, have been reported to inhibit pentagastrin-stimulated gastric acid secretion, but not histamine stimulated secretion.[110] These results suggest that these agents may inhibit the release of histamine in the stomach.



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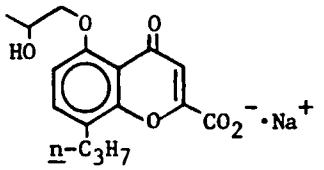
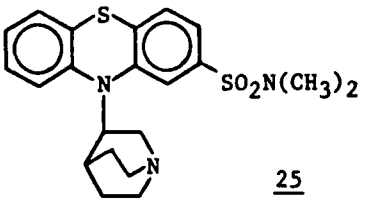


24

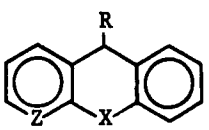
In a clinical evaluation, 23 duodenal ulcer patients were treated with LM 24056 (25), at 100, 200, and 300 mg/dose, which resulted in a reduction of nocturnal acid output by 31, 70, and 81%, respectively.[111] Overnight pepsin secretion was also inhibited. The mechanism of inhibition of acid by LM 24056 remains to be elucidated. It does not block H_2-receptors and is devoid of anticholinergic activity.



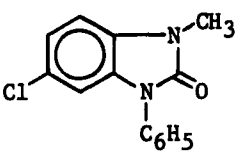
25

Another class of antisecretory agents, represented by 26, 27, and 28, was reported which inhibit histamine-stimulated gastric acid secretion in Heidenhain pouch dogs following intravenous but not oral administration.[112,113] Compounds 26 and 27 also inhibit gastric secretion in dogs stimulated with insulin hypoglycemia, dimaprit, and feeding.

A series of 3-arylbenzimidazolin-2-ones represented by 29, the most potent member of the series, had antisecretory and antiulcer activity in the rat.[114]



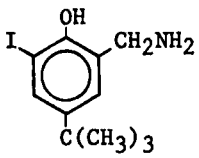
	X	Z	R
26	O	N	$CH_3NCONHCH_3$
27	S	N	$CH_3NCONHCH_3$
28	O	CH	$S(CH_2)_3NH_2$



29

MK447 (30) is a free radical scavenger that reduces the accumulation of PGG_2 and enhances the formation of PGH_2 in the arachidonic acid cascade. MK447 has been shown to reduce gastric acid secretion in the pylorus-ligated rat and to protect the rat mucosa against ethanol- and aspirin-induced lesions. In dogs, MK447 also reduced betazole-stimulated gastric acid secretion.[115]

The synthesis of Y-8894 (31) has been reported.[116] In animal models, Y-8894 inhibited stimulated gastric acid secretion, while exhibiting no effect on basal secretion. Y-8894 also inhibited stress-induced ulcers in rats.



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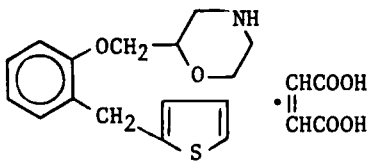

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Chapter 11. Hemorheologic Agents

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Introduction - Copley defined hemorheology in 1952 as "the study of deformation and flow (i.e., rheological) properties of cellular and plasmatic components of blood in macroscopic, microscopic and submicroscopic dimensions and the rheologic properties of vessel structure with which blood comes into direct contact."[1] A more restrictive though more suitable definition from a pharmacologist's point of view would be the study of flow properties of blood, including deformation of cells. This will allow the use of the term "hemorheologic agents" for those drugs that affect the flow properties of blood. The all-encompassing definition of hemorheology by Copley would include a wide variety of drugs, e.g., peripherally acting antihypertensives, as hemorheologic agents.

The importance of hemorheology in clinical medicine has been recognized by the new journal entitled Clinical Hemorheology (Pergamon Press, N.Y.) which itself is a companion journal to Biorheology by the same publishers. Additionally, in the last five years a number of symposia on hemorheology, blood viscosity and cell deformability have been organized, perhaps stimulated by the availability of new hemorheologic agents, or at least the recognition of hemorheologic effects of several marketed drugs.

The understanding of a hemorheologic mode of action of a drug may help explain the observed usefulness of certain drugs in apparently disparate clinical indications. Since hemorheology is currently receiving wide attention, it is worthwhile to review briefly the various factors affecting the flow of blood in the vascular tree and particularly the methods employed to determine changes in these factors.

Hess in 1915 discovered that blood was a non-Newtonian fluid, i.e., its viscosity changes with shear stress.[2] Thus, to define the rheological characteristics of blood, determinations of viscosity have to be carried out over a range of constant definable shear rates. Since viscometers were unavailable until the 1960's, most of the clinical data in this field has been obtained recently.

Since blood is a suspension of cells in plasma, its rheological behavior depends on the properties of both components and their interactions. Thus, the fluidity of blood (reciprocal of viscosity) is determined by plasma viscosity, cell concentration (mainly hematocrit), cell aggregation (mostly RBC's, but also platelets) and cell deformability.[3] Blood viscosity is also affected by driving pressure, vessel radius, temperature and geometry of the vessel. However, because of their high number, the red blood cells are the primary determinants of locally effective blood viscosity values.

Schmid-Schoenbein et al. mentions the enormous difficulties that nature overcomes in pumping of very concentrated suspensions of blood cells through a complex network of blood vessels, the majority of which are of equal or smaller size (capillaries 4-14μ) than the suspended

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particles (RBC's 5-8μ).[4] The study of hemorheology elucidates some of the key mechanisms in operation, may explain some forms of circulatory insufficiency, and could offer new tools for their remedy.

Disease States in Which Altered Hemorheology is Important - Altered hemorheology has been causally implicated in a wide variety of clinical states. Essentially all diseases in which impaired blood flow to an organ produces signs/symptoms are affected by changes in hemorheology.

A number of clinical conditions are presumed to result from organ/tissue ischemia following obstructed blood flow in the major arteries which supply the heart, brain and legs. In the last few years, many studies have been reported which associate blood rheology changes with clinical arterial events, not only retrospectively but also prospectively.[5-7] Changes in blood viscosity and its major determinants (hematocrit, cell aggregation and deformability, plasma viscosity and plasma fibrinogen level) have been related to changes in cerebral and limb blood flow in man. Improvement of the flow properties of blood appears to be a possible therapeutic approach.

The clinical conditions in which there is a possibility of hemorheologic intervention have been discussed:[8]

1. Hyperviscosity states may result from plasma abnormality, e.g., in congenital hyperfibrinogenemia, macroglobulinemia, myeloma, some collagen diseases; high hematocrit, e.g., in polycythemia, stress, and neonates; cellular abnormality, e.g., in sickle cell disease, spherocytosis, some hemoglobinopathies and leukemia.

2. Conditions which are partly or occasionally associated with hyperviscosity include: post-surgical thrombosis, peripheral vascular diseases (including intermittent claudication, Raynaud's phenomenon and ischemic ulcers), malignancy, diabetes, myocardial ischemia, dehydration, hypovolemic shock, certain cerebrovascular conditions and hypertension.

Hemorheologic agents have been used or are being evaluated in many of the above clinical indications. Of particular interest are the clinical conditions in which no agent has been approved for use in the U.S. (e.g., sickle cell disease, peripheral and certain cerebrovascular diseases).

Measuring of Hemorheology Parameters - Determination of macroscopic blood viscosity as a scientific method to quantify flow *in vivo* is questionable since the discovery of the so-called Fahreus and Fahreus-Lindqvist effect[9,10] (hematocrit and apparent viscosity are much lower in microvessels than in macrovessels). Blood viscosity *in vivo* is a physical quantity that eludes measurement as it varies greatly with changing flow conditions. Hence, the measurements of blood viscosity have to be conducted over a range of constant definable shear rates. As the viscosity of a fluid is due to the internal friction between adjacent layers moving parallel to one another, the velocity difference is a measure of the shearing within the flowing fluid; and this velocity gradient is termed shear rate. Shear stress is the force which maintains the velocity of flow and the shearing velocity. Shear stress is expressed as force per unit area in Pascals (Pa) units. The viscosity of blood (shear stress/shear rate) is expressed as Pascal seconds (Pa.s).

Viscometers - Several rotational or capillary viscometers are available. The performance of three different viscometers has been compared while determining whole-blood viscosity at low shear rates. Sampling

techniques, use of anticoagulants, temperature, storage of blood or delay before measurement, hematocrit, and the calibration of the equipment were the factors that needed to be controlled. Viscometers may be used to measure viscosity of suspended and washed RBC's in buffered isotonic solutions as an indicator of deformability. However, in these measurements, the effective shear stresses are usually so high that only the behavior of RBC's in rapid flow through large vessels is simulated.

Red Cell Deformability - Though a number of techniques are available to measure red cell deformability, none actually duplicates the conditions in nutritive capillaries (diameter 3-5μ) of the microcirculation. Microfiltration methods are the most common ones used to assess RBC deformability.[12-14] RBC flow rate is measured by testing the ability of red cells to pass through a filter membrane (e.g., from Nucleopore Corp.) of defined pore width (5μ), length (10μ), and density (4×10^5 pores/cm^2) at a constant pressure gradient. The principal limitation of this method with the use of whole blood is that the less deformable leucocytes and platelets plug many of the pores.

Micropipette Methods - A single red blood cell is aspirated partially or completely in a micropipette (1μ ID). The negative pressure required to deform the red cell is recorded.

Centrifuge Technique - Normal red cells can be packed at higher concentrations than are possible for less deformable, more rigid erythrocytes.[15] Centrifugal acceleration (200g) and the differences between red-cell columns (or packing rate) before and after centrifugation was used as an index of red-cell deformability.[16]

Determination of RBC Geometry in Well-Defined Shear Fields - Various instruments, e.g., rheoscope, filtrometer, ectacytometer, and other techniques have been used to determine the shear stresses required to deform the erythrocyte.[17]

Therapeutic Approaches - Review of the clinical conditions mentioned above in which altered hemorheology plays a role suggests that hemorheologic agents with the ability to improve the flow properties of blood would have wide therapeutic implications.

The possible approaches to therapeutic lowering of blood viscosity may be summarized as follows:[18] 1) Hemodilution, e.g., blood letting, exchange transfusions with low-molecular weight dextran; 2) Reduction of plasma fibrinogen - a) by drugs to normal level, e.g., with clofibrate or to a desired low level with Arvin®, or possibly by fibrinolytic agents; b) by plasma exchange; 3) Improvement of red cell flexibility - a) by drugs, b) by plasma exchange.

Hemodilution by blood letting[19] or isovolemic exchange[20] is known to cause extensive alterations in blood rheology by reduction of the hematocrit (to at least 30%), and to therapeutically benefit a number of vascular conditions. The use of low molecular weight dextran has been well established in acute cerebrovascular conditions.[21]

Plasma Exchange mainly produces its beneficial effect, e.g., in Raynaud's syndrome, by a reduction of plasma fibrinogen with subsequent reduction in blood viscosity at low shear rates and an increase in the deformability of red blood cells.[22]

Defibrinating Agents are not available in the U.S. Ancrod (Arvin®) and

batroxobin (Defibrase®), both available abroad, are purified proteolytic enzymes from snake venoms.[23] Ancrod has been more widely investigated and, when given IV, reduces blood and plasma viscosity by about 5-10% at high shear rates;[24] with decreasing shear rates the fall in blood viscosity is even greater. A reduction of plasma fibrinogen to at least 20% of the normal level is associated with marked lowering of blood viscosity and a commensurate increase in blood flow. However, the earlier positive results in peripheral vascular disease have not been confirmed in double-blind placebo controlled studies.[25,26]

Xanthine Derivatives - Pentoxifylline (Trental®) (1) is an extensively investigated xanthine derivative for its hemorheologic action and efficacy in a wide variety of clinical conditions.[27] Although it is not available in the U.S. at present, it is likely to be commercially available in the near future.[28] Hess et al. described its hemorheologic activity in patients with peripheral vascular disease.[29] Their findings of the reduction of blood viscosity, particularly at low shear rates, have been confirmed by others.[30-32]

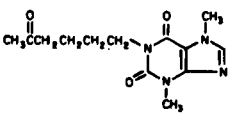
Ehrly showed that the blood viscosity lowering effect of pentoxifylline was mainly due to its effects on red blood cells.[33] Red cell flexibility depends on the internal viscosity and membrane flexibility, and these factors are decisively influenced by the content of energy-rich phosphates, e.g., ATP, as well as plasma osmolarity and pH.[34-36] Increased plasma osmolarity and decreased pH occur in ischemic muscle under load, and reduced ATP concentrations in red cells will cause a reduction of red cell deformability and affect the passage through capillaries.[37] Pentoxifylline increases the low red cell flow rate caused by hyperosmolarity,[38] as well as the ATP content of red cells in patients under treatment for vascular diseases.[39]

Pentoxifylline also prevents red cell aggregation which mainly depends on fibrinogen. The reduction of fibrinogen concentration and increased fibrinolytic activity observed with pentoxifylline antagonize the tendency of red cells to aggregate.[40] In addition to its effect on red cells, pentoxifylline has a platelet anti-aggregation effect,[41] which has been confirmed in clinical studies.[42-46]

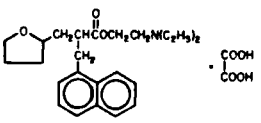
Recent investigations have revealed that pentoxifylline stimulates prostacyclin synthesis and release.[41] By this mechanism, platelet cAMP increases via platelet adenylcyclase activation.[42] Pentoxifylline also inhibits platelet membrane cAMP-phosphodiesterase,[43] which increases intracellular cAMP, a phenomenon demonstrated in incubated platelets. The raised levels of cAMP inhibit prostaglandin cyclo-oxygenase and thus reduce the synthesis of the aggregator thromboxane.[43]

The myriad actions of pentoxifylline provide some rationale for the clinical use of this hemorheologic agent in a variety of circulatory disorders. Pentoxifylline has been employed in the treatment of patients with intermittent claudication and other signs/symptoms of peripheral vascular disease.[47-49] It has been reported to be effective in chronic cerebrovascular conditions, e.g., following acute hemorrhagic stroke as well as in senile dementia.[50-53] More recently, the efficacy of pentoxifylline in cardiac insufficiency and angina pectoris has been studied.[54-56] The reported clinical efficacy of pentoxifylline in the prevention as well as management of an acute crisis in sickle cell disease might be predicted on the basis of its mode of action on the erythrocyte.[57,58] Pentoxifylline also has been reported to be effective in the management of specific circulatory disorders of the skin,[59] eye,[60] and the ear.[61] Confirmation for

the efficacy of pentoxifylline in peripheral vascular diseases has been obtained in U.S. studies.[28]



(1)



(2)

Adrenergic Beta Activators/Alpha Antagonists - Nylidrin HCl (Arlidin®) and isoxsuprine (Vasodilan®) are structurally related and derivatives of epinephrine. Nafronyl oxalate (2), buflomedil (3), cetiedil (4), as well as ergot alkaloids, are other well known drugs in this group.

Nylidrin has been recommended for a wide variety of circulatory disorders of the extremities and of the inner ear. The NAS/NRC review in 1966 classified it as "possibly effective." Since then, FDA's Advisory Committee has recommended approval of the drug only for senile dementia, based on recently available data from U.S. clinical studies.[61,62] There are no publications suggesting a hemorheologic mode of action.

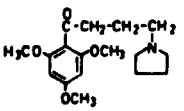
Isoxsuprine has recently been shown to have an effect on platelet adhesiveness,[63] on sickle cell disease,[64] and on hemorheologic parameters.[65]

Nafronyl oxalate is a direct acting papavering-like vasodilator, which is under clinical investigation in the U.S., with antinicotine and antibradykinin effects. Its mode of action appears to include vasodilation and effects on glycolysis and intracellular ATP. Several double-blind placebo-controlled studies have failed to demonstrate the efficacy of the drug in patients with intermittent claudication.[66-68]

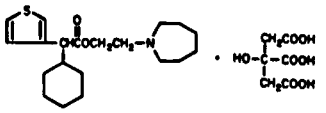
Ergoloid mesylates is a mixture of the methanesulfonates of the dehydrogenated derivatives of three ergotoxine alkaloids - ergocornine, ergocristine and ergocryptine. The mixture has potent α-adrenoceptor blocking actions and has been shown to improve cognition and affect variables in well-controlled double-blind studies.[69,70] However, there is no suggestion that their action is due to any hemorheologic activity.

In contrast, buflomedil (3), another α-adrenoceptor blocking agent, has also been reported to inhibit platelet aggregation, to improve deformability of red blood cells and reduce blood viscosity.[71] Several publications have compared its efficacy with that of other vasoactive drugs in peripheral vascular[72,73] and cerebrovascular disease.[74,75] Confirmation of these findings in double-blind placebo controlled studies is not yet available.

Cetiedil (4) has an agonist effect on the β_2-adrenergic system and an antagonist effect towards calcium ion exchange through the membrane.[76] During the last several years, it has been extensively investigated in Europe for various clinical indications, including sickle cell disease, peripheral and cerebrovascular diseases.[77-79] Its action on red cell deformability, blood viscosity and other hemorheologic parameters has been confirmed.[77,80] The drug is being studied clinically in the U.S. in the treatment of sickle cell disease.



(3)

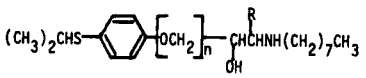


(4)

Miscellaneous Drugs - Cinnarizine and its defluorinated derivative, flunarizine, are calcium antagonists and block 5-HT and histamine receptors. Probably because of their effect on calcium, they are potent antagonists of several endogenous vasoconstrictor substances.[81] They also decrease whole blood viscosity by improving red cell flexibility in patients with peripheral vascular disease.[82-84] However, the clinical evidence of efficacy in peripheral vascular disease is insufficient.[85]

Tipropidil (MJ12880, 5) has been investigated because of its expected antihyperviscosity properties.[86] The drug tends to normalize blood viscosity whether it is elevated or low. Interestingly, the reduction of blood viscosity was observed to a greater extent in blood samples from blood group O than blood group A subjects. The action of the drug is attributed to an effect on rigidity and deformability of the red blood cell, rather than to an effect on aggregation. There are no publications on the clinical use of this drug.

Suloctidil (6) has been reported to have effects on platelet aggregation, blood viscosity and lipid metabolism, as well as vascular antispasmodic activity.[87,88] Several double-blind controlled studies demonstrate its effect in the treatment of patients with peripheral vascular disease,[89,90] as well as cerebrovascular diseases.[91]



(5) n = 1, R = H
(6) n = 0, R = CH_3

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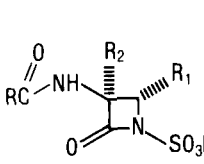
Section III - Chemotherapeutic Agents

Editor: Leslie M. Werbel, Warner-Lambert Company
Ann Arbor, Michigan 48105

Chapter 12. Antibacterial Agents

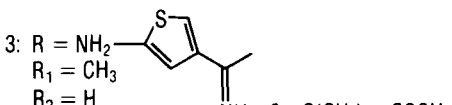
M. Debono and R. S. Gordee
Lilly Research Laboratories, Indianapolis, IN 46285

Monobactams - A novel group of monocyclic β-lactam antibiotics has been isolated from bacteria.[1] Sulfazecin, its epimer isosulfazecin, and SQ 26,180, produced by Pseudomonas sp. and Chromobacterium violaceum, are highly active against gram-negative bacteria.[2-4]



1: R = HOOC—C(NH$_2$)H—(CH$_2$)$_2$—C(=O)—NH—C(CH$_3$)H— ; R_1 = H ; R_2 = OCH$_3$ (Sulfazecin)

2: R = CH$_3$; R_1 = H ; R_2 = OCH$_3$ (SQ26,180)



3: R = NH$_2$—(thiazole)—C(=NH—O—C(CH$_3$)$_2$—COOH) ; R_1 = CH$_3$; R_2 = H (Azthreonam)

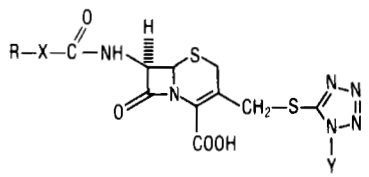
Substituted α-acylamino and α-piperazinedionecarbonylaminoacyl monobactams, in some cases, showed potent broad-spectrum antibacterial activity and exhibited properties similar to other β-lactam antibiotics.[5] When bearing the same side chain as a penicillin or a cephalosporin the monobactam exhibited greater similarity to the cephalosporin.[6] Azthreonam (SQ 26,776), which is active against gram-negative bacteria including Pseudomonas and highly stable to the action of β-lactamases, possesses a mode of action similar to cephalosporins.[7-11] Azthreonam administered parenterally to laboratory animals showed high and prolonged serum levels, excellent distribution in most body tissues and cerebrospinal fluid, 50 percent urinary excretion in 6 hours, and a low order of toxicity.[12-14] Intravenous infusion in man produced serum levels considered therapeutic and high urinary recovery.[15]

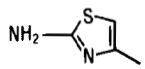
Cephalosporins and Related Analogs - Cefodizime (HR 221) and cefonicid (SKF 75073) are new broad-spectrum cephalosporins with high β-lactamase stability and a long-lasting pharmacokinetic properties.[16-19] E-0702 is a new parenteral cephalosporin with potent in vitro and in vivo anti-pseudomonas activity.[20]

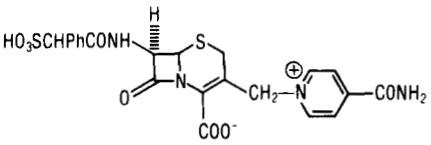
Cefotiam (CGP 14221/E), a broad spectrum cephalosporin was well tolerated in man and achieved therapeutic serum levels, as well as high urinary excretion.[21,22] 42,980 RP is reported to have greater potency than "third generation" cephalosporins.[23] Cefsulodin in combination with cefazolin or mecillinam showed synergistic in vitro antibacterial activity and clinical efficacy against Pseudomonas infections.[24-25]

ISBN 0-12-040517-2

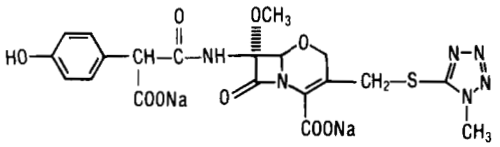
Clinical studies with moxalactam, a 1-oxa-cephalosporin analog, include septicemia, osteomyelitis, pneumonia, gonorrhoeae, and urinary tract infections in combination with tobramycin.[26-29] Ceftizoxime is a new cephem derivative with broad-spectrum activity.[30]



	R	X	Y
Cefonicid (SKF 75073):	phenyl	>CHOH	$-CH_2SO_3H$
Cefotiam:		$-CH_2-$	$-(CH_2)_2-N(CH_3)_2$

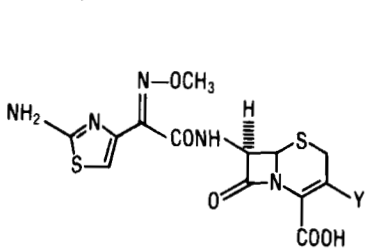


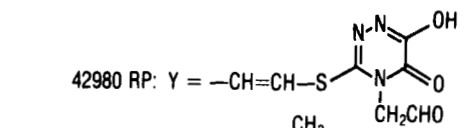
CEFSULODIN



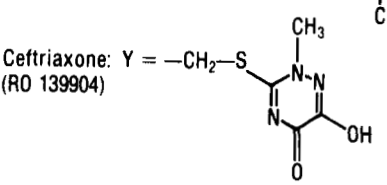
MOXALACTAM DISODIUM

Ceftriaxone (RO 13-9904), a potent broad spectrum cephalosporin with unique pharmacokinetic properties, is being evaluated clinically.[31-36] Clinical studies indicate that cefotaxime is effective against susceptible bacteria at most body sites.[37] Ceftazidime (GR 20263) continues to be of particular interest because of the potent in vitro and in vivo activity against Pseudomonas which is comparable to aminoglycoside antibiotics.[38,39] Initial human pharmacokinetic and clinical efficacy studies with ceftazidime (GR 20263) are encouraging.[40] Ceftizoxime (FK 749) showed in vitro synergy with tobramycin against Ps. aeruginosa and was effective against bacteremia caused by multiresistant organisms.[41,42]

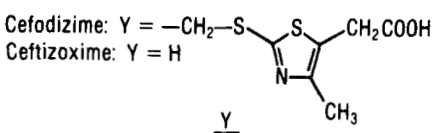




42980 RP: Y = $-CH=CH-S-$ CH_2CHO

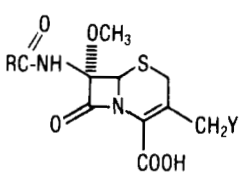


Ceftriaxone: Y = $-CH_2-S-$ (RO 139904)



Cefodizime: Y = $-CH_2-S-$ CH_2COOH

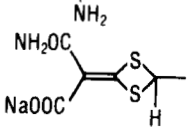
Ceftizoxime: Y = H

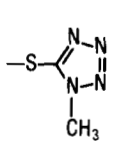


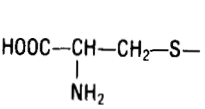
Oganomycin A:

	R	Y
		$-OCO-CH=CH-\phi-p-SO_3H$

YM 09330:

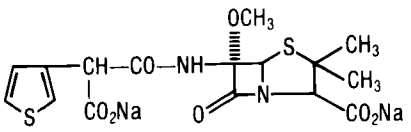




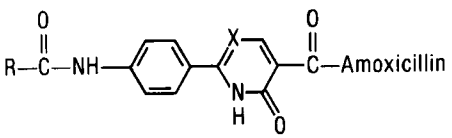
MT 141:  " "

Clinical studies with cefuroxime indicate effectiveness in pneumonia, meningitis, and gonorrhoea.[43-45] The broad-spectrum antibacterial activity, human pharmacokinetics, and clinical effectiveness of cefmenoxime (SCE 1965) have been investigated.[46,47] Oganomycin A, Cefotetan and MT-141 are new cephamycin antibiotics that have broad-spectrum activity.[48-51]

Penicillins and Other β-Lactams - BRL 17421 is a semisynthetic β-lactam antibiotic that is stable to β-lactamase action and possesses high activity against gram-negative bacteria with the exception of Pseudomonas and Bacteroides.[52,53]



BRL 17421

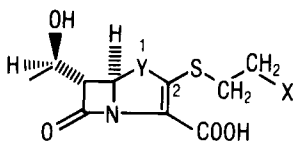


A

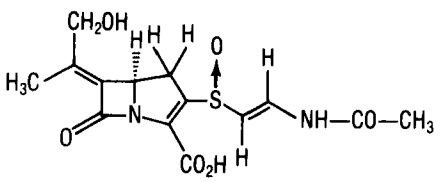
FCE 21420 (threo-trans-(5R)-6-hydroxyethyl-2-acetoxymethyl-2-penem-3-carboxylate) showed broad-spectrum antibacterial activity with the exception of Pseudomonas and Haemophilus.[54] A series of acylated amino acid derivatives of amoxicillin (A) showed little difference in in vitro and in vivo potency, but a change of the chirality of the amino acid resulted in significant differences in the acute LD_{50} of the derivatives.[55] Azlocillin is a semi-synthetic acylureido-penicillin that possesses enhanced activity against Pseudomonas strains and desirable pharmacokinetic properties for treating Pseudomonas infections.[56,57]

Sch 29482 is a novel orally absorbed semisynthetic 2,6-disubstituted penem antibiotic that is highly active against most gram-positive and gram-negative bacteria except Pseudomonas sp. 58-62 This antibiotic is highly stable to β-lactamases and was well-tolerated in human studies. 63-65

Carbapenems - The stereocontrolled introduction of the hydroxyethyl side chain in synthesis of thienamycin was reported and the preparation and antibacterial activity of Δ'-thienamycin described.[66,67] Studies on the human pharmacokinetics of N-formimidoyl-thienamycin (MK0787) are reported.[68]



Sch 29482: X = H, Y = S
Thienamycin: X = NH_2, Y = $-CH_2-$
MK0787: X = $-NHCH=NH$, Y = $-CH_2-$

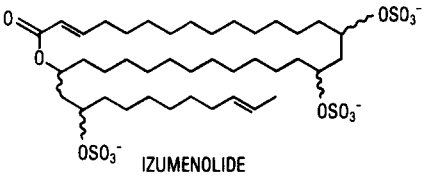


Asparenomycin A

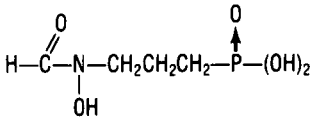
Further efforts were reported to enhance the urinary recovery of N-formimidoyl-thienamycin (MK0787) in man by the co-administration of 3(L-cysteinyl-S-butyl) homolog, MK0791, a renal dipeptidase inhibitor.[69] At least six distinct antibiotics in the epithienamycin family are produced by a strain of Streptomyces flavogriseus.[70,71] Carpetimycins A and B (C-1939-S_2 and -H_2), new antibiotics related to thienamycins and olivanic acids, are potent β-lactamase inhibitors.[72-74] A series of 6-unsubstituted olivanic acid analogues were synthesized and showed moderate antibacterial activity.[75] The total syntheses of 1-carbapen-2-em and 1-carbacepha-1,3-diene systems, as well as a stereoselective synthesis of carbapenem antibiotic PS-5, were reported.[76,77] Asparenomycin A is a new broad spectrum carbapenem produced by Streptomyces spp.[78]

Beta-Lactamase Inhibitors - UK 38,006 (6β-Iodopenicillanic acid) has poor intrinsic antibacterial activity, but when combined with ampicillin potentiated the in vitro and in vivo activity of ampicillin.[79] Human pharmacokinetic studies with sulbactam (CP-45,899) combined with cefoperazone showed that blood levels exceeded the antibiotic activity required to eliminate many resistant organisms susceptible only to the combination.[80] Augmentin® (amoxicillin plus clavulanic acid) has been used clinically to treat respiratory and urinary tract infections.[81,82] BL-P2013, potassium 2B-chloromethyl-2α-methyl-penam-3α-carboxylate sulfone, was synergistic when combined with amoxicillin or ampicillin.[83]

Izumenolide, isolated from Micromonospora, is a novel macrolide β-lactamase inhibitor.[84] (7-α-2-Hydroxyimino-2-thienylacetamido) cephalosporanic acid is a potent inhibitor of the Type I β-lactamase.[85]



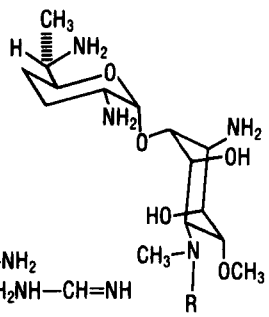
IZUMENOLIDE



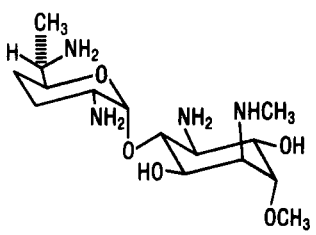
FR31564

Cell Wall Inhibitors - FR 31564, a phosphonic acid inhibitor of cell wall synthesis in gram-negative bacteria, showed complete synergy when combined with penicillins or cephalosporins.[86]

Aminoglycosides - The search for a modified aminoglycoside with increased activity, low toxicity and a low potential for resistance development continues to be a major goal. The fortimicins (FM) -A and -B



FM-A: R = —$COCH_2$—NH_2
Dactimycin: R = $COCH_2NH$—CH=NH



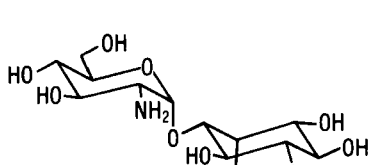
FM-B

continue to be subjects of a large number of chemical modifications. O-Demethyl-FM inhibited organisms that enzymatically deactivated other aminoglycosides.[87] A number of FM-analogs were synthesized by glycosidation of the FM hydrolysis product-fortamine, but were less active than FM-A.[88] The various amino groups of FM-A were substituted with alkyl and acyl groups; a 4-amino-2-hydroxy-butyl group at the 2'-NH_2 group improved the antimicrobial activity of FM-A.[89,90]

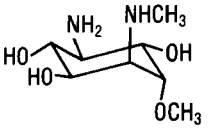
FM-B was chemically modified by selective protection of the various amino groups.[91] Reductive alkylation at the 6'-NH_2 position and the acylation of the 2'-NH_2 group of FM-A or -B gave compounds with weak activity.[92,93] The 1,2; 4,5 - biscarbamate and the three monocyclic carbamates of FM-B have been reported and their utility in the synthesis of glycosyl analogs at O-6 was discussed.[94] The variation of the stereochemistry of the C-1 amino group, C-2 OH-group, as well as the C-2 deoxy group, offered no advantage against strains known to deactivate FM-A, while 6'-epi-FM-A and -B (from FM-B) have reduced antibacterial activity.[95-98] A number of interesting aminoglycosides were prepared by glycosylation of gentamine-C_1 and -C_2 which were obtained by hydrolysis of the appropriate gentamicin.[99-100] Novel 1 and 3-substituted derivatives, as well as the 1 and 3-episisomicin and gentamicin compounds, were synthesized with the 1 epi modification giving superior bioactivity.[101,102]

A new pseudodisaccharide, lysinomicin, (3-epi-2'-N-(L-β-lysinyl)-4', 5'-didehydro-6'-C-demethyl-FM-B) is active against strains harboring aminoglycoside-3-acetyl transferase.[103,104] Glycosylation of apramycin gave derivatives that retained activity except against Ps. aerugi-nosa.[105] Habekacin, 1-N-[(S)-4-Amino-2-hydroxybutyl]-dibekacin, was highly active against a broad spectrum of organisms and was effective against experimental Pseudomonas infections.[106,107] Dactimycin, a new FM-A type bearing a formimino-glycine moiety at the 4-CH_3-NH group was reported as active as FM and less toxic than the latter.[108,109] Hybrids of gentamicin and kanamycin (3',4'-dideox KM), the combimycins, are produced by mutasynthesis using GM producing wild type or mutant organisms.[110]

Semi-synthetic deoxyparomomycin analogs were as active against strains with deactivating enzymes.[111] The structure of X-14847 has been established.[112] Additional members of the sporacin family (-C and -D) showed broad spectrum antimicrobial activity.[113]



X-14847

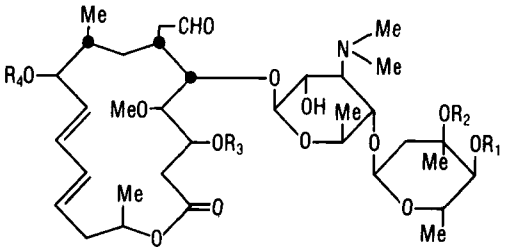


Fortamine

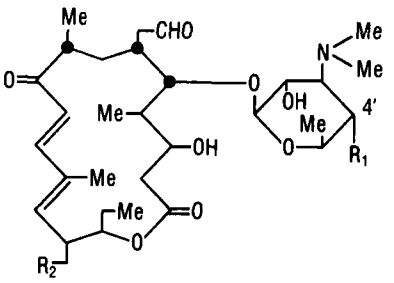
The 2" deoxy KM derivative, synthesized from neamine, were less potent than KMB.[114] The site of transition metal directed acylation of apramycin was altered by changing the nature of the metal cation employed.[115] 7-Hydroxy-tropolone, a fermentation product, is a novel inhibitor of an aminoglycoside inactivating enzyme.[116,117]

Macrolides - Interest remains high on new semi-synthetic macrolides. The anti-mycoplasmal activity of de-epoxy-rosaramicin (M-4365G_2) was

greater than several other macrolides.[118] 3"-O-propionyl leucomycin A_5 (TMS-19-Q), had improved in vitro antibacterial activity and gave higher serum levels and these data correlated well with preliminary clinical results.[119,120] In other semi-synthetic studies, 4" -(p-substituted phenacetyl)-deltamycin derivatives showed a correlation between increased electronegativity of the aryl substituent with increase in antimicrobial activity.[121] 3-Acetyl-4"-isovaleryl tylosin inhibited tylosin resistant strains.[122] Antibacterial activity of tylonolides was enhanced by removal of the 4'-OH group and by esterification of the C-23-OH group.[123-125]



TMS-19-Q: $R_1 = C_3H_7CO-$, $R_2 = C_2H_5CO-$
$R_3 = R_4 = H$

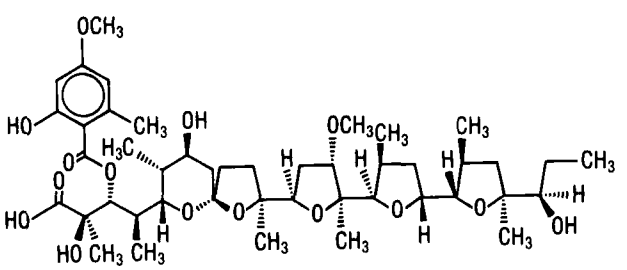


M4365G_2: $R_1 = R_2 = H$
Demycarosyl-tylonolide: $R_1 = OH$, $R_2 = OH$

Derivatives of josamycin and isojosamycin at C-9 and C-13 showed comparable activity to parent compounds.[126] Introduction of an aryl sulfonamido function at C-4 in oleandomycin resulted in activity greater than erythromycin and in vivo activity in animals but not in humans.[127] Blocked mutant strains of S. fradiae produced tylosin biosynthetic intermediates that may be sources of modified macrolides.[128]

The total synthesis of macrolide antibiotics can be considered one of the major achievements of modern organic chemistry. Total synthetic studies on carbomycin B, leucomycin A_3, and the aglycone of tylosin have been reported.[129-131] The total synthesis of erythromycin has been achieved by the research group of the late Professor R. B. Woodward.[132]

Ionophores - The ionophore family of antibiotics continues to expand. Antibiotics X-14667-A and -B have recently been reported to be 2-phenethylurethanes of monensins-B and -A, respectively.[133,134]

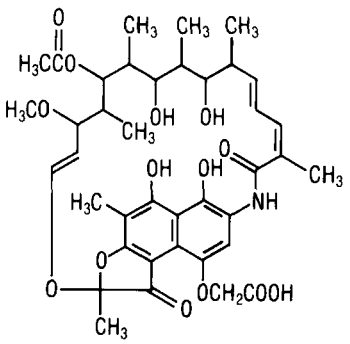


CATIONOMYCIN

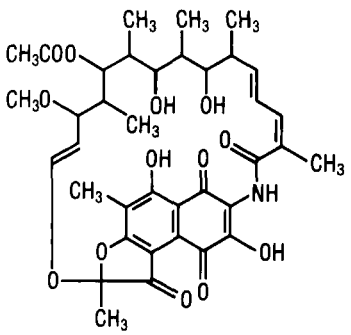
Halopolyether X-14766A has monovalent complexing ability.[135] Two new polyethers, TM-531B and TM-531C, were shown to be 4'-O-demethyl and 3'-hydroxydianemycin, respectively.[136] These are the first polyethers that contain sugars other than 4-O-methylamicetose. Cationomycin and CP-51,532 are polyether antibiotics isolated from Actinomadura.[137-138]

Total synthetic studies of ionophores remain of great interest. The syntheses of keto-pyrole ionophore X-14547A, narasin and salinomycin, were described.[139,140]

Rifamycins - Current studies of the rifamycins involve the search for new derivatives by genetic manipulation of Nocardia mediterranei.[141] Recombinant strain R-21 produced seven novel variants of rifamycin of which 3-hydroxyrifamycin S had the best antimicrobial activity. Other variants included rifamycins of the W and G types.[142]



Rifamycin B

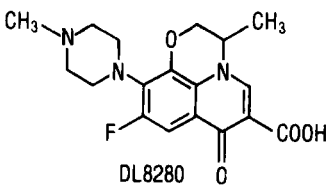


3—Hydroxyrifamycin S

Rifamycin Z was produced by another variant of this organism.[143] Aminorifamycin-S derivatives (modified at position 3 and/or 4) showed in vitro antibacterial activities comparable to rifampicin (RAMP).[144] The semi-synthetic RAMP, DL 473, was more active against clinical isolates of M. tuberculosis, less toxic than RAMP and was effective in vivo against experimental infections.[145]

Glycopeptides - The vancomycin-ristocetin group of antibiotics has recently been reviewed.[146] The chief advance in the current review period continues to be the refinement in the structural definition of vancomycin, avoparcin and epi-avoparcin.[147,148] Degradation products upon which the vancomycin x-ray structure was based had undergone a subtle rearrangment which was delineated by H'-NMR studies. Teichomycin has comparable activity to vancomycin but is more inhibitory against enterococci.[149,150]

Synthetic Agents - DL-8280 was highly active against gram-positive and gram-negative cocci and anaerobes, and also showed potent in vivo activity against experimental rodent infections and oral absorption in man.[151,152]



DL8280

MK-366, structurally related to nalidixic acid, was effective in urinary tract infections against multiple-resistant organisms.[153-155]

New Antibiotics - A number of new antibiotics that are not readily classified by structure type or function have been recently reported. These are listed in Table I along with their source, producing organism and observed activity.

TABLE I

Antibiotic	Producing Organism	Activity	Reference
LL AB 664 LL AC 541	Streptovert. olivoreticuli	G-	(156)
BM 782	Nocardia	G-, AF	(157)
Glysperins-A,-B and -C (Bu 2349)	Bacillus cereus	G+, G-	(158,159)
Cairomycin A	Streptomyces AS-C-19	G+	(160)
Nodusmicin A	Saccharopolyspora hirsuta	G+, G-, AA	(161)
Reductiomycin	Streptomyces griseorubiginosus	G+, AF	(162)
Grahamimycins (A,A_1,B)	Cytospora sp Ehrenb. WFPL 13A	G+, G-	(163)
Pyrrolomycin -A and -B	Streptomyces SF 2080	G+, G-	(164)

G+ (gram-positive), G(gram negative), AA (anaerobic), AF (Acid Fast)

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Chapter 13. Mechanisms of Antibiotic Resistance

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Introduction. Bacterial resistance to antibiotic therapy constitutes a serious problem in human medicine. Resistant organisms have been responsible for many fatal hospital infections and several epidemics which have taken a heavy toll of human life.[1] An important tool in overcoming this problem is an understanding of the mechanisms underlying this resistance. There are several relevant reviews covering different aspects of antibacterial resistance mechanisms.[2] This report will review those mechanisms which have been identified in clinical isolates of human pathogens. These mechanisms generally fall into one of three categories: (1) altered target site, (2) altered permeability, and (3) enzymatic drug inactivation. The recent application of recombinant DNA techniques has greatly facilitated the elucidation of many aspects of the mechanisms of antibacterial resistance and their regulation at the molecular level. Drug inactivation mechanisms will be reviewed at a molecular level, while other mechanisms will be discussed at a more general level of detail.

β-Lactams. Bacteria resist the antibiotic effect of β-lactams by all three of the basic defensive mechanisms. The most extensively studied of the three is enzymic inactivation via the β-lactamases. This work has been the subject of a book[3] and recent reviews.[4,5,6] Based on the primary amino acid sequences known for several β-lactamases,[7] these enzymes can be grouped into three classes of homologous proteins. Class A enzymes are represented by the *Staphylococcus aureus* PC1 penicillinase and the *Escherichia coli* R-TEM enzyme, class B by the β-lactamase II enzyme of *Bacillus cereus,* and class C by the inducible cephalosporinases from *Enterobacteriaceae* and *Pseudomonas aeruginosa.* The TEM enzyme has been the most thoroughly studied because of its considerable clinical importance in reducing penicillin effectiveness against many Gram-negative infections.[8]

Several workers have provided persuasive evidence that class A β-lactamases are serine amidohydrolases which become acylated on an active site serine by the β-lactam, followed by hydrolytic enzyme regeneration. The Knowles group found that the kinetics of hydrolysis of a poor substrate, cefoxitin (**1**), by the TEM enzyme were consistent with an intermediate which slowly breaks down to give product, and their observation of a 1753 cm^{-1} IR band supports an acylated serine residue as this intermediate.[9] Using another poor substrate, methicillin (**2**), for the *B. cereus* I enzyme, Waley's group obtained kinetic evidence of not only an acyl-enzyme intermediate, but also a conformational unfolding which delays its hydrolysis.[10] Anderson and Pratt used a fluorescent cephalosporin derivative to provide evidence for an acyl-enzyme intermediate and its cleavage from the *S. aureus* PC1 penicillinase.[11] Knowles and coworkers have made use of a number of suicide inactivators of the TEM enzyme: clavulanic acid and various penicillanic acid sulfones. Their data on the interaction of clavulanic acid (**3**) with the TEM enzyme are consistent with an acyl-enzyme intermediate which goes on to three inactivated forms of the enzyme.[12] Their work with Pfizer's sulbactam (**4**) and its di-deuterio analogue has established that the acyl-enzyme intermediate can undergo a number of subsequent reactions:[13] hydrolysis, tautomerization to a transiently inhibited form of the enyzme, and inactivation, possibly by covalent attachment to a lysine residue. They also found that

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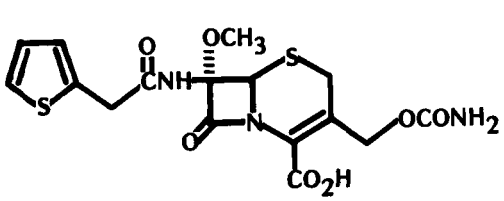
quinicillin sulfone (**5**) remains covalently bound to the TEM enzyme permitting tryptic digestion and analysis of the labelled fragment.[14] Based on the known amino acid sequence of the TEM enzyme,[15] the labelled residue was shown to be serine 70. Knowles has related this result to earlier work by showing that the same tryptic peptide is labelled by cefoxitin. Both Waley[16] and Pratt[17] have shown that β-Br penicillanic acid (**6**) labels the homologous serine 70 of the *B. cereus* I enzyme. Cartwright and Coulson have shown that α-Cl penicillanic acid sulfone (**7**) labels the analogous serine or the adjacent threonine of the *S. aureus* penicillinase.[18] When the ongoing X-ray work on these enzymes[19] is complete, a detailed picture of their mechanisms can be constructed.



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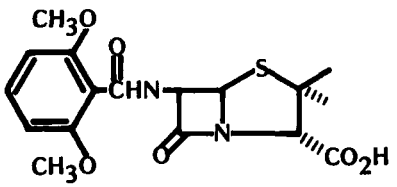

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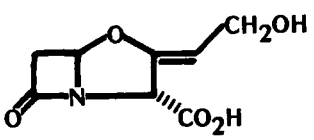

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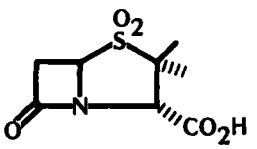

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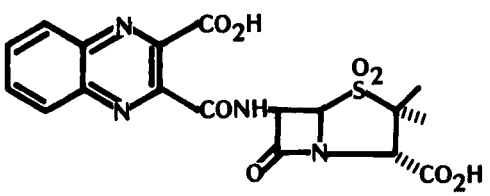

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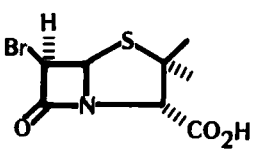

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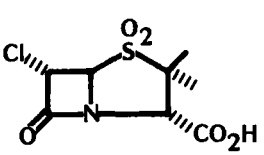

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Greater understanding of the processing and evolution of the class A enzymes has become available through consideration of their primary amino acid sequences. Strominger has shown that significant sequence homology exists between these β-lactamases and a *B. subtilis* DD-carboxypeptidase penicillin binding protein when the active site serine labelled by penicillin G of the latter is aligned with serine 70 of the former.[20] A similar observation has been made in *E. coli* leading to the hypothesis that class A β-lactamases are derived evolutionarily from carboxypeptidases. How this process may have occurred is being studied by Spratt[21] and Baty,[22] who have elucidated the mechanism of post-transcriptional processing of these two enzymes. The carboxypeptidases and β-lactamases are secreted through the cytoplasmic membrane and anchored to the membrane by their carboxy terminal portion.

This anchor is cleaved off the β-lactamases, freeing the enzyme to float into the periplasmic space. Work on the *Bacillus licheniformis* β-lactamase has revealed analogous processing.[23]

Ambler has assigned the *B. cereus* β-lactamase II to class B on the basis of its marked dissimilarity to class A enzymes.[7] Not only is its primary amino acid sequence completely non-homologous, but also it requires zinc as a cofactor.[24] Its mechanism remains unknown, although its active site has been shown to consist of three histidines and one cysteine holding the zinc ion which catalyzes the reaction.[16]

Class C is comprised of chromosomally-encoded cephalosporinases from Gram-negative bacteria. Jaurin and Grundstrom have recently provided support for this classification by sequencing the *E. coli* K12 chromosomal gene for the ampC cephalosporinase, a member of this group.[25] They found no significant sequence homology to members of the other two classes of enzymes. Their experiments with ampC gene probes show that extensive sequence homologies exist with chromosomally-encoded β-lactamases of other *Enterobacteriaceae. Although class C* β-lactamases may differ in evolutionary origin from the class A enzymes, their mechanism appears to be similar. Waley has shown that the inducible cephalosporinase from *P. aeruginosa* reacts with cloxacillin to form an acyl-enzyme intermediate which is stable enough to be tryptically digested.[26] The label was found to be on a serine in a 14 amino acid peptide which showed very nearly complete homology to a sequence in the ampC gene.

One of the mechanisms of non-β-lactamase mediated resistance to penicillin is alteration of the essential target sites. Zighelboim has reviewed work of Tomasz and co-workers which showed that the changes in penicillin-binding proteins observed in progressively more resistant clinical isolates of *Streptococcus pneumoniae* parallel those seen in the laboratory in resistant strains grown in the presence of progressively higher concentrations of penicillin.[27] In the most resistant isolate, some binding proteins had disappeared completely, while others had decreased affinity for penicillin, and new proteins labelled by penicillin at high concentration had appeared. A similar mechanism underlies methicillin resistance in *S. aureus*.[28] Several groups of workers have observed varying changes in the penicillin sensitive target proteins depending on the strains isolated.[29] Tomasz' group observed decreased affinity to methicillin of several proteins, while Brown and Reynolds found a new penicillin-binding protein in addition to decreased sensitivity of the other targets.

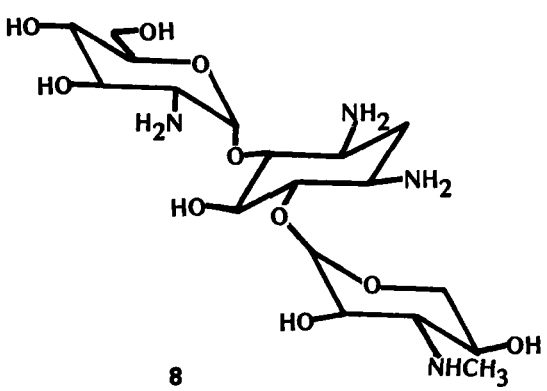
Intrinsic resistance to β-lactams in Gram-negative organisms is usually attributed to non-specific permeability barriers, but altered targets may be involved here as well. Following up on a preliminary study of Rodriguez and Saz.[30] Tomasz showed that two levels of penicillin resistance in *Neisseria gonorrheae* were due to decreased penicillin binding of two target proteins.[31] Two recent reports have described clinical isolates of *P. aeruginosa* with singly or multiply changed penicillin-binding proteins.[32]

Tolerance to the killing action of β-lactams is a mechanistically related but physiologically distinct phenomenon. Tolerant organisms are growth inhibited by normal concentrations of β-lactams (and other antibiotics of related mechanism of action), but are killed at a reduced rate in comparison with normal organisms. Sabath has shown that tolerance in clinical isolates of *S. aureus*[33] is due to suppression of the autolytic system believed to be involved in cell growth,[34] triggering of which by penicillin leads to lytic death.[35] The suppression is due to an excess of the autolysin inhibitor, which is produced in such large amounts that it can protect susceptible strains in the medium. The recent confirmation of the consistent occurrence of low levels of tolerant organsisms in all populations of *S. aureus*[36] may help explain the stubbornness of their infections. Tomasz has recently reviewed his group's work on tolerant streptococcal mutants produced in the laboratory which possess a defective autolytic enzyme system, in contrast to the normal one in staphylococcus.[37] Their

natural counterparts are *Streptococcus sanguis*, which lyse in the presence of penicillin only with added exogenous murein hydrolase.[38] The picture has been complicated by the isolation of tolerant laboratory mutants with no deficiency in their autolytic system.[39]

Permeability barriers to β-lactams due to the outer membrane of Gram-negative bacteria were demonstrated long ago by showing that added EDTA sensitized normally resistant organisms to pencillins by disrupting this membrane.[40] A recent study illustrated this phenomenon by mutating a resistant *P. aeruginosa* strain to a sensitive strain which had no change in inactivating enzymes or targets.[41] Plasmids may encode certain specific permeability barriers, as shown recently in a clinical strain of *Serratia marcescens*.[42] Even resistance to specific β-lactams only can be found in mutants lacking one of the porins necessary for transport through the outer membrane.[43] Finally, the complex interaction of these many resistance mechanisms and its implications for therapy have been recently discussed.[44]

Aminoglycosides. Aminoglycoside resistance is generally mediated by a variety of mechanisms aimed at interfering with drug transport.[45] Aminoglycosides enter cells in three stages: (1) initial rapid binding to the cell surface, (2) a slow uptake across the cytoplasmic membrane, termed EDP I, and finally, (3) a rapid influx phase, termed EDP II, following drug binding to the ribosome, which is associated with cell death.[46] Two basic mechanisms mediate resistance to the crucial EDP II step. The most clinically prevalent one is the plasmid coded enzymic modification of the drug via three types of inactivating functionalization:[47] (1) transfer of an AMP moiety from ATP to a hydroxyl group on the drug, (2) transfer of a phosphoryl moiety from ATP to a hydroxyl group on the drug, and (3) transfer of an acetyl moiety from acetyl CoA to an amino group on the drug. The enzymes are associated with the cytoplasmic membrane and inactivate drug by one of the three pathways as it enters the cell in EDP I.[48] Inactivated drug cannot bind to the ribosome to initiate EDP II. Kagan and Davies have demonstrated the importance of inactivating the incoming drug faster than its EDP I rate by showing that an altered acetyl transferase with an elevated dissociation constant for gentamicin (**8**) no longer protected its host, even though it still modified the drug.[49] Harford found that a normal acetyl transferase was unable to protect its host against two new semi-synthetic aminoglycosides for the same reason, and suggested that an inhibitor of this enzyme which interfered with substrate binding should be synergistic with the natural substrate.[50] This proposal has recently been borne out by workers at Lilly, who discovered that fermentation-derived 7-hydroxytropolone (**9**) is a competitive inhibitor of 2''-O-adenylyltransferase.[51] This compound competes with ATP in the initial binding step,[52] slowing down inactivation to permit lethal drug accumulation.



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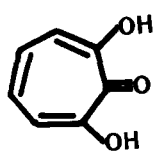

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The second major pathway for interfering with aminoglycoside transport is not plasmid encoded. Bryan and coworkers have provided extensive evidence that aminoglycoside transport across the cytoplasmic membrane is driven by its electrical potential gradient and that respiratory quinones are involved in this process.[53] Resistance can arise if either of these two components is inadequate or missing. For example, the anaerobes *Bacteroides* and *Clostridium* are naturally resistant to aminoglycosides because they lack the quinones which mediate drug transport.[54,55] Anaerobically grown *E. coli* and *Klebsiella pneumoniae* lack an electrical potential gradient sufficient to drive drug across the membrane.[55] The anaerobic growth observed in recent clinical isolates of *S. marcescens*[56] and *Enterobacter aerogenes*[57] resulting from specific defects in their oxidative metabolism is apparently responsible for their aminoglycoside resistance as well. Finally, resistance in laboratory mutants by virtue of ribosomal defects resulting in decreased drug binding[58] has been shown to result from decreased drug accumulation and not just decreased amino acid misincorporation.[59]

Even though most of the observed permeability barrier-mediated resistance to aminoglycosides can be attributed to a defective electrical membrane gradient, the outer cell wall seems to contribute as well.[60] Surface properties of certain gentamicin resistant strains of *P. aeruginosa* were shown to differ from their sensitive counterparts.[61]

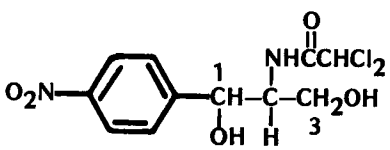
Macrolides. Modification of the target site constitutes the most significant form of erythromycin resistance, which is generally plasmid-borne and can be constitutive or inducible by the drug. Weisblum and coworkers showed some time ago that this modification consists of one or more N(6),N(6)-dimethyladenines in the sequence AAAG present in the 23S ribosomal RNA,[62] thus reducing the affinity of erythromycin for its target. They demonstrated that this dimethylation, which is induced by erythromycin, is the cause and not the result of resistance.[63] Recent information on the genetic sequence of one of the erythromycin resistance determinants, pE194, has dramatically illuminated the mechanism underlying its regulation.[64] The coding region for the erythromycin-inducible 29,000 MW methylase[65] is preceded by a region coding for a 19 amino acid peptide. Weisblum has proposed a model for induction of resistance based on the secondary interactions which can be predicted for this segment of the gene. They suggest that the mRNA forms stem and loop structures which mask the ribosome-binding site and start codon for the methylase and expose only the initiation site of the short polypeptide. When a sensitive ribosome is inhibited by erythromycin binding, it becomes stalled in the middle of this short sequence, thus dissociating the stem and loop structure and freeing the initiation site of the methylase. The small amount of constitutively resistant ribosome generally present can then translate the methylase, in turn forming more resistant ribosome. The process is halted either when too little sensitive ribosome is left to unmask the methylase initiation site or possibly by the methylase binding to an AAAG site present in the loop structure obscuring its initiation site. Since erythromycin normally stalls ribosomes after translation of about ten amino acids, the short polypeptide at the beginning of the gene is the right length to ensure induction of resistance. Erythromycin resistant organisms which have mutated from inducible to constitutive resistance have single base pair changes in the regions believed to be necessary for proper stem and loop structure formation masking the methylase initiation site, thus supporting the above model. Weisblum has also proposed a model for the changes in RNA secondary structure which occur to remask the initiation site to account for the return to normal ribosomes upon removal of the drug.[66] The model may also be useful in explaining the varying efficiencies of different macrolide analogues[67] since the ability to unmask the methylase initiation site would depend on where ribosomal translation is inhibited.

In order to determine the generality of this mechanism, other resistance determinants have been examined for possible DNA sequence homology with pE194. While pE194 showed

considerable homology to determinants from *S. sanquis*[68] and *Staphylococcus epidermidis*,[69] other streptococci and a *S. aureus* carried determinants showing no such similarity.[68] A resistance determinant from the *B. licheniformis* chromosome was shown to be non-homologous to pE194 and to produce a 35,000 M.W. protein when induced by erythromycin.[70] Thus at least three different resistance determinants must exist. Gram-negative strains are generally impermeable to erythromycin by virtue of their outer membrane.[71]

Tetracycline. As reviewed extensively by Chopra,[72] the picture of the mechanism of tetracycline resistance is still fairly incomplete. Plasmid-mediated reduced accumulation of the drug is the basis of resistance, and has been shown to consist of four genetically distinct resistance determinants.[73] Plasmids representative of two of the groups, R100 and pSC101, have been studied at the genetic sequence level. pSC101 is associated with low levels of resistance and codes for several proteins, two of which are located in the membrane and prevent tetracycline accumulation.[74] The high level resistance of transposon Tn 10 from R100 is due to at least two proteins.[75] One of these is located in the cytoplasmic membrane and promotes active efflux and reduced influx of the drug,[76] reversing the pattern seen in sensitive strains where greater influx than efflux leads to drug accumulation. All four genetic determinants of tetracycline resistance involve an efflux mechanism.[77] The function of the second protein is presently unknown. It has been speculated that ribosomal protection contributes the balance of the high level of resistance observed.[78] Tn 10 also codes for a trans-acting repressor which regulates the inducible resistance.[79] One way in which tetracycline penetration could be inhibited is suggested by recent work,[80] which showed that resistant strains are more sensitive to lipophilic chelators. Since tetracycline passage across the membrane seems to require divalent cations, reduction of their concentration in the membrane would lead to reduced permeability of drug but increase susceptibility to chelators. Thus while the exact mechanism of tetracycline transport is only partly known,[81] it is clear that its alteration results in decreased accumulation and resistance. While most of the work already cited has been done in *E. coli*, the situation may be different in *N. gonorrhoeae*, where resistance is likely chromosomally encoded.[82] Tetracycline resistant ribosomes seem to be confined to laboratory mutants,[83] and there is no evidence of modification of the drug.

Chloramphenicol. The major form of bacterial resistance to chloramphenicol (**10**) is enzymatic deactivation of the drug via acetylation of one or both hydroxyl groups, using acetyl CoA as the acyl source, by chloramphenicol acetyl transferase (CAT).[84] Since CAT is



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only able to effect 3-OH acylation, diacetylation results from acyl migration from the C-3 to the C-1 hydroxyl followed by reacylation at C-3.[85] There are at least fifteen naturally occurring variants of the enzyme.[86] In Gram-negative bacteria the plasmid-borne enzymes are constitutive and comprise types I-III. In Gram-positives, the enzymes are also plasmid-mediated but inducible, and comprise types A-D. All the enzymes have approximately 22,500 molecular weight (except type III, 24,500) and are composed of four subunits. The monomers of types I-III will hybridize with each other, but not with type A-D monomers, which also associate. Although there is little overall amino acid sequence homology between types I-III and types A-D enzymes, the active site sequences of the type I and type C

enzymes are almost identical, suggesting that similarity in mechanism may prevail.[87] In his mechanistic studies of the type A-D enzymes, Shaw has found that a histidine imidazole ring seems to be involved, but that an acyl-enzyme intermediate is not.[88] His studies of the type I enzyme revealed that two cysteine residues were involved, one to bind the drug and the other to bind the acetyl CoA, but again without forming an acyl-enzyme.[89] The predicted folding of the known primary amino acid sequence of the type I enzyme also shows a potential binding site for the adenine nucleotide portion of acetyl CoA in the region of this latter cysteine residue.[90] Labelling studies have provided evidence that lysine residues are involved in ion-pair interactions to hold the subunits together, although different lysines are involved for the type I and type III enzymes.[91] When the ongoing X-ray work is completed, a more detailed picture of the enzymatic mechanism will be available.[92]

Although plasmids specifying chloramphenicol resistance usually code for CAT, Shaw and others have suggested that some plasmids mediate permeability barriers for the drug, since antibiotic inactivation and ribosomal insensitivity were not involved.[93] Recent work has shown that resistance in *P. aeruginosa* can be due to impermeability resulting from alteration of a membrane function essential to amino acid transport.[94] Ribosomes resistant to the polypeptide elongation inhibitory effect of chloramphenicol seem to be confined to laboratory mutants.[95]

Trimethoprim. Resistance to trimethoprim, which has been recently reviewed,[96] is mediated mostly by target site alteration, via two basic mechanisms. The major mechanism observed in one study of clinical isolates was chromosomal production of an altered dihydrofolate reductase (DHFR), the enzyme in the folic acid synthesis pathway inhibited by trimethoprim.[97] This altered DHFR seems to result from a single mutation, since its properties differ somewhat from the normal enzyme, but it retains the same molecular weight and ability to bind dihydrofolate.[98] This situation differs from that observed in trimethoprim-resistant laboratory mutants, where a mutation in the DHFR regulatory gene leads to elevated levels of DHFR,[99] although overproduction of a resistant DHFR has been reported in clinical strains.[100] Whereas chromosomally coded resistant DHFR mediates an intermediate level of resistance, the second basic mechanism, plasmid coded trimethoprim resistant DHFR, results in high level resistance. The plasmid encoded enzymes are of two types. Both types bind dihydrofolate as well as the normal enzyme, but have molecular weights twice as high. The type I enzyme is 10,000-fold less sensitive to trimethoprim and is made up of 18,000 M.W. dimers.[101] The type II enzyme is almost totally insensitive to the drug and is made up of 8,500 M.W. tetramers.[102] The primary amino acid sequence of the type II tetramer shows no homology with known DHFRs,[103] so its origin is unknown. Long term use of cotrimoxazole (trimethoprim plus sulfamethoxazole) can lead to thymine-dependant organisms resistant by virtue of a mutation in the thymidylate synthetase gene which makes them unable to use the folic acid pathway inhibited by trimethoprim.[104] Though easily selected for in the laboratory, these strains are rare in the clinic. Finally, reduced permeability to the drug has been implicated only rarely in clinical isolates,[97] and modification of trimethoprim is not known.

Sulfonamides. Bacterial resistance to sulfonamides is generally due to plasmid mediated synthesis of a less sensitive target enzyme. The plasmid coded dihydropteroate synthetase (DHPS), the enzyme in the folic acid pathway inhibited by sulfa drugs, binds its substrate, p-amino benzoate, normally but is several thousand times less sensitive to the drug.[105] In contrast, laboratory sulfa-resistant mutants produce an altered DHPS which is 10-fold less efficient at substrate binding and only 150-fold less sensitive to the drug.[106] More recent work has shown that different plasmids can code for different resistant enzymes.[107] In addition, two groups have shown that some strains whose resistance is plasmid borne contain only the normal DHPS, thus leading to the suggestion that reduced permeability to the drug may be involved.[107,108] Modification of the drug has not been demonstrated.

Conclusion. The study of resistance mechanisms has led to two basic approaches to overcoming them: discovery of new antibiotics impervious to the resistance mechanism and development of agents which render resistant bacterial pathogens susceptible to a known antibiotic by disabling their resistance mechanism. Examples of the former class include cefoxitin (**1**) and methicillin (**2**), both of which resist enzymatic deactivation by β-lactamases. Examples of the latter include clavulanic acid (**3**) and sulbactam (**4**) which deactivate β-lactamases and render resistant organisms susceptible to penicillins. Work on this latter approach is still at an early stage, but it offers considerable promise in that it might be applied successfully to many other classes of antibiotics. An understanding of the mechanisms responsible for antibacterial resistance should be an important step in the direction of developing agents to control this problem.

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Chapter 14. Antiparasitic Agents

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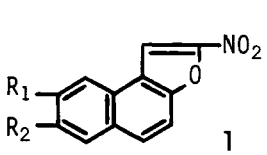
PROTOZOAL DISEASES

General - The pharmacokinetics, metabolism and adverse reactions of 5-nitroimidazoles have been reviewed,[1] together with detailed reviews of their mode of action on protozoa and bacteria.[2,3] Acetamide and N-(2-hydroxyethyl)oxamic acid were the two major metabolites of metronidazole in rat and man.[4,5] Novel 2-nitronaphtho[2,1-b]furans (1) were as good as or better than metronidazole against both Trichomonas and Entamoeba, whereas 2-nitrobenzofurans showed good activity only against Entamoeba.[6] The succinyl esters of o-tertbutylphenol (2) were more active than metronidazole against sensitive or drug-resistant strains of these same two species when given orally, topically or parenterally.[7] A structure/activity study of the activity of congocidine derivatives 3 against Trypanosoma congolense and Leishmania tropica has been published.[8] Antibiotic A-33853 4 from Streptomyces had a wide spectrum of antimicrobial activity including activity against coccidia and Trichomonas.[9]



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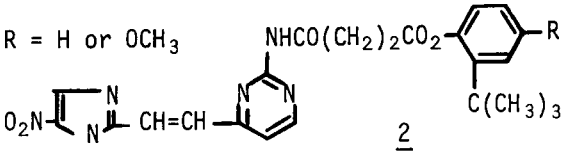

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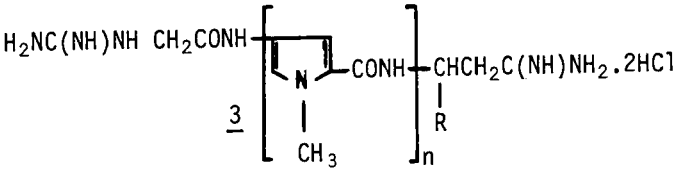

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Trichomonas - Substituted quinazoline derivatives of 5-nitro-2-imidazoles were effective against metronidazole-resistant strains of Trichomonas vaginalis.[10] Systemic activity was shown by a new class of anti-trichomonal agent, the pyrido[2,3-b]quinoxalines 5,[11] while one of a series of 2-alkylamino-methylbenzisoxazolin-3-ones 6 showed in vitro activity against T.vaginalis 7-12 times greater than metronidazole.[12] In vitro activity was also shown by a series of 2,7-diazabicyclo [4,1-0]hept-3-enes 7.[13]

Amoebae - A comprehensive review of the chemotherapy of amoebiasis has appeared.[14] The bromoacetamide group is contained in two compounds active against Entamoeba, one a tetrahydro-2,3,4,5-1H-pyrido[3,2-b]azepine (8), and the other a derivative of homoveratrylamine.[15] A rapid agar disc diffusion assay to test the drug susceptibility of Acanthamoeba castellani, causing keratitis, showed that the polyene antibiotic pimaricin, related to amphotericin B, was an effective agent against this species.[16]

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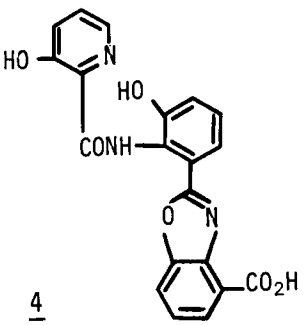

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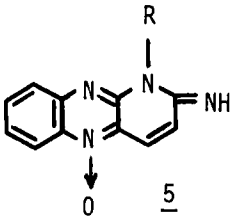

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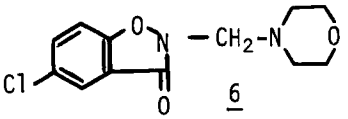

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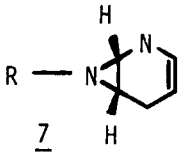

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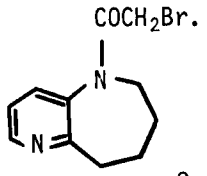

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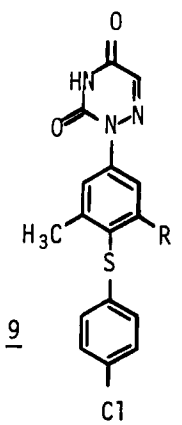
Coccidia - The polyether ionophorous antibiotics continue to dominate the therapy of coccidiosis, and many novel molecules of this type have been described.[17] The polyether antibiotics have been used successfully in rabbit coccidiosis, but many of the older anti-coccidials are still recommended for rabbit infections.[18] Sulphonamides and lasalocid were effective against Isospora laidlawi in mink,[19] and lasalocid and halofuginone were successful against I. suis in pigs.[20] A detailed report on the possible mode of action of arprinocid-N-oxide, and its parent, arprinocid, showed interaction of the compounds with cytochrome P450 of mammalian microsomes, and it was suggested that a similar interaction might lead to the destruction of intracellular parasites.[21]

A continuing study of anti-coccidial derivatives of 6-azauracil has led ultimately to 9, which has a wide species spectrum of activity, 4000-fold greater than azauracil, and a short plasma half life, but toxicological symptoms related to inhibition of RNA synthesis were seen.[22] A related triazine from Bayer has proved effective against mammalian coccidiosis.[23] The optimized substituents from the triazine series noted above have been used to produce a series of 1-substituted phenyluracils which also showed anti-coccidial activity.[24] Analogues of emimycin (2-(1H)-pyrazinone-4-oxide) showed in vivo activity against E.tenella, and such activity was reversed by orotic acid or adenine. Active compounds from the series included "nucleoside" and orotic acid analogues, but much information on structure/activity relationships can also be obtained from the negative data.[25] 1,4-Naphthoquinone antibiotics 10a, c from Streptomyces showed high activity against E.tenella in chickens, and an inactive carboxylic acid metabolite 10b can be lactonized to the active 10c by treatment with trifluoroacetic anhydride.[26]



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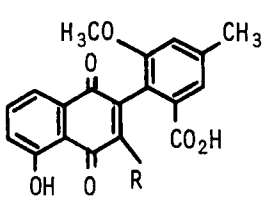
R = Cl or CH_3

10 a R = H

b R = OH



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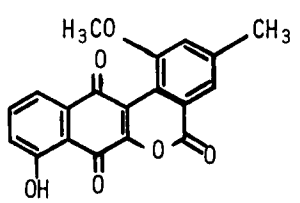
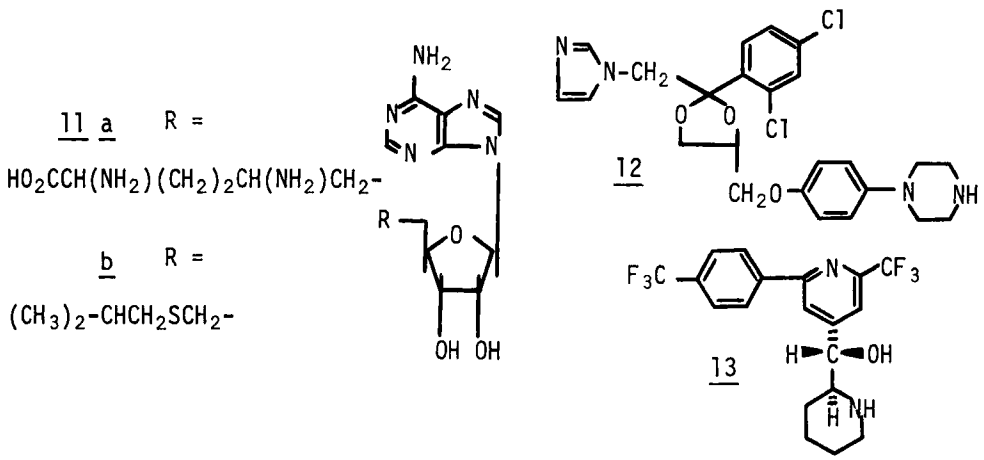


African Trypanosomes - Two major reviews have covered the chemotherapy and prophylaxis of human[27] and animal[28] trypanosomiasis of the Eastern hemisphere. Despite the early promise that a mixture of salicylhydroxamic acid (SHAM) (as an inhibitor of the α-glycerophosphate oxidase system) and glycerol (as a feedback inhibitor of anaerobic glycolysis) would give in vivo cure of T.brucei infections, this hope has not been fulfilled.[29] Both compounds were rapidly eliminated from the host, with only a narrow margin between therapeutic and lethal doses. A systematic search for alternatives to this combination showed that no other polyol could substitute for glycerol, and that only primary and secondary aromatic hydroxamates were inhibitory. Two other iron chelators, 3-bromo-4,5-benzotropolone and 3,4-dihydroxybenzaldehyde, were as active as SHAM as inhibitors of α-glycerophosphate oxidase, and a lipophilic aromatic iron chelating agent might be a substitute for SHAM in combination therapy.[30] A rapid in vitro screen for Fe-binding chelators, based on the growth of Crithidia fasciculata has been described, and of 161 chelators examined, 32 inhibited growth at low concentrations.[31] Polyamine biosynthesis has been suggested as an area of potential chemotherapy for trypanosomes.[32] α-Difluoromethylornithine, an inhibitor of ornithine decarboxylase, blocked putrescine biosynthesis in T.brucei, and inhibited its growth in vivo[33]; an effect which was reversed by polyamines.[34] Administration of buthionine sulfoxime to T.brucei-infected mice caused a 50% fall in glutathione levels within the trypanosomes due to inhibition of γ-glutamylcysteine synthase, but gave only partial clearance of the infection.[35]

Trypanosoma cruzi - The combination of the nitrofuran nifurtimox with a corticosteroid not only destroyed parasites within the heart, but prevented the myocardial inflammation which this produced.[36] The occurrence of a glutathione-S-transferase was demonstrated in epimastigotes of T.cruzi.[37] Such enzymes are potentially capable of forming conjugates with 2-substituted 5-nitrofurans, but did not do so with the trypanocidal compound 2-(1-methyl-5-nitroimidazolyl)-4-(thiomorpholinoiminomethyl)-thiazole-1',1'-dioxide, which was instead an inhibitor of the enzyme. Such interactions with glutathione-S-transferases of parasite and host are of importance for the design of potential therapeutic compounds. Although allopurinol showed promising results in mice against some strains of T.cruzi[38] other strains were not at all inhibited by the drug.[39]

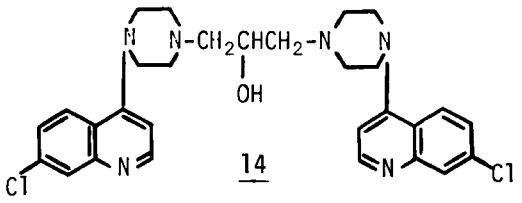
Leishmania - Activity of allopurinol and its ribonucleoside against intracellular amastigotes of Leishmania donovani confirmed that these forms posess the same purine metabolic pathways as the extracellular promastigotes,[40] and activity against human infections of L.donovani has now been demonstrated.[41] Unlike mammalian cells, Leishmania phosphotransferases converted formycin B to its ribonucleotide, which was an inhibitor of adenylosuccinate synthetase in parasite extracts. Formycin B was an inhibitor of the in vitro growth of both amastigotes and promastigotes, and was effective in treating established L.donovani infection in hamsters.[42] 4-Amino-5-imidazole- carboxamide (AICA) [43] and thiopurinol and its ribonucleoside [44] showed in vitro activity against several Leishmania species. Promastigotes of L.donovani and L.mexicana were not directly inhibited by 6-methylpurine-2'-deoxyriboside, but this compound killed mouse macrophages infected with amastigotes, leaving non-parasitised cells intact. A 2'-deoxyribonucleosidase, unique to Leishmania, produced 6-methylpurine in infected cells, which was then toxic to the macrophage.[45] Two inhibitors of polyamine biosynthesis (11a, sinefungin and 11b, SIBA), showed activity against promastigotes and amastigotes of Leishmania in vitro.[46] Four anti-fungal imidazoles, which interact with sterol metabolism in fungi, showed activity against intracellular amastigotes of L.tropica, but only one, deacetylated ketoconazole 12, was active at concentrations (>4μg/ml) achievable in human sera.[47] The enhanced activity of liposome-entrapped compounds against visceral leishmaniasis has enabled two further anti-fungal drugs, 5-fluorocytosine, and griseofulvin to be added to the list of active anti-leishmanial drugs.[48]



Malaria - A summary of scientific work on malaria during the past 5 years has been compiled by the WHO.[49] A 3-volume publication entitled "Malaria", edited by Kreier, was published in 1980, and the chapter on chemotherapy by Peters is important as a compendium of practical methods for detecting anti-malarial activity of all types.[50] The proceedings of a WHO meeting dealing with "Tissue Schizonticidal Drugs against Malaria" have been published, and most of the 9 papers dealt with the pharmacology, metabolism, toxicity and assessment of the 8-aminoquinolines, but one paper described the ten distinct chemical series which show causal prophylactic activity in rodent malarias.[51]

Pharmacokinetic studies in man of WR 180409 (dl-threo-13) showed that a single 750 mg dose produced blood levels for over 2 weeks which exceeded the ID_{50} for chloroquine-resistant Plasmodium falciparum.[52] Mefloquine was shown to be effective in supressing natural infections of P.vivax and chloroquine-resistant P.falciparum in a semi-immune Thai population when given at a weekly dose of 180 mg/50kg.[53] Hydroxypiperaquine phosphate from China, a 4-aminoquinoline 14, was more active than chloroquine in an extensive field trial against P.falciparum in man, and was also effective against chloroquine-resistant strains.[54] Similar compounds have been patented in Russia.[55] 4-Aminoquinolines 15 were effective against normal and drug-resistant strains of malaria, and acted as a drug repository when given parenterally, or could be used orally at 0.5-5mg/kg daily.[56] Continuing studies of anti-folates as anti-malarials indicated that 6-[(aralkyl)amino]-2,4-diaminopteridines show good in vivo activity against rodent and primate malarias.[57] Promising activity was present in a series of N^2-aryl-N^4-[(dialkylamino)alkyl]-2,4-quinazolinediamines, but the best compound 16, and others, showed phototoxicity and could not be put forward for human studies.[58] The sesquiterpene lactone quinghaosu is being evaluated in the Western world. The ED_{90} against P.berghei in mice treated subcutaneously was 2.3mg/kg, and the compound was effective against all drug-resistant (except chloroquine-resistant) strains tested. No toxicity was seen with daily doses of 300mg/kg.[59] A re-examination of other natural products using the P.falciparum in vitro assay indicated that the quassinoids, especially simalikalactone D, show activity at low concentrations (0.002 µg/ml). [60] Activity against P.berghei in mice was shown by the antibiotic kijanimycin[61] and by a polyether ionophore antibiotic (X-14766A) unique in having a halogen substituent.[62]The P.falciparum in vitro system was used to identify anti-malarial activity in many varied types of inhibitor. 5-Substituted 8-hydroxyquinolines, and 2-mercaptoquinoline-N-oxides and 2-mercaptopyridine-N-oxide all showed inhibition of growth proportional to their chelating properties.[63] The anti-fungal agents ketoconazole, miconazole and amphotericin B showed activity against chloroquine-resistant strains of P.falciparium.[64] Coordination complexes of several "quinine-like" anti-malarial drugs with ferriprotoporphyrin

were studied in vitro[65], and it was concluded that their anti-malarial activity, and patterns of cross-resistance, may be determined by the dissociation constant of the drug/haemin complex[66].



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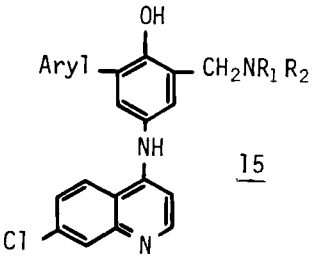

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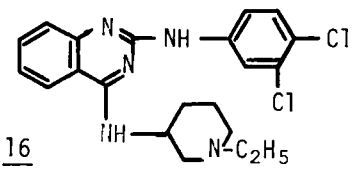

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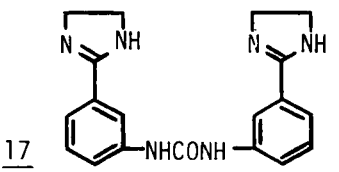

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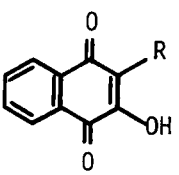
Tick-borne Haemotropic Diseases - Theileria, Babesia, Anaplasma and Cowdria are the important tick-borne microorganisms which make up the haemotropic disease complex, common in domestic animals and occasionally (Babesia) in man. An ideal chemotherapeutic agent would be effective against all four organisms; imidocarb 17 is active against both Babesia and Anaplasma, using a single dose treatment.[67,68]



18 R =

a $-(CH_2)_8-C_6H_{11}$

b $-C_6H_{11}$

Theileria - A comprehensive review of the chemotherapy of theileriosis is provided in "Advances in the Control of Theilerosis".[69] Great advances in chemotherapy were the in vivo successes of naphthoquinones such as menoctone 18a [70] and 993C (parvaquone) 18b, [71] and the quinazoline derivative halofuginone.

Babesia - Imidocarb dipropionate provides a specific therapeutic and prophylactic treatment for babesiosis in cattle[72] and dogs.[73] 1-(Chlorophenoxyalkyloxy)-4,6-diamino-2,2-dimethyl-1,3,5-triazine hydrobromides previously shown to be active anti-malarials, showed good activity against B.rodhaini in mice. A related chlorophenylalkyltriazine active against cycloguanil-resistant P.berghei, is the only member of that series active against B.rodhaini.[74] Ticks carrying B.microti or B.bovis can infect man,[75] and poor diagnosis often leads to treatment with the ineffective chloroquine, and the poorly effective and toxic berenil or pentamidine[76,77]. The small number of human Babesia infections probably make it uneconomic to make the highly effective imidocarb available for medical use.

HELMINTH DISEASES

As in 1981, the wide spectrum anthelmintics will be dealt with separately before turning to specific disease applications.

Praziquantel - Proceedings of a recent symposium on the use of praziquantel against African schistosomiasis have been published and provide up-to-date information on the results of clinical trials and toxicology.[78] When the in vitro uptake of

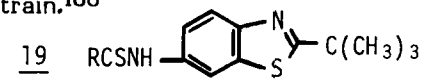
^{14}C-praziquantel by several helminth species was examined, it was shown that susceptible worms such as Schistosoma mansoni or Hymenolepis nana took the drug up very quickly, and above a threshold of 0.3µg/ml, an instantaneous contraction and paralysis occurred.[79] This primary effect caused schistosomes to be shifted to the liver where secondary effects, leading to the death of the worms were initiated. Intensive vacuolization occurred at distinct sites on the tegument which allowed host granulocytes to attach, and penetrate into the interior of the worm. In 14-18 days, the worms have disintegrated and are recognised only as a granuloma.[78] In cestodes the site of vacuolization is the neck region of the strobila and mature proglottides were not affected.[80] Even though effective it is not economic to use praziquantel for some veterinary cestode infections such as Stilesia hepatica of sheep,[81] but the compound has proved particularly useful in the treatment of Clonorchis/Opisthorchis in man.[82]

Avermectins- "An Introduction to Avermectins" was published which dealt with their structure, spectrum of activity against both helminth and arthropod parasites, and their mode of action on invertebrate nervous systems.[83] Details of the general structural determination of these macrocyclic lactones,[84] and the absolute stereochemistry and conformation of avermectin B_{2a} aglycone and avermectin B_{1a},[85] were reported. Parenteral administration of a single dose of avermectin B_{1a} or ivermectin (22,23-dihydroavermectin B_1) was effective against migrating Strongylus vulgaris larvae in ponies.[86] Ivermectin also showed anti-filarial activity, and a single dose cleared adult Setaria equina, and microfilariae of Onchocerca cervicales from ponies,[87] and Dirofilaria immitis larvae from dogs.[88] Paralysing effects of avermectins on susceptible nematodes in vitro can be detected at concentrations of 3.6×10^{-18}M.[89] In rat brain synaptosomes they appeared to irreversibly modify benzodiazepine receptors at a GABA-chloride recognition site.[90]

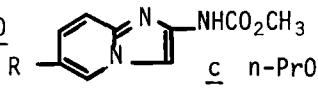
Benzimidazoles- Many reports appeared during the year confirming the wide-spectrum anthelmintic activity of benzimidazoles, and extending their action to new hosts and parasites. Oxibendazole, the n-propoxybenzimidazole carbamate, was active against the filarial worm Brugia pahangi in cats when given by parenteral routes, but was inactive orally.[91] This compound was more effective than the regularly used thiabendazole and cambendazole against Strongyloides westeri in foals, even when used at a dose level of 10mg/kg.[92] The n-propylthio derivative, albendazole, showed in vivo activity against Ostertagia ostertagi[93] and larval cestodes,[94] but is noted for its activity against trematodes such as Fasciola hepatica[95] and Paragonimus in cats.[96] The majority of current papers concerning benzimidazoles relate to the benzoylbenzimidazole carbamate, mebendazole, and its 4-fluoro analogue, flubendazole. Mebendazole was shown to be as effective as, and better tolerated than, diethylcarbamazine for the treatment of human onchocerciasis,[97] and was generally effective against adult cestodes of cats[98] and dogs.[99] Poor drug absorption from oral dosing, and the short half-life of mebendazole, make it difficult to achieve the plasma concentration of 0.25µmole/L required to treat cystic echinococcosis in humans,[100] but several reports noted the success of mebendazole or flubendazole in treating hydatid disease in man and animals.[101-103] There is now widespread resistance to benzimidazoles amongst small strongyles of the subfamily Cyathostominae, and switching of drug types is recommended to avoid resistance problems.[104]

Activities against S. mansoni and micro- and macro-filariae were shown by a group of benzoxazoles, benzothiazoles and benzimidazoles, typified by 19, containing an acylamino substituent in the benzene ring.[105] A series of 6-substituted imidazo[1,2-a]pyridine-2-carbamates 20a-f have been synthesised as analogues of the most effective benzimidazole carbamates, and showed equivalent activity to benzimidazoles against nematodes in both mouse and sheep tests. The most active imidazopyridine compound in the mouse, 20f, had no benzimidazole equivalent, and

when this was synthesised it showed higher activity than any of the known benzimidazoles. Whereas this latter compound showed side-resistance to benzimidazole-resistant Haemonchus, 20a was fully active against the resistant strain.[106]



19 R = 1-piperazino or $HO_2C(CH_2)_2S$-



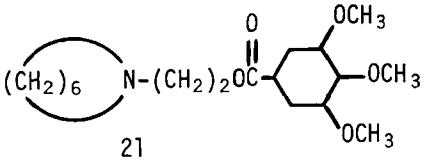
20 R = a PhSO- b PhS- c n-PrO- d n-PrS- e PhCO- f CCl_2=Cl-

Nematodes - The problems of drug resistance in sheep and other farm animals, particularly in Australia, have received much attention in veterinary publications. The best introduction to this literature is the report[107] of a New South Wales Committee on drug resistance topics (and current papers by the members of that committee), which classified the major sheep anthelmintics into 4 groups, based on mode of action, and gave guidelines for testing the effectiveness of anthelmintics against resistant strains.

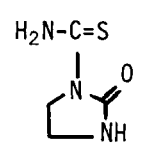
The most efficient treatment for mixed infections of the important human soil-transmitted nematodes (Ascaris, Trichuris and the hookworms Necator and Ancylostoma) was to use oxantel/pyrantel (10mg/kg of each) plus 100mg of mebendazole as a single mixed dose.[108] Experimentally, few novel compounds have shown good anti-nematode activity, but the finding that long chain aliphatic secondary and tertiary amines, known to disrupt development in arthropods, are effective against O.ostertagi in vitro, may lead to inhibitors of nematode exsheathment.[109] The hexahydroazepine ester of 3,4,5-trimethoxybenzoic acid 21 showed activity as great as piperazine and pyrantel against Aspiculuris tetraptera in the mouse.[110] The structural features required for inhibition of ribosomal peptide-bond formation by 4-aminohexosylcytosine antibiotics, which include the anti-nematode agent anthelmycin, were reviewed.[111]

Filariasis - Diethylcarbamazine and its major N-oxide metabolite were quantified after oral dosing in rat and man,[112] and in vitro both these compounds enhanced the adhesion of leucocytes to microfilariae.[113] 1,2-Ethylenebis(2-nitroimidazole) and 2-nitro-1-vinylimidazole were claimed as micro- and macro-filaricidal agents.[114] A concise summary of the metabolism of filarial worms appeared.[115]

Cestodes - A detailed review of the chemotherapy of cestode infections was published,[116] while a book entitled "Biology of the Tapeworm Hymenolepis diminuta"[117] contained chapters on "Chemotherapy of Hymenolepiasis" and "Energy Metabolism of Adult H.diminuta". A long-term study which reported the success of metrifonate against human cysticercosis (T.solium) with CNS and musculocutaneous involvement [118] was confirmed by independent workers.[119]



21



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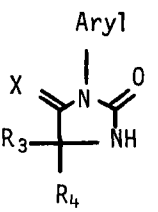
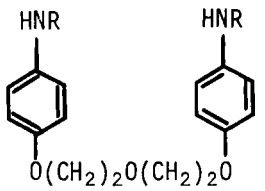


23

Schistosomes - The in vivo production of reduced aromatic compounds with potential mutagenic or carcinogenic properties, and also of the cellular immunosuppressor molecule 1-thiocarbamoyl-2-imidazolidone (22), from niridazole was shown to be due to the gut bacterial flora.[120] During niridazole treatment 22 is responsible for the prevention of granuloma formation around S.mansoni eggs. Imino-oxo and dioxo-imidazoline derivatives 23 have been patented as anti-schistosomal compounds.[121] Extensive field trials with oxamniquine against S.mansoni infections

in Egypt,[122] and in Brazil[123] indicated that chemotherapy using single dose treatment protected the community for a limited period. Oxamniquine and praziquantel showed synergistic activity against S.mansoni.[124] The CNS-stimulating side effects shown with oral dosing of oxamniquine occur because its hydrophobicity parameter (log P) lies very close to the optimal value shown by neutral molecules which can penetrate the blood/brain barrier.[125] Metrifonate gave a good cure rate and reduction in egg production in human populations infected with S.haematobium,[126,127] and is a cheap drug for mass therapy. Ouabain was shown to bind to Na^+-K^+-ATPase located in the tegument of S.mansoni and S.japonicum, and this binding was inhibited by praziquantel and the benzodiazepine Ro-11-3128. Analogues of benzodiazepines which have no anti-schistosomal activity do not compete for this binding site and it is suggested that the mode of action of such anti-schistosomal agents is to affect ion movements by blocking the Na^+-K^+ pump, and hence affect neuromuscular activity.[128]

Fasciola - The diamine 24a is the active metabolite of diamphenethide 24b. Detailed studies on the mechanism of action of 24a against F.hepatica at the biochemical level indicate that the most sensitive indicator of metabolic inhibition is a rise in the level of malate within the worm. Serotonin protects flukes from diamphenethide inhibition in vitro, and other biogenic amines, especially dopamine, also do this and also prevent the rise in internal malate. The site of action of diamphenethide and the biogenic amines might involve membrane-related events other than neurotransmission, such as transport.[129]



24

R = a H, b $COCH_3$

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Chapter 15. Antifungal Chemotherapy

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INTRODUCTION - Preliminary clinical results obtained with ketoconazole have been confirmed in many intensive trials, and the introduction of this compound into clinical practice may be considered as a new milestone in the treatment of fungal diseases.

The antimicrobial spectrum of ketoconazole and its potential in the therapy of dermatomycoses, candidosis of the mouth and the vagina, systemic candidosis, chronic mucocutaneous candidosis (CMC) and candiduria, as well as of deep and percutaneous mycoses such as histoplasmosis, coccidioidomycosis and paracoccidioidomycosis have been the subject of a book[1] and of one symposium.[2]

Antifungal chemotherapy has been reviewed in a book edited by Speller.[3] Various forms of coccidioidomycosis and aspects of its treatment are discussed in a publication[4] and in the outstanding book of Stevens.[5]

Comprehensive reviews have been published, dealing with chemistry, mode of action and clinical use of various classes of antifungal agents.[6-8] Intravenous miconazole in the treatment of systemic mycoses has been the subject of a symposium[9] and a review.[10] Topics of recent reviews are: fungal infection of the heart,[11] fungal infections in the immuno-compromised host,[12] systemic candida infections,[13] mucor mycoses[14] and agents for systemic treatment.[15] The role of fungi in the pathogenesis of human corneal ulcers has been the subject of another review.[16] An overview on ciclopiroxolamin (Hoe 296) has been published, dealing with synthesis, structure-activity relationships, antimicrobial spectrum, mode of action, metabolic, pharmacological and toxicological data.[17]

Approaches have been made to the diagnosis and therapy of dermatophytosis,[18] onychomycosis[19] and other fungal infections.[20]

NEW ANTIFUNGAL AGENTS - Fruitful research efforts in the imidazole field have resulted in new antifungals. Oxiconazole (1) proved to be active against many fungi of clinical importance;[21] topically it is active against both trichophytosis in guinea-pigs and vaginal candidosis in rats.[22]

Imidazole derivatives 2 and 3, structurally related to clotrimazole, have been described.[23] The activity of 2a, 2b and 2c against Candida albicans (C.a.) and Trichophyton asteroids (T.A.) ranges from <0.1 to 12.5 µg/ml, whereas imidazole derivative 3 has weak activity.

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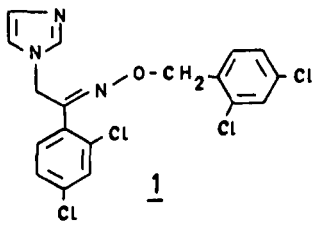

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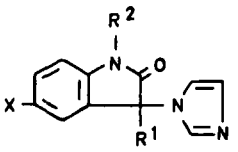

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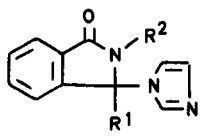
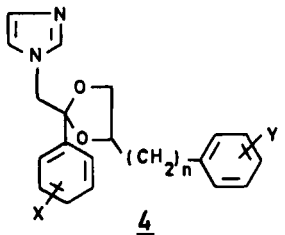


3

	X	R^1	R^2
a.	H	$CH_2CO\varnothing$	H
b.	H	$\varnothing$	$-CH_2CH_3$
c.	Cl	$\varnothing$	CO-(thienyl)



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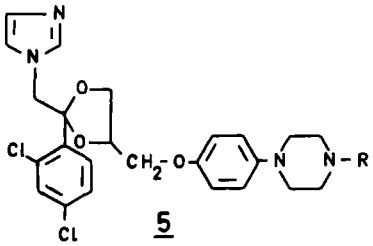


5

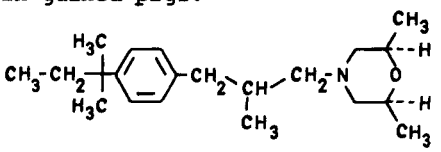
	X	Y	n
a.	4Cl	4Br	0
b.	$2,4Cl_2$	$2,4Cl_2$	0
c.	4Cl	4F	0
d.	$2,4Cl_2$	4Cl	1

	R	
a.	$COCH_3$	KTZ
b.	CHO	
c.	$COOCH_3$	
d.	$CONHCH_3$	

In a series of imidazole ketals (4), broad spectrum activity was found,[24] e.g., oral activity against vaginal candidosis in rats (4a,b) and skin candidosis in guinea pigs (4c,d) at doses comparable to miconazole.

In a series of ketoconazole analogs (5), the structure-activity relationship was discussed with reference to the influence of the acyl moiety on in vitro and in vivo activity.[25] The oral activity of 5b,c,d against vaginal candidosis in rats is comparable to 5a (ketoconazole KTZ); the activity decreases with more lipophilic acyl groups.

Ro 14-4767/002 (6) shows high in vitro activity against C. albicans (0.27 µg/ml), Candida sp. (0.35 µg/ml), Cryptococcus neoformans (0.27 µg/ml) and against various dermatophytes (0.25 µg/ml),[26] and good topical activity against vaginal candidosis in rats and dermatophytosis in guinea-pigs.



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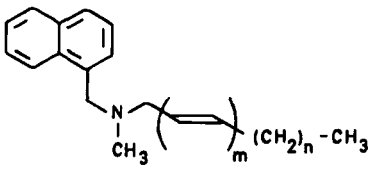
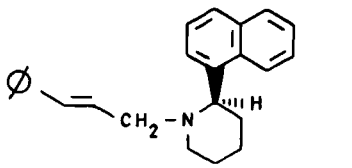


7

	m	n
a.	2	3
b.	2	4

Naftifine analogs (7a,b) have been reported with good in vitro activity against various dermatophytes (0.05-0.2 µg/ml) against Sporothrix schenkii (1.6-3.1 µg/ml) and Candida parakrusei (1.6 µg/ml).[27] Compound 7a was reported to cure guinea-pigs infected with Trichophyton mentagrophytes after topical or oral treatment. However, a rather high dose of 150 mg/kg was needed.[27] Another representative of the naftifine family (8 code no. 85190) is active against dermatophytes (0.05-0.2 µg/ml), Aspergillus sp. (0.78-1.56 µg/ml) and Candida sp. (0.78-100 µg/ml). Guinea-pigs, infected with T. mentagrophytes were cured following topical treatment; in contrast the corresponding S-enantiomer is completely inactive.[28]



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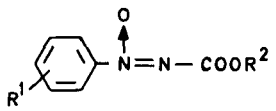
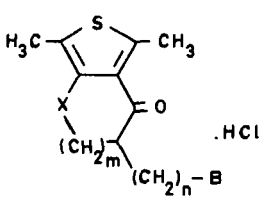


9

The synthesis of a series of phenyldiazenecarboxylate-2-oxides (9) has been reported. One compound (9a: R^1 = 4Br, R^2 = CH_3) shows a good in vitro activity against C. albicans (0.09 µg/ml), T. mentagrophytes (1.56 µg/ml) and Aspergillus niger (0.09 µg/ml); unfortunately it is found to be toxic in mice (10-80 % mortality).[29]

A series of Mannich bases (10) with broad-spectrum activity has been described;[30] 10a (X = S, n = m = 1, B = -N⟨⟩CH_3) is active against dermatophytes (<3 µg/ml) and against C. albicans, but in experimental vaginal candidosis in hamsters the compound was less active than clotrimazole.



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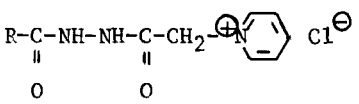
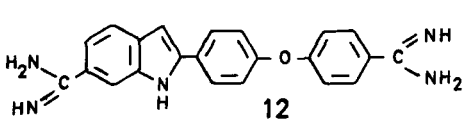


11

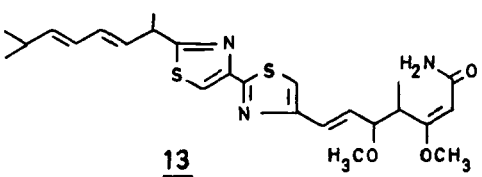
R

a. $C_{13}H_{27}$
b. $C_{15}H_{29}$

In a series of 2-N-(pyridineacetyl) fatty acid hydrazides (11), activity has been found against C. albicans, A. niger and T. mentagrophytes (1.8-3.5.10^{-5} M).[31] Compound 12, a representative of a series of 79 diarylamidines, is active in vitro against dermatophytes, Candida sp. and Aspergillus sp. (0.3-3 µg/ml).[32] In mice, intravenously infected with C. albicans, the latter compound was found inferior to both miconazole and amphotericine B.



12



13

The absolute configuration of ambruticin, with regard to the cyclopropane ring, has been established by chiral synthesis.[33] Water soluble N-D-lysyl and N-D-ornithyl amphotericine B methylesters show in vitro activity against yeasts (0.02-0.75 µg/ml).[34]

A new antibiotic - myxothiazole (13) - isolated from the myxobacterium Myxococcus fulvus inhibits growth of many yeasts and fungi at doses of 0.01-3.0 µg/ml. In C. albicans, S. cerevisiae and Mucor hiermalis growth inhibition was neutralized by glucose.[35-37]

MECHANISM OF ACTION - The mode of action of antifungal drugs - with special reference to the imidazoles - has been reviewed.[38,39]

An overview on the mode of action of 5-fluorocytosine (5-FC) has been published.[40] 5-Fluorocytosine (0.1 mg/ml) inhibits the germination of the conidia of A. fumigatus.[41] This may be inter-related with an effect of 5-FC on DNA synthesis, a pre-requisite for germination in A. fumigatus. DNA synthesis is not required by C. albicans for the production of hyphae from blastopores. As a consequence 5-FC does not inhibit hyphal formation from C. albicans blastospores.[41]

Amphotericin B was found to stimulate Na^+ transport across the rabbit corneal epithelium, loading the stromal compartment with NaCl.[42] In response to the NaCl gradient, water is translocated across the epithelium. Ouabain stops the active flux of Na^+ into the stromal compartment and hence the active water translocation across the epithelium.[42]

Morphological effects of ketoconazole on the yeast phase of H. capsulatum and P. brasiliensis include surface changes, abnormal membrane proliferation, degeneration of cytoplasm fat and lysis of subcellular organelles.[43] Ultrastructural changes in C. immitis, caused by miconazole and by ketoconazole,[44] are localized at the cell periphery and the vacuolar system; both drugs prevent the transformation of arthroconidia into mycelium. Studies with the concanavalin A-peroxidase method clearly showed the presence of a surface coat surrounding C. albicans.[45] It is thought that this coat may represent an external capsular or slime layer and might play an important role in the phagocytosis phenomenon and adherence capacity to host cells.[45] Factors responsible for the uptake of ^{3}H-miconazole may be associated with this cell wall coat, probably a glycoprotein,[46] that can be separated from the cell wall by treatment of the cells with pronase and mercaptoethanol.[45]

In T. mentagrophytes, C. albicans and C. parapsilosis naftifine induces morphological changes, presenting an intracytoplasmic accumulation of lipids. This fact suggests an interference with lipid metabolism.[47] Naftifine affects in C. albicans a decrease in ergosterol synthesis with a concomitant accumulation of squalene. This may be related to an inhibition of the conversion of squalene into 2,3-oxidosqualene and in addition an inhibition of the 4α-demethylation step.[48]

Ketoconazole affects, like miconazole, ergosterol synthesis in yeast cells resulting in an accumulation of C-14 methylsterols, known to disturb membrane and cell properties.[49] These effects were observed after 4 h of contact with ketoconazole concentrations as low as 10^{-9} M.[49] It is of interest to note that sterol synthesis in C. albicans grown in the same medium was already affected after 4 h of contact with 5×10^{-10} M of miconazole.[50] At miconazole doses up to 10^{-8} M and

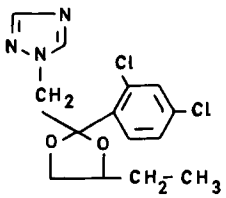
a contact time of 16 h, the fatty-acid pattern of the triglycerides, free fatty acids, sterol esters and phospholipids, revealed a shift from oleic acid (18:1) to linoleic acid (18:2).[51] This may reflect an attempt by C. albicans to increase membrane fluidity, compensating for the miconazole-induced alterations of ergosterol synthesis. However, at miconazole doses $>10^{-8}$ M an enrichment in shorter and saturated fatty acids (mainly palmitic acid) was found.[50] Similar results were obtained with ketoconazole (Van den Bossche et al., in preparation).

In the presence of ergosterol and an unsaturated fatty acid S. cerevisiae is able to grow anaerobically. At a concentration of miconazole and clotrimazole 30 to 60 times the usual aerobic minimal inhibitory concentration (MIC) these imidazoles are fungicidal both aerobically and anaerobically,[52,53] whereas ketoconazole is only active aerobically.[52] This may indicate that in addition to affecting aerobic ergosterol synthesis and fatty acid desaturation and elongation, miconazole and clotrimazole affect another target, whereas the basis for ketoconazole's antifungal activity seems to be only interference with both aerobic processes.[52,54]

Using differential scanning calorimetry it has been shown that high concentrations of miconazole shift the lipid transition temperature of multilamellar vesicles to lower values without affecting the enthalpy of melting; ketoconazole induces a broadening of the main transition peak only.[54] It is suggested that high doses of miconazole change the lipid organization without binding to the lipids, whereas ketoconazole is localized in the multilayer without a significant direct effect on lipid organization.[54] The miconazole-induced change in lipid organization may be at the origin of its inhibitory effect on cytochrome oxidase,[54] microsomal- and plasma membrane ATPase,[54,55] of the induced leakage of potassium ions[56,57] and of its direct effect on liposomes.[52,54,58]

It is of interest to note that due to the inhibitory effect of clotrimazole and triadimefon on ergosterol synthesis and the concomitant accumulation of ergosterol precursors, e.g., lanosterol, a "feed-back" inhibition of 3-hydroxy-methyl-glutaryl-CoA-reductase was observed.[59]

Other compounds that interfere with the demethylation at the C-14 position of the sterol nucleus are buthiobate[60] (Denmert ®) and etaconazole, 14.[61]



14

Tridemorph (N-tridecyl-2,6-dimethylmorpholine) also inhibits ergosterol synthesis, possibly at the conversion of fecosterol into episterol.[60]

In vitro [25,62] and in vivo[49] experiments have shown that cholesterol biosynthesis in mammalian cells is much less sensitive to miconazole and ketoconazole than ergosterol synthesis in C. albicans and S. cerevisiae.

Ciclopiroxolamine (Hoe 2L6) is an antifungal compound without effect on the permeability barriers of C. albicans protoplasts or lecithin liposomes.[63] It is presumed that the ciclopiroxolamine-induced growth inhibition and death of fungal cells is primarily caused by intracellular depletion of some essential substrates and/or ions and that such effects are brought about through blockage of their uptake from the medium.[63]

THERAPEUTICAL AND PHARMACOLOGICAL STUDIES - Failures with sulfonamide therapy in paracoccidioidomycoses can be associated with primary or "de novo" resistance of the infecting fungus.[64] In T. rubrum infections, griseofulvin MIC values ≥ 3 µg/ml have resulted in poor clinical effectiveness.[65] Hypersensitivity to miconazole, econazole and tolciclate has been reported in a patient.[66]

Oral candidosis in children[67,68] and adults[69] has been treated successfully with miconazole gel, even in the presence of predisposing factors.[69] Both clotrimazole troches and nystatin tablets are effective in oropharyngeal candidosis, however more nausea has been reported with nystatin.[70] Salivary concentrations of clotrimazole[71] and miconazole[72] above MIC values for Candida sp. are still present 2-3 hr after oral administration of a 10 mg troche or 5 ml 2 % gel respectively. Oral clotrimazole,[71] intravenous miconazole[73,74] and amphotericin B[74] are effective in candidal oesophagitis. A 13-year-old boy with candidal granuloma was successfully treated with oral miconazole.[75]

The clinical pharmacology of miconazole, econazole and clotrimazole in topical treatment has been reviewed.[76] A short-term, 3-day treatment of vaginal candidosis with miconazole,[77,78] econazole[79-87] and clotrimazole[83-86] has resulted in high and comparable cure rates; this schedule was equivalent to a 14-day regimen of nystatin.[88] Combined vaginal and vulval treatment of vaginal candidosis with econazole has resulted in high cure rates without mycological relapse over a 4-week observation period.[87] This schedule is very attractive in cases with a predictable recurrence risk,[87] e.g. pregnant women.[86] Boric acid powder capsules have produced the same results as nystatin in the treatment of vaginal candidosis.[89]

Miconazole tampons - a cosmetically attractive presentation that is more acceptable to patients[90-96] - give high cure rates, comparable to clotrimazole,[91,93,94,97] in vaginal candidosis. In pregnant women candidal vaginitis is frequently associated with Streptococcus agalactiae, which is often responsible[98,99] for the early onset of group B neonatal infections, as well as for perinatal pyrexia in the mother. In vitro assays showed miconazole to be highly active (MIC <3 µg/ml) against most strains of Str. agalactiae.[98,99]

Topical treatment of tropical dermatomycoses with haloprogin has shown the drug to be effective in candidiosis, Tinea cruris and Tinea pedis.[100] Infections of the scalp with Trichophyton soudanese and T. tonsurans are resistant to haloprogin treatment, and in cases of Pityriasis versicolor disappointing results have been obtained.[100]

Miconazole,[101], econazole,[102,103] clotrimazole,[102,104], tolciclate[101,105] and naftifine[104] are equipotent in dermatophytoses, the imidazoles still being the first choice in Candida dermatomycoses. A long-term follow-up study in patients with dermatomycoses, treated with topical econazole, has indicated low relapse rates.[106] Miconazole[107] and econazole[108] cream have been effective in erosive candidosis,

caused by C. albicans[107] and tinea caused by Microsporum gypseum,[108] respectively.

Patients with T. rubrum, T. mentagrophytes, T. verrucosum[109] and C. albicans[110] infections have been treated with oral ketoconazole[109] and topical econazole[110] and skin samples have been investigated. In these skin preparations, the ultrastructural changes in the parasites closely resemble the degenerative changes seen following in vitro exposure of the parasites to the drugs.

Intravenous miconazole has been able to cure a severe candidosis of the eye[111] and to improve a Torulopsis glabrata retinitis.[112] In endogenous coccidioidal endophthalmitis,[113,114] amphotericin B may be more effective than miconazole. Topical and subconjunctival miconazole has been successfully used in seven patients with keratomycosis, caused by C. albicans and Aspergillus sp.[115] Fusarium keratomycosis responds moderately to either miconazole, pimaricin and amphotericin B or to drug combinations of miconazole and the two polyenes.[116]

In cutaneous cryptococcosis beneficial therapeutic results have been obtained with a combination of amphotericin B and 5-FC,[117] miconazole[118] and ketoconazole.[119] The value of intravenous and intraventricular miconazole has been confirmed in cryptococcal meningitis.[120-125]

Clinical isolates of Phialophora richardsiae, resistant to 5-FC, miconazole and amphotericin B, are found to be susceptible to cycloheximide.[126]

Following amphotericin B treatment, failures have been reported in patients with invasive Trichosporon beigellii infections.[127] In one responding patient lesions were resolved following treatment with amphotericin B and 5-FC and oral ketoconazole was administered to prevent relapse.[128] Intravenous amphotericin B has been effective against Kluyveromyces fragilis infections,[129] disseminated candidosis,[130] cerebral mucormycosis[131] and blastomycotic meningitis;[132] however, in the latter disease therapy failures were also reported.[132] Intracavitary amphotericin B may be beneficial in symptomatic pulmonary aspergillosis, particularly when surgical resection is not feasible.[133] A pregnant woman with coccidioidomycosis[134] and a case of disseminated sporotrichosis,[135] refractory to potassium iodide and to ketoconazole, has responded to amphotericin B. Oral histoplasmosis[136] has successfully been treated with intravenous miconazole, while in coccidioidomycosis[137] the results are precarious.[138] Miconazole therapy is favourable against Candida septicaemia,[139] cutaneous alternariosis[140] and peritonitis[141] due to Alternaria sp.[140,141] Miconazole cream is effective against tinea nigra palmaris caused by Exophiala werneckii.[142]

The antimycotic activity of nystatin[143] is reduced by strongly acidic gastric juice (pH < 2). Overall mortality in immunocompromised patients with Candida fungaemia is high despite amphotericin B treatment.[144] Sodium depletion[145] enhances the nephrotoxicity of amphotericin B. Leukocyte transfusions combined with amphotericin B may be associated with severe pulmonary reactions.[146]

Evidence continues to be amassed on the effectiveness of ketoconazole in the treatment of both superficial and disseminated fungal infections. Recent developments in antimycotic research with ketoconazole has

been reviewed.[147-149] The bioavailability of ketoconazole is dose related[150,151] and average plasma levels in 40 patients following intake of an oral dose of 200, 400 and 600 mg/day are 2.67, 3.42 and 4.77 µg/ml respectively.[150] For optimal resorption of the drug, administration with food[147] or orange juice[152] is advocated to enhance gastric acidity.

Oral ketoconazole gives high cure rates in dermatophyte[153,154] and yeast[147,148,155] infections of the skin, in pityriasis versicolor,[147,148,156] in onychomycosis,[147,148,157] in oral candidosis[147,148] and in vaginal candidosis,[147,148] and has been able to clear griseofulvin resistant dermatophyte infections.[158] Double-blind studies with ketoconazole and griseofulvin indicate ketoconazole to be at least as effective[159] as - but to act faster than griseofulvin in dermatophyte infections of the skin[147,158] and nails.[134] The clinical outcome of ketoconazole in chronic mucocutaneous candidosis[147,160-163] is favourable. Encouraging clinical responses are obtained in systemic candidosis,[147,164,165] oesophageal candidosis,[147] paracoccidioidomycosis,[147,165,166] disseminated and pulmonary histoplasmosis,[147,164,166,167] aspergillosis,[147,168,169] Alternaria infection,[147] chromoblastomycosis,[147,165,170,171] cryptococcosis,[147] blastomycosis,[171] and sporotrichosis.[147,171]

No relapses or development of active mycoses has been observed in follow-up studies (12 months or more) of patients with paracoccidioidomycosis, treated with a complete course of ketoconazole.[172] Ketoconazole has been successfully used in a patient with petriellidiosis.[173] Encouraging results with ketoconazole are reported in patients with coccidioidomycosis.[147,174] In predisposed patients, prophylactic ketoconazole has been more effective than placebo and the polyenes amphotericin B and nystatin in reducing the incidence of fungal infections.[175-178]

In general ketoconazole is well tolerated. A multicentre-study of 2500 patients with various fungal infections have shown that ketoconazole is both effective and without significant adverse effects.[1,179] Signs of hepatitis have been reported in two cases.[180,181] However, based on a careful analysis of liver function tests and enzymes it can be stated that the incidence of this adverse reaction must be very low.[147,180,182]

CONCLUSION - During the last two years, antifungal chemotherapy has made major progress. The introduction of many topical antifungal agents and of an orally absorbed broad-spectrum antifungal drug represent important advances in antifungal chemotherapy.

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Chapter 16. Interferon Inducers

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Introduction - Research on the interferon system is now beginning its third decade. The past twenty years of research have demonstrated that interferons are glycoproteins found in many cell types, whose production can be induced by a chemically diverse group of agents, and whose activities include antiviral, anticancer, and immunomodulatory, among others. Clinically, exogenous interferon has been examined as a prophylactic antiviral agent against such localized infections as vaccinia, rhinovirus, and influenza and therapeutically against vaccinia, herpetic keratitis and genital herpes. Systemic viral infections including varicella-zoster, cytomegalovirus, and chronic hepatitis-B have also been challenged with interferon. Interferon therapy has been administered to patients with acute leukemia, recurrent breast cancer, multiple myeloma, juvenile larynx papilloma, and osteosarcoma. Apparent success in many of these early studies has stimulated more extensive multicenter studies, which are ongoing, to more fully establish the clinical utility of interferon.

Interferons and the interferon system have received in-depth review and cogent analysis in several recent books.[1-3] Recent issues of this series have included comments on interferons under the topics of antiviral agents[4] and immunostimulants.[5] Recent, general reviews of interferon inducers have also appeared.[3,6-10] The purpose of this review is to update and highlight the area of interferon inducers with particular emphasis on low molecular weight inducers. Since this has not received recent emphasis in this series, some historical development will be included. The more general topic of biological response modifiers or immunomodulators will not be covered.

The Induction Process - Uptake of virus by a host cell begins the viral infection process of uncoating the protein coat of the invading virus. The viral nucleic acid (RNA or DNA) can then be reproduced, stimulate translation of more protein, and thus the replication of more viral particles. It can also stimulate the mRNA of the host cell for translation into interferons. How a non-viral "interferon inducer" initiates this process is poorly understood and may not even involve penetration by the inducer to initiate interferon synthesis. The interferon is then secreted and interacts with a neighboring cell membrane to trigger, presumably by a derepression process to initiate mRNA synthesis, the synthesis of at least three proteins that act to prevent viral reproduction in that host cell after viral infection occurs.[36] One protein phosphorylates an initiating factor utilized in the synthesis of viral protein. A second protein catalyses the formation of the pppA2'p5'A2'p5'A trimer from the ATP which activates an endonuclease for RNA cleavage. Thus apparently this protection process proceeds at both the viral nucleic acid level and protein synthesis with a specificity for viral rather than host cell protein synthesis. Other factors involved in protection effected by interferons include activation of macrophages, production, activation, and maturation of T and B lymphocytes, and natural killer (NK) cells. Thus, the release

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of interferon appears to offer the possibility of host resistance of a very broad scope. Lastly, high interferon levels appear to effect the appearance of a protein inhibitor for further interferon synthesis, perhaps through suppression at the mRNA level.

The complexity of the interferon system is further underscored by the recognition of the multiplicity of types of interferon and the apparent heterogeneity of these types. Many types of interferons can be produced depending on the species, tissue, cell-type, and inducer. In the mouse and human interferon systems, which have received the most study thus far, two basic categories of interferons have been loosely classified (Table I). The type I or viral induced interferons are the most widely studied. Type II interferon, produced as a lymphokine in a CMI response or by mitogen stimulation (e.g., PHA, CON-A), differs from Type I physicochemically (e.g., pH and thermal stability), biologically, and antigenically. For example, immune (γ) interferon exhibits delayed kinetics in inducing an antiviral state relative to viral induced interferon.

Additional evidence for heterogeneity of interferon type was disclosed recently by the isolation of a multigene family for human leukocyte-derived interferons.[12-14] These discoveries will most certainly allow not only generation of homogeneous interferons, but also provide mechanisms to prepare analogs.[15-17,57]

TABLE I: Classification of Interferons

TYPE I (Antiviral)	TYPE II (Immune) [γ]
1) Antigenic	1) Antigenic
- fibroblast derived [β]	2) Induced
- leukocyte derived [α]	- antigen
2) Induced	- mitogen
- virus	
- polymers (nucleic acids)	
- low molecular weight compounds	

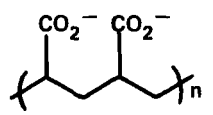
There exists a diverse and expanding group of substances which induce interferon.[18,19] These have been generally classified into high molecular weight and low molecular weight inducers. In some cases the interferons induced have not been classified according to type. However, one phenomenon is common to all inducers and that is hyporeactivity. This involves the loss of an interferon response on the part of the host upon sequential administration of an inducer.[3] The onset and degree of hyporeactivity is inducer dependent.

High Molecular Weight Inducers - In 1957 Isaacs and Lindenmann employed the first known interferon inducer, heat-inactivated influenza virus, in demonstrating the existence of interferon as a factor transferring viral resistance.[20] Since these early studies a number of substances have been reported to induce interferon including viruses, bacterial and fungal organisms and products, nucleic acids, polymers, and mitogens (Figure I).[2,3] The number of viruses inducing interferon now includes every major virus group: single- and double-stranded RNA viruses as well as DNA viruses.[19,21] A variety of microorganisms and their products have also been reported as interferon inducers including fungi and fungal

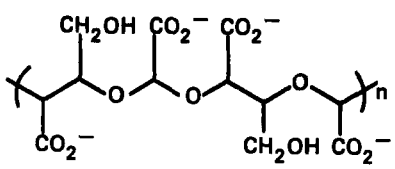
Figure I: High Molecular Weight Interferon Inducers

NATURAL PRODUCTS	EXAMPLES
Viral	
- RNA	influenza A
- DNA	varicella-zoster
Fungi	
- mycophage	Asperigillus foetidus
- extracts	Candida albicans
Bacteria	
- intact	Staphlococcus aureus
- products - endotoxin	Brucellus abortus
Chlamydia	conjunctivitis agents
Rickettsiae	
Mycoplasma	M. arthritidis
Protozoa	Plasmodium berghei
Mitogens	PHA
Antigens	Bacterial BCG

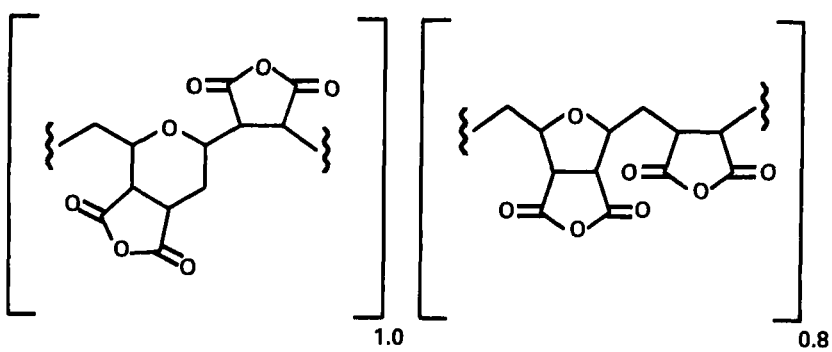
SYNTHETIC POLYMERS	STRUCTURES
Polyvinylsulfate	
Dextran phosphate	
Pyran	
Polyacrylic acids	
Chlorite oxidized oxyamylase (COAM)	
Poly IC (poly rI•poly rC)	



Polyacrylic acid



COAM



Pyran

extracts, intact bacteria and bacterial products such as endotoxins and lipopolysaccharides. Also trachoma-inclusion conjunctivitis agents, rickettsia, mycoplasmas, and various protozoa have demonstrated interferon induction both in animals and cell culture. Substances which induce lymphocyte blastogenesis such as phytohemagglutinin, conconavalin-A, pokeweed mitogen, and mycobacterium tuberculosis BCG also induce interferon, and, in the latter example, apparently type II interferon.[22]

There is another group of large molecular weight substances which are synthetic in origin. One subgrouping in this category is anionic polymers which include pyran, polyacrylic acids, chlorite oxidized oxyamylose (COAM), polyvinylsulfate, dextran phosphate,[23,24] and fractions of sulfated polygalactan (carrageenan).[35]

Most of these ionic polymers have been reported to induce circulating levels of interferons in mice, and several have been examined in humans. These agents also are antiviral, antimicrobial, antitumor and immunomodulatory and these activities were not always correlated with circulating interferon. The best studied agent is the maleic anhydride-divinyl ether copolymer -- pyran.[25] With pyran the antiviral and interferon inducing capacities increase with increasing molecular weight of the polymer. Unfortunately the toxicity parallels these activities.[26,104] Efficacy in a clinical study with advanced cancer patients was limited by toxic side effects.[27,30] The maleic anhydride-divinyl ether copolymer, pyran, is a preparation that contains polymers with a broad distribution of molecular weight. Its structure has recently been revised.[31] Since most of the biological properties are molecular weight associated, more recent work has focused on preparations of controlled molecular weight. Results with a copolymer of average molecular weight of 15,000 called MVE-2 show a diminished toxicity relative to effects on the reticuloendothelial system (RES), interferon induction, antiviral, and antitumor activity.[28,29,32,33] The sodium salt of MVE-2 is now in Phase I clinical trials in advanced cancer patients. Preliminary results include enhanced immunological responses (e.g., ADCC assays, NK cells) with good tolerance.[34] Interferon was not detected.

A second group of synthetic, large molecular weight interferon inducers is polynucleotides. The initial discovery of viral interference (interferon) suggested that nucleic acids may act as inducers of interferon. It was subsequently observed[37] that natural and synthetic polyribonucleotides did act as immunomodulators[39] and induce interferon, and, particularly that the double-stranded homopolyribonucleotide of inosine and cytidine (poly I:C) was a potent inducer.[38] This stimulated a substantial research effort into structure-activity requirements.[23,41] It appears that necessary but not exclusive requirements for effective induction include high molecular weight ($>2 \times 10^5$), a stable secondary structure with a Tm > 60°, double-strandedness,[40] a 2'-hydroxyl, and some resistance to nucleases.

Several examples of maintaining the necessary structural requirements and enhancing the stability of poly I:C include the substitution of the 2'-hydroxyl of the polyriboionisinic acid with a fluorine or chlorine.[44] Conformational hypotheses of polyribonucleotides correlating with interferon induction have been offered including requirements for type A structures (C-3' endo)[42] and other intrinsic glycosidic orientations.[43] The addition of poly L-lysine and CMC to poly I:C, called poly ICLC, also fits the apparent structural and stability requirements.[45] Poly I:C and most analogs, although they are the most potent interferon

inducers known, also exhibit toxicological problems such as pyrogenicity, leukopenia, and hypotension.[46] Poly I:C and poly ICLC exhibit antiviral and immunomodulatory activities and are being examined for interferon induction in terminal cancer patients (Max. tolerated dose ~ 350 μg/kg ⟹ 2000 U/ml serum interferon) and in a herpes genitalis study via topical application.[45] Poly I:C and poly ICLC are pleiotropic; they are effective adjuvants with weak vaccines -- with strong antigens they inhibit Ab production. Poly ICLC has passed the DN2A level at the NCI and is currently in patients with acute lymphocytic leukemia, laryngeal papilloma, multiple myeloma, and recurrent breast cancer.

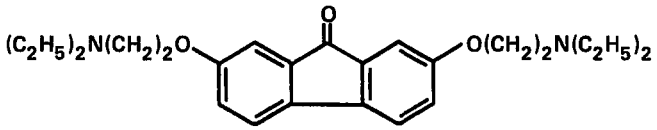
An alternate approach to minimize the toxicity of poly I:C has been to prepare analogs which would be more susceptible to RNase activity. One example of these analogs is a poly $rI_n:r(C_{13}U)_n$,[47] a so-called mix-matched analog wherein uridylic acid has been incorporated in the polyribocytidylic acid in a ratio of 1/13. It is indeed less resistant to nuclease activity and less thermally stable.[48] It appears to induce interferon and has less pyrogenicity and lymphopenia in rabbits and rodents. Preliminary Phase I-II clinical results in cancer patients indicated fever as the only significant side effect and positive immunoaugmentation including interferon induction and antitumor effects.[49]

Low Molecular Weight Interferon Inducers - Although therapeutically useful agents may yet come from some of these classes of large molecular weight interferon inducers, such as pyran or modified poly I:C, problems associated with toxicity, antigenicity, lack of oral activity, economics, and other factors have stimulated a search for other agents. That low molecular weight compounds might be possible candidates as interferon inducers was suggested by early work on two low molecular weight natural products, cycloheximide[50] and kanamycin.[51] In fact, these agents (RNA or protein synthesis inhibitors) can delay hyporesponsiveness to interferon induction[52] and thus prolong interferon production (referred to as superinduction).[53]

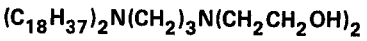
In 1970 a fluorenone, named tilorone, was reported to induce interferon.[56] Since that time a variety of low molecular weight compounds have been reported as interferon inducers. Some of these are shown in Figure II. There are other structural subgroups not depicted, which include antibiotics such as streptimidone, thiazines such as methylene blue, 5-methyltryptamine, and alkylamino thiaphosphates.[54,55] The diversity of structures suggests multiple mechanisms of action. Of these compounds, only tilorone, the diamines (CP-20,961 and CP-28,888) and a pyrimidinone have reached clinical trials as interferon inducers (the antibiotics, quinacrine, and methylene blue have been in man for other purposes; interferon levels were not monitored).[55]

Perhaps the most studied to date is tilorone and its analogs, of which over 800 have been synthesized.[58,104] Tilorone induces high levels of interferon in mice when administered orally, i.p., or s.c. Both spleen and thymus contained high levels of interferon. However, neither tilorone nor its analogs induced interferon in monkeys, rabbits, cats, dogs, or in man. Tilorone does exhibit positive effects on antibody and CMI responses[59] and exhibits antitumor activity in the rat mammary adenocarcinoma model (p.o.).[60] Similarly, protection is afforded against murine B16 melanoma in mice.[61] Activity was attributed both to cytotoxic effects (DNA intercalation?) and enhanced macrophage function (interferon?). Oral administration to advanced cancer patients (Phase I-II) caused nausea and reversible corneal inclusions; some clinical response was noted in melanoma patients. Intraocular administration caused a decrease in visual acuity.[62] Interferon was not detected. Suggested future uses include adjuvant therapy.[104]

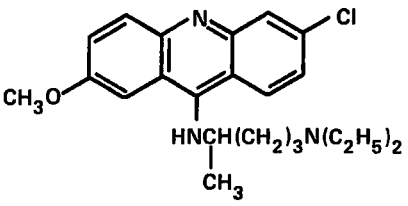
Figure II: Low Molecular Weight Interferon Inducers



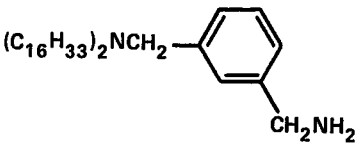
Tilorone



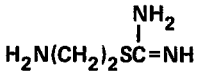
CP 20,961



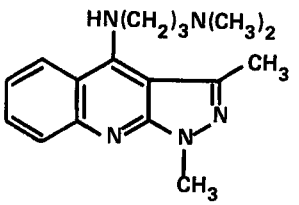
atabrine



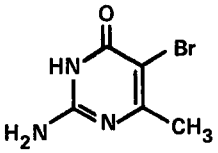
CP 28,888



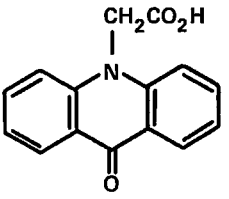
(s, 2-aminoethylisothiouronium)



BL 20,803

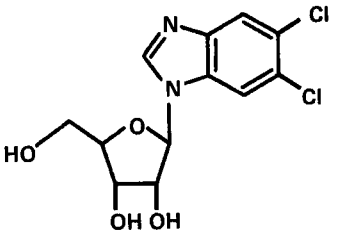


ABMP



10-carboxymethyl-9-acridone

carboxyethyl germanium sesquioxide



DRB

Although tilorone exhibits DNA intercalation effects, which it shares with other structurally related, tricyclic interferon inducers, it is not clear this is part of its mechanism for induction of interferon. Related compounds include a series of pyrazolo-quinolines, represented by BL20,803. These induced interferon in mice when administered i.p. or orally. This was correlated with antiviral activity against vaccinia; the mediation of macrophages was implicated.[63-65] Similarly, acridines such as atabrine and acranil induced interferon, albeit at lower levels, when orally administered to mice.[66] A series of substituted 1,5-diaminoanthraquinones exhibited antiviral activity against encephalomyocarditis virus (EMC) and induced interferon when given orally, i.p. or s.c. to mice.[67] Recently, 10-carboxymethyl acridinone was reported to induce high titers of circulating interferons in mice by several routes of administration as well as to protect against RNA and DNA viruses including Japanese encephalitis virus.[68-69] No other data have as yet been forthcoming regarding these various tricyclic interferon inducers to establish any clinical utility.

A structurally unrelated class of low molecular weight interferon inducers, represented by a propanediamine, CP-20,961, and a xylylenediamine, CP-28,888, were reported to be inducers.[70] The former compound was reported to induce interferon in mice i.p., in humans when given intranasally, and appeared to have an effect on rhinovirus infections in man. The latter compound, although a more potent interferon inducer in mice, was less active in man and devoid of antirhinovirus effects.[71-73] CP-20,961 exhibits immunoadjuvant activity for humoral and cellular immune responses with efficacy as an adjuvant in monkeys with a merozoite antigen.[74] Both diamines have received some evaluation as antitumor agents;[75] in vivo data in sarcoma 180J-bearing mice (i.p.) showed moderate increase in life span and examination (i.v.) in a murine pulmonary metastasis model showed reduction and/or prevention of lung metastases. CP-28,888 enhanced the activity of cyclophosphamide (mice, P388 lymphoma) and L1210 leukemia vaccine in combination therapy. Analogs studied had lower interferon induction but often retained significant antisarcoma activity.[75]

Several halogenated benzimidazole ribosides have been reported as interferon inducers.[76-77] The 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) is a representative structure; it exhibits antiviral activity (influenza) and inhibits RNA synthesis and cell proliferation (reversible). Cells pretreated or primed with interferon showed enhanced interferon induction in the presence of DRB (super-induction).

An intriguing carboxyethyl germanium sesquioxide[78] (Ge-132) was recently disclosed as an oral interferon inducer with low toxicity[79] (six month chronic toxicology in mice and dogs). Although only moderate titers of serum interferon were seen in mice, the interferon was determined to be of the immune (γ- or type II) type in contrast to all other low molecular weight interferon inducers. Antitumor activity (Ehrlich ascites) in mice with the peritoneal exudate cells from germanium sesquioxide-treated mice was also demonstrated (increase in % ILS and % survivors), as well as antimetastatic activity in Lewis Lung carcinoma in mice.[80] Preliminary human tolerance studies in healthy volunteers (single dose, oral, 25 and 75 mg/kg) showed no side effects with Ge-132 and low, but dose-related serum interferon levels.[81]

In 1976, 2-amino-5-bromo-6-methyl-4(3H)-pyrimidinone (U-25,166; ABMP) was reported to induce interferon in mice, rats, and cats (p.o. and i.p.).[82] Although it compared favorably with poly I:C and tilorone in a

competitive study, with the added advantage of a slower onset of hyporeactivity,[83] it exhibited a toxicity-limiting crystal deposition in the renal papillae of rats upon chronic administration.[86] A second-generation 6-phenyl analog (ABPP) exhibited increased antiviral and interferon-inducing capabilities without the aforementioned toxicity.[84] An expanded SAR study disclosed that (1) there was no correlation with interferon induction and general antiviral activity [e.g., the 5-iodo-6-phenyl analog (AIPP) exhibited good antiviral activity but poor interferon induction relative to ABPP]; (2) maintenance of either activity precluded analog substituent changes from the parent compound (ABPP) at positions 1-4 of the pyrimidine ring; (3) alkoxy and halogen-substituted 6-aryl analogs optimized antiviral activities; and (4) a steric parameter appeared to be the controlling element for the substituent at C-5.[85]

ABPP and/or AIPP exhibited antiviral activity against RNA viruses such as Semliki forest and encephalomyocarditis viruses in mice (i.p., p.o., s.c.)[87] and influenza A and parainfluenza-3 in hamsters (i.p.). Activity against DNA viruses include: herpes simplex type-1 (i.p., mice), type-2 (i.vag., mice and guinea pigs), cytomegalovirus (i.p., mice), pseudorabies (i.p., mice),[88] and infectious bovine rhinotracheitis virus (i.n., calves).[89] Serum interferon responses were noted with i.p., p.o., s.c., i.m., or i.n. administration of ABPP and some analogs (not AIPP) in mice, rats, cats, dogs, and calves, and in human tonsillar tissue cocultured with confluent monolayers of human amnion cells.[94] Generally these pyrimidinones do not exhibit antiviral activity *in vitro* although some interferon can be detected.[93] Duration of an antiviral effect upon a single i.p. dose of AIPP and selected analogs was up to two weeks.

The antiviral activity of ABPP or AIPP against HSV-1 could be abolished by addition of anti-thymocyte serum, implicating a T-lymphocyte role. Immunomodulatory activities of ABPP and selected analogs (including AIPP) include enhanced background antibody formation (polyclonal Ab to SRBC), inhibition of the inflammatory phase of the GVH reaction, enhanced natural killer cell (spleen) and macrophage activity, reduced response capacity of spleen cells to alloantigens, and increased colony-forming unit potential of bone marrow. They are non-mitogenic.[90-92]

The pyrimidinones also exhibited antitumor activity against P388 leukemia and B16 malignant melanoma in mice[95,97] (i.p.). The activity was tumor-load dependent; weekly administration post tumor inoculum was as effective as daily. Synergistic effects were noted in combination therapy with surgery or cyclophosphamide (B16 melanoma). Good activity was also reported in several artificial lung metastases in mice.[96] ABPP is currently in Phase I clinical trials in patients with advanced cancers. Various immune parameters are being monitored including interferon response and NK cell activity.

An alternative approach to producing interferon related effects is the use of 2-5A (2'-5'-pppApApA) analogs as "interferon mediators". The goal is the preparation of metabolically stable 2-5A mimics which would activate a resident endoribonuclease and consequently inhibit protein synthesis.[102] Although inhibition of translation has been attained, recent results indicate that 2,5-A synthetase activity does not correlate directly with antiproliferative activity.[103]

SUMMARY - The role for interferon in anticancer, antiviral, and immunotherapy is still in the early stages of being defined. That it is not a panacea for cancer is evident from several recent editorials[98,99] and

updates.[100,101] Although the role of interferon inducers is linked to interferon, their other activities have to be taken into consideration in interpreting their actions. The augmentation of various immune functions unrelated to interferon, resulting in antiviral and antitumor effects is a potential advantage over direct interferon therapy. Economics, oral activity, and acceptable bioavailability/pharmacokinetics are other, currently advantageous characteristics of interferon inducers. The apparent drawback of hyporeactivity may be overcome by allowing longer intervals between successive administration of the inducer.[97]

The biological data available on both high and low molecular weight interferon inducers is becoming substantial and impressive in terms of antiviral, antitumor, and immunoregulatory effects. Translation of this into analogous clinical effects is just beginning. The preliminary clinical results with MVE-2, modified poly IC, and several low molecular weight inducers support an optimistic perspective regarding prophylactic, broad-spectrum antiviral therapy and combination antitumor therapy.

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Chapter 17. Antineoplastic Agents

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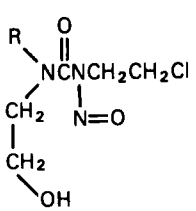
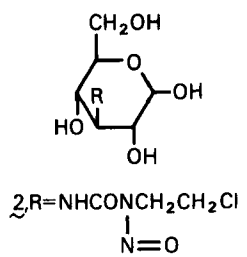
Introduction - A continued progress in the understanding of the mechanism of action of antitumor drugs has resulted in the rational design of new structures and in the improved utilization of the available arsenal of drugs during 1981. The proceedings of a symposium on the molecular actions and targets for chemotherapeutic agents have been published.[1] Biochemical programs of cancer cells which could aid the medicinal chemist in the design of enzyme-targeted inhibitors continued to be unravelled. The activities of purine and pyrimidine enzymes in slowly and rapidly growing human colon carcinoma tumors have been studied.[2] The similarities between the *in vitro* and *in vivo* enzymic profiles should expedite the use of appropriate cell culture systems as predictive models for *in vivo* response.[3] The chemotherapy of inhibitors of the *de novo* pyrimidine pathway has been reviewed[4] and an update on the biochemistry of 5-FU has been published.[5] Knowledge of the mechanisms of chemical carcinogenesis also represents a useful avenue of research for the prevention and treatment of cancer as discussed in two important reviews.[6,7] Liposome encapsulation of antitumor drugs continues to be used extensively. The prospects of this method for selective drug delivery to tumor cells have been evaluated.[8] Nature's unending versatility of chemical architecture continues to be an important source of drugs and ideas for drug design. The structures and preclinical activities of 26 potential antitumor agents from natural products have been reported[9] and the NCI's program for testing new antitumor natural products has been reviewed.[10]

Alkylating Agents - A new class of very potent nitrosoureas (1) showed therapeutic ratios 3-5 times greater than that of CCNU against L1210 leukemia in mice. The β-hydroxyl group activates the molecule non-enzymatically by intramolecular cyclization, without the generation of isocyanates.[11]



1, R=alkyl



2, R=NHCONCH$_2$CH$_2$Cl
|
N=O

A chlorozotocin analogue (2) with low bone marrow toxicity was curative at doses between 20-80 mg/kg in S180 and leukemic mice after a single injection.[12] C-13 and N-15 NMR, as well as O-18 exchange studies of labelled BCNU and CCNU, revealed the formation of a tetrahedral intermediate resulting from hydration of the carbonyl group. The conformation of this intermediate controls the formation of decomposition products that are ultimately responsible for activity.[13] A postulated intermediate (3) in the decomposition pathway of chloroethylnitrosoureas has been

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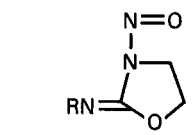
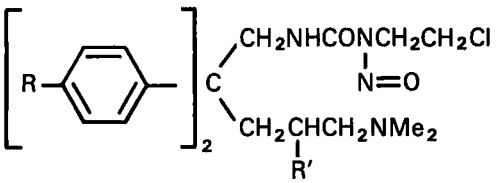
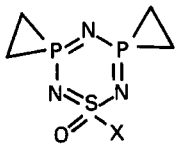
synthesized and studied.[14] The metabolism of BCNU by rat liver microsomes revealed that inactivation by denitrosation was the major pathway.[15] The possible role of nitrosoureas as having immunoassociated cancerostatic properties may be related to their carbamoylating activity.[16] Several novel lipophilic nitrosoureas (4) with superior L1210 antitumor activity over that of BCNU, CCNU and MeCCNU were developed.[17]



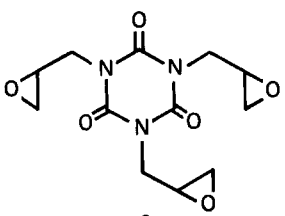
3, R=C_6H_{11}, p-MeC_6H_{10}, CH_2CH_2Cl



4, R=H,F
R'=H,Me



5a, X=N
b, X=C_6H_5
c, X=F



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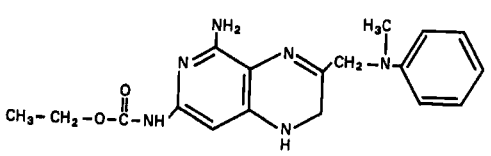
The synthesis of 3-hydroxycyclophosphamide and its role in the metabolism of cyclophosphamide (CPA) was studied. Its activity against L1210 was comparable to that of CPA.[18] Trans 5-F- and 5-Cl-CPA, which were expected to be more efficiently metabolized to the phosphoramide mustard, failed to increase the yield of the active metabolite after microsomal hydroxylation.[19] Derivatives of the thiatriazadiphosphorine ring system exhibited significant activity against L1210 and P388 leukemias. Following a single injection of a dose of 200 mg/kg, the aziridino derivative (5a) produced ILS values of 195% with 40% cures against P388 leukemia in mice.[20] Phase I clinical trials were initiated with α-1,3,5-triglycidyl-s-triazinetrione (6), in view of its lack of cross-resistance with CPA.[21]

Folic Acid Antagonists - Resonance Raman spectroscopy and C-13 NMR have been used to study the mode of binding of methotrexate (MTX) to dihydrofolate reductase.[22,23] Improved MTX therapy was achieved with probenecid via inhibition of MTX efflux from tumor cells.[24] The dianilide and dihydrazide derivatives of the glutamate side chain of MTX showed promise as MTX prodrugs due to their lower toxicity.[25] Bridge elongation of the C^9-N^{10} region of active 10-deazaaminopterin and 10-oxaminopterin produced analogues with lower activity than MTX.[26] A new synthesis of 8-deazafolic acid was reported.[27] 5,11-Methenyltetrahydrohomofolate, a suspected metabolite of homofolate, was



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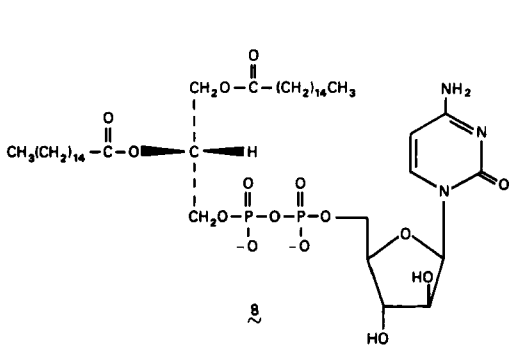
synthesized. This compound may be responsible for inhibition of de novo purine synthesis.[28] Antimitotic activity similar to that of vincristine has been reported for the 1-deaza-7,8-dihydropteridine derivative 7. The compound belongs to a new class of mitotic inhibitors with activity against P388 strains resistant to vincristine.[29]

Purine and Pyrimidine Antagonists - 5-FU continued to be an intense focus of attention both in combination chemotherapy and as a model for prodrug synthesis. In combination with N-(phosphonoacetyl)-L-aspartate (PALA), increased amounts of 5-FUTP were formed via the salvage pathway.[30] Recent Phase I clinical trials support further evaluation of this combination.[37] Enzymatic cleavage of 1-(tetrahydro-2-furanyl)-5-FU (ftorafur) catalyzed by thymidine phosphorylase represents a possible mechanism of activation of this 5-FU prodrug.[32] The metabolic pathway of 1-hexylcarbamoyl-5-FU proceeded via oxidation and scission of the side chain with final release of 5-FU.[33] 1-Methoxycarbonylmethylcarbamoyl-5-FU was shown effective in mice by oral administration. The compound produced ILS values of more than 30% against L1210 when given orally at 100 mg/kg.[34] The antitumor activity of methyl 1-(5-fluoro-1H-2-oxopyrimidin-4-yl)-β-D-glucopyranouronate was found superior to that of 5-FU and ftorafur. Daily injections (30 days) of 400 mg/kg of the drug produced ILS values of 92% without marked loss of body weight.[35] The 5'-O-glucuronide of 5-fluorouridine (5-FUrd) was prepared as a 5-FUrd prodrug selective for tumor tissues rich in β-glucuronidase activity.[36] Carbocyclic analogues of 5-FU-nucleosides were synthesized to be more selective than 5-FUrd and 5-FdUrd and to avert liberation of 5-FU by the phosphorylases. These compounds showed borderline activity against P388 in vivo.[37] Two syntheses of the acyclonucleoside derivative of 5-FU have been reported. The two reports disagree as to the activity of the compound against L1210 leukemia.[38,39] A facile synthesis of 5-alkynyl-2'-deoxyuridines allowed a thorough evaluation of these compounds as thymidylate synthetase inhibitors.[40] Ara-FUMP also behaved as a powerful inhibitor of this enzyme.[41] Ara-C was a subject of much attention when used in combination and as a candidate for prodrug design. In combination with cis-dichlorodiammineplatinum (DDP), it produced a dramatic increase in lethality over DDP alone. This effect may have resulted from inhibition of the DNA repair mechanism.[42] Sustained release of ara-C by gradual hydrolysis of N^4-palmitoyl ara-C provided sufficient cytotoxic levels of the drug.[43] The ara-C prodrug (8) showed increased efficacy relative to ara-C irrespective of the schedule used. The compound resisted deamination by cytidine deaminase (CDA).[44] The synthesis of 9-deazaadenosine (9), a C-nucleoside isostere of adenosine, was achieved. The compound possessed marked cytotoxicity against several mouse and human leukemia cell lines.[45] The pyrazolopyrimidine nucleoside 10, which resisted deamination by adenosine



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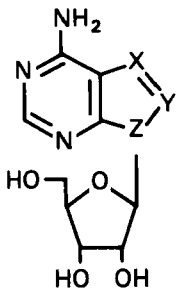
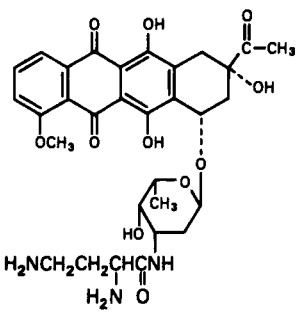


9, X=N Y=Z=C

10, X=C–CONH$_2$ Y=Z=N

deaminase (ADA), produced values for ILS of L1210-bearing mice as high as 135% with long term survivors.[46] The ara-A analog arazide (2'-azido-2'-deoxy-ara-A) proved more water soluble than ara-A and its activity was superior to that of the parent drug, especially in combination with ADA inhibitors.[47] The 2-chloro and 2-bromo analogues of 2'-deoxyadenosine were good inhibitors of cell growth in vitro. Their activity did not increase with ADA inhibitors.[48] The 7-deaza analogue of 5'-methylthioadenosine (MTA) was an inhibitor of MTA phosphorylase with superior inhibition of cell growth relative to other MTA analogues that behaved as substrates for this enzyme.[49] Selective killing of cells deficient in MTA phosphorylase can be achieved by inhibition of de novo purine synthesis with MTX.[50] 5'-[(Bromoacetyl)amino]-5'-deoxyinosine, designed as an active-site-directed inhibitor, showed significant cytotoxicity against H.Ep-2 cells.[51] The cordycepin oligonucleotide (2'-5')ppp3'dA-(p3'dA)n was synthesized as a metabolically stable (2'-5')pppA(pA)n analogue. These oligonucleotides inhibit mitogen-stimulated DNA synthesis in a manner similar to interferon.[52]

Anthracyclines - The semiquinone radicals of adriamycin (ADM) and radicals of the reduced forms of ADM do not need to intercalate to produce DNA strand breaks.[53] A mechanism other than direct DNA intercalation may play an important role in the action of non-DNA binding derivatives of ADM.[54] Replacement of daunosamine by non-basic sugar moieties linked to carminomycinone, daunomycinone and ε-pyrromycinone reduced the activity against L1210.[55] A qualitative SAR study of doxorubicin and aminoanthraquinone derivatives revealed the importance of the N-O-O-triangular pharmacophore.[56] 5-Iminodoxorubicin showed comparable efficacy to 5-iminodaunorubicin against P388 leukemia but the dose required was ten times greater.[57] Improved antitumor activity and reduced toxicity was achieved with basic amino acid and dipeptide derivatives of daunorubicin. The diamino butyric acid derivative 11 was the most effective of these compounds producing 80% cures in EL4 lymphoma tumor-bearing mice.[58] Aclacinomycin A, a compound less cardiotoxic than ADM,was studied in Phase I clinical trials.[59]



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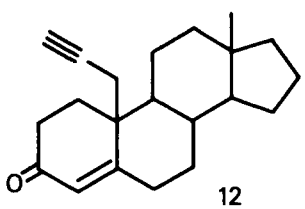
Aminoacids and Peptides - α-Difluoromethylornithine (DFMO), an ornithine decarboxylase inhibitor, produced G_1-phase arrest in normal cells, whereas their transformed counterparts accumulated in S-phase. Further treatment with S-phase specific agents (i.e. ara-C) resulted in preferential synergistic killing of transformed cells.[60] Significant increases in ILS were obtained when DFMO was used in between courses of chemotherapy with vindesine and ADM.[61] DFMO was active per se against cultures of human small-cell lung carcinoma by virtue of its reduction of polyamine levels.[62] An enhanced uptake of methylglyoxal bis(guanylhydrazone) induced by DFMO, led to rapid therapeutic responses in childhood leukemias.[63] Cis-hydroxyproline, an inhibitor of collagen production, inhibited growth of a rat mammary tumor that required type IV collagen for growth.[64] Modification of the luteinizing hormone-releasing hormone decapeptide by replacing the 6-position glycine with various D-amino acids produced compounds which significantly reduced the growth of chemically induced squamous cell carcinoma in rats.[65]

Steroids, Prostaglandins and Analogs - Tumor regression induced by tamoxifen occurred via c-AMP mediated events.[66] The drug was metabolized to 4-hydroxytamoxifen which possessed high estrogen-receptor affinity.[67] Meso-1,2-dialkyl-1,2-bis(3'-hydroxyphenyl)ethanes inhibited a DMBA-induced rat mammary carcinoma in a manner that correlated with their affinity for estradiol receptors.[68] Similar correlations were found for other nonsteroidal antiestrogens such as α-{p-[2-(1-pyrrolidino)ethoxy]-phenyl}4-methoxy-α'-nitrostilbene and cis-3-[p-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol.[69] 10-β-Propynyl-substituted steroids (12) functioned as mechanism-based enzyme-activated irreversible inhibitors of estrogen biosynthesis.[70] Glucocorticoids inhibited growth by increasing the cell's doubling time rather than by a cytolytic effect.[71] Stable analogs of PGA and PGE prostaglandins inhibited DNA synthesis in B16 amelanotic melanoma and LL carcinoma cells.[72] Prostacyclin behaved as a potent antimetastatic agent, possibly by virtue of its antiplatelet aggregation effect.[73]

Miscellaneous Synthetic Agents - Degradation studies of 1-(2-chloroethyl)-3-(4-substituted-2,3-dioxo-1-piperazinyl)alkyl-1-nitrosoureas led to the discovery of antitumor-active 2,3-dioxopiperazine derivatives.[74] An ensuing SAR study led to the synthesis of very potent compounds of general structure 13. When the heterocyclic moiety was 2-pyrimidinyl, T/C values against murine L1210 leukemia were as high as 262.[75]



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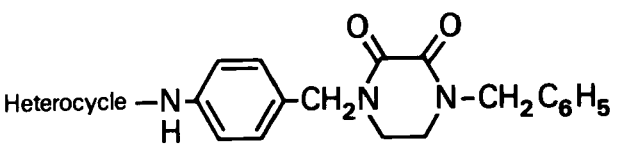
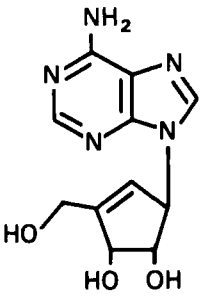


13

Tricyclic analogs of bis(dioxopiperazine) ICRF-159 with different geometrical orientations were studied for their stereoselective antimetastatic activity.[76] Marked inhibition of spontaneous lung metastases was observed for 1-p-(3,3-dimethyl-1-triazeno)benzoic acid.[77] Sulfone analogues of 1,4-naphthoquinone designed as "bioreductive alkylating agents" showed marginal activity against P388 leukemia.[78] Among several polyamine derivatives synthesized, N,N'-bis(3-dimethylaminopropyl)-N,N'-bis(palmitoyl)-trans-1,4-diamino-2-butene was active against B-16 melanoma at several doses with the highest T/C of 177 at 12.5 mg/kg.[79] Trihydroxy benzylamine derivatives were more potent than their dihydroxy counterparts in inhibiting thymidine incorporation in P388 and L1210 leukemias.[80] These compounds are probably active through o-quinone formation.[81] A dimeric analogue of the intercalator ethidium bromide (EB) with a ten methylene connecting bridge was 5-20 times more potent than EB against P388 leukemia.[82] The tetra-O-acetylated chloroacetamido analogue in the mannose series had significant activity against Ehrlich tumors in vivo, whereas the galactose analogue was inactive.[83] Naloxone, the opiate antagonist, was effective in modulating neoplasms. In mice receiving daily sc injections of 20 mg/kg for 2 weeks, prior to the inoculation with neuroblastoma, 30% failed to develop tumors within the 91 day postinoculation period. Naloxone post-treatment produced ILS values of 20-40%.[84]

Metal Complexes and Polymers. Cis-diamminedichloro Pt(II) (DDP) was the subject of numerous reports. Its mode of binding to DNA has been studied with circular DNA.[85,86] The specific action of DDP toward some tumor cells appeared to be due to a repair deficiency in these cells.[87] The repair mechanism that operated in repair-proficient cells after exposure to chloroethylnitrosourea did not function when the cells were treated with DDP.[88] In a study of the relationship between conformation and activity of cis- and trans-Pt(II) complexes of 1,2-cyclohexanediamine and 2-(aminomethyl)cyclohexylamine it was proposed that the rigidity of the former pair sterically prevents the cis-isomer from interacting with DNA. In the latter pair, the coplanarity of the trans- and flexibility of the cis-isomer allowed both to approach DNA efficiently.[89] Seleno-guanosine- and thioguanosine-Pt(II) complexes were less potent than the parent compounds.[90] Various substituted titanocene dichlorides modified only at one cyclopentadienyl ring showed optimum cure rates of 80-100% against Ehrlich ascites tumor in mice. A weakening effect of antitumor activity correlated with increased ring substitution.[91] Auranofin surpassed the effect of 5-FU in screening trials against murine P388.[92] A 15,500 mw fraction of a pyran copolymer inhibited up to 85% of the growth of B-16 melanoma cells *in vitro*.[93]

Fermentation Products and Synthetic Analogs - The structure of Tallysomycin, an antibiotic related to bleomycin (BLM), was shown to contain 1,4-diaminobutane as the terminal moiety instead of spermidine.[94] ESR spectrometry revealed that the conformation of the BLM-Fe(II)-NO complex was not perturbed with RNA as it was with DNA. This finding correlated with the selective scission of DNA by BLM.[95] The DNA-cleavage reaction required Fe(II) and oxygen and proceeded with liberation of B-CH=CH-CHO (B = purines or pyrimidines).[96] BLM was complexed with isotopes of Ru without loss of its chemotherapeutic properties. With the proper isotope, it provided a compound capable of combining radiotherapy and chemotherapy.[97] The acetyl derivative of the cationic terminal dipeptide of BLM-A_2, a portion that mimics most of the DNA-induced perturbations, was synthesized.[98] The first chemically characterized bioreductive product of Mitomycin C (MTC) was isolated.[99] Substitution at the 7-position of the mitosane ring gave analogs that were more potent than MTC.[100] A series of 1-substituted mitosane analogs with good leaving group character supported the bifunctional alkylation hypothesis of the mode of action of MTC.[101] A systematic structural modification of Actinomycin D revealed that modifications at C-7 and N-2 retained tumor inhibitory properties.[102] Two new syntheses of AT-125 (acivicin) have appeared.[103,104] Combinations of acivicin and PALA are efficient against PALA-resistant strains.[105] The reductive release of a CN group from Saframycin C is responsible for the formation of a carbinolamine intermediate which is ultimately involved in the interaction with DNA.[106] Likewise, in pyrrolo-[1,4]benzodiazepine antibiotics a covalent linkage to guanine residues of DNA occurred via a carbinolamine carbon.[107] Significant L1210 activity was displayed by Neplanocin (14), a new nucleoside antibiotic.[108] The complex structures of active antitumor antibiotics, such as Gilvocarcins,[109] BBM-928,[110] and CC-1065 (15),[111] have been elucidated. The first synthesis of the potent macrocyclic antibiotic Verrucarin A was reported.[112]



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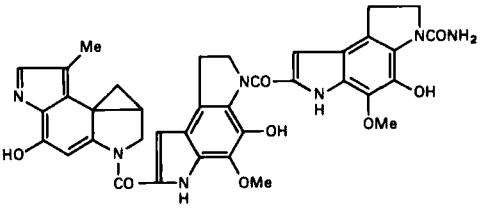
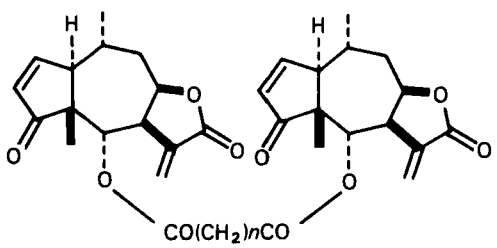


15

Natural Products and Analogs - For a series of active sesquiterpene lactones, the corresponding bis malonate and succinate esters proved more active than the parent compounds.[113] This type of sesquiterpene lactone when transformed into bifunctional alkylating structures by a diester linkage (16) produced increased activity and reduced toxicity.[114] A new mycophenolic acid derivative, ethyl O-[N-(p-carboxyphenyl)carbamoyl]mycophenolate, was found orally active against L1210 and P388.[115] 4-Formyl colchicine surpassed colchicine in activity and therapeutic range against P388 murine leukemia.[116] Ellipticine was bioactivated to 9-hydroxyellipticine, a compound that may continue oxidation to the quinone stage. Among ellipticine analogs, only those which were oxidized by peroxidase-H_2O_2 exhibited high cytotoxic effect against L1210 in vivo.[117] The nature of the ester moiety at C-3 in ansamitocins and maytansinoids played an important role in increasing cell permeation and tubulin binding.[118] A new class of polypeptides (didemnins A, B and C), isolated from a Caribbean tunicate, demonstrated activity against P388 and L1210 leukemias.[119]



16, n=1-4

Immunotherapeutics and Anticarcinogens - Mice with advanced tumors showed a very high cure rate when treated with BCNU followed by immunotherapy with micrococcus, BCG, or related polysaccharides.[120] The activity of bestatin, an orally active immunomodulator, was reviewed.[121] Alveolar macrophages were rendered selectively tumoricidal by in vitro incubation with muramyl dipeptide.[122] Phorbol ester tumor promoters were reported to be potent modulators of macrophage function.[123] These compounds also induced cell differentiation in HL60 cells. The HL60-treated cells lysed various tumor cell lines, including the parent HL60 line with very little cytotoxicity to nonmalignant cells.[124] Other compounds reported as inducers of cell differentiation include alkyl lysophospholipids,[125] vitamin D_3,[126] busulfan,[127] N,N'dimethyltetramethyleneurea,[128] N-isopropyl-2-pyridone,[129] and L-histidinol.[130] 5-Fluoro-12-methylbenzylanthryl-7-acetic acid, a non-carcinogenic analogue of DMBA, when combined with bovine serum albumin, produced reactive antibodies that neutralized the in vitro cytotoxicity of DMBA.[131] A SAR correlation of naturally occurring flavonoids as inhibitors of benzo(a)pyrene hydroxylation revealed the required presence of hydroxyl ring substituents.[132] Butylated hydroxyanisole (BHA) inhibited adduct formation between DNA and benzo(a)pyrene metabolites.[133] As expected, retinoic acid (RA) analogues have received a great deal of attention as inhibitors of tumor promotion. Moderate to excellent activity was observed with ring-modified RA analogues designed

to avoid metabolic degradation.[134] p-Carboxyphenyltrienes synthesized as aromatic RA analogues also displayed moderate to good activity.[135] A new synthesis of (E,Z,E,E)-3,7-dimethyl-4-fluoro-9(4-methoxy-2,3,6-trimethylphenyl)nonatetraenoate was reported.[136] The chemotherapeutic effect of the less toxic retinamides appeared equal to or greater than that of 13-cis RA.[137]

Drug Delivery - Liposomal entrapment was widely used as a selective means of drug delivery in animal experiments. In most instances a superior life-prolonging effect was observed when compared to the free drugs, and toxic effects were often diminished. Some of the drugs studied after liposome encapsulation included daunoblastin,[138] doxorubicin,[139] ADM,[140] mechlorethamine,[141] DDP,[142] thalicarpine,[143] MTX,[144] actinomycin D,[144] alkyl lysophospholipids,[145] vinca alkaloids,[145] and MeCCNU,[146] among many others. Other means of selective drug transport used with MTX therapy were represented by covalent linking of the drug to agglutinins,[147] polylysine,[148,149] serum albumin,[150] bovine fibrinogen,[151] IgG antibodies,[152] and monoclonal antibodies.[153] Other drugs, such as MTC, given as a dextran conjugate showed increased antitumor activity.[154] MTC and ADM have also been linked to immunoglobulins and acted as sustained release forms.[155] The potency of vinca alkaloids was remarkably increased in anti-CEA IgG-conjugates by the ability of the antibody to deliver the drug to tumor cells.[156] Doxorubicin entrapped in magnetically responsive (Fe_3O_4) albumin microspheres increased the drug's efficacy due to selective accumulation in the tumor after the application of an extracorporeal magnetic field.[157]

Biological Methods - An electronic cell-counting technique was devised for determining L1210 cell populations in mice.[158] A soft agar clonogenic assay for human tumor cells was proposed as an *in vitro* technique to predict clinical activity.[159] This medium was also adapted for measuring the cytotoxicity of drugs that required microsomal bioactivation.[160] The *in vitro* growth of colony-forming units from human tumor xenografts appeared to be a stable and reproducible system to study the biology of tumors with the potential of being used as a screening technique.[161]

QSAR - A QSAR study of 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-substituted-phenyl)-s-triazines was performed to make inferences about the mechanism of resistance of dihydrofolate reductase *in situ* as compared to *in vitro*.[162] Factors influencing the dose-potency of m-AMSA analogues in L1210 have been investigated by multiple regression analysis using DNA binding constants.[163] A model consistent with the intercalation of this agent was derived.[164] In a semiempirical molecular orbital study, various parameters such as frontier electron densities, electronic charges, electric field, and bond energies, were correlated with the activity of some aryltriazenes.[165] A QSAR study derived for 30 colchicine analogs indicated the existence of a parabolic dependence of antitumor potency on the partition coefficient. The most potent compound of the series was the 7-fluoroacetamido analog.[166]

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Section IV - Metabolic Diseases and Endocrine Function

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Chapter 18. Inhibitors of Connective Tissue Degradation and Their Potential as Antiarthritics

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Introduction - Breakdown of connective tissue occurs under a variety of physiological and pathological conditions including cartilage degradation in degenerative joint disease and rheumatoid arthritis, bone resorption, differentiation or remodeling of tissues, chronic periodontal disease, wound healing, skin or corneal ulcerations and a variety of other conditions.

The pathogenesis of rheumatoid arthritis (RA) is initiated by unknown stimuli and progresses to the active proliferative stage wherein polymorphonuclear leukocytes (PMN) and eventually mononuclear cells including T and B lymophocytes, plasma cells, and macrophages become predominant in the joint tissues. The PMN's and macrophages may act as effector cells producing enzymes capable of destroying connective tissue macromolecules.[1-14] Macrophages, in addition, may act as mediator cells secreting soluble factors that stimulate fibroblasts,[15-16] synovial cell populations,[17-19] or chondrocytes,[20-23] to produce abnormal quantities of degradative enzymes. The function of macrophages as mediator cells may furthermore be influenced by activated lymphocyte populations.[19] Synovial tissue and specific synovial cell populations also produce soluble factors, such as catabolin,[24-26] which are capable of inducing resorption of cartilage macromolecules by action on chondrocytes in intact tissue.

Connective tissue degradation therefore involves destruction of joint tissue elements, collagen and proteoglycan, mediated by neutral pH enzymes derived from infiltrating PMN's,[1-4] macrophages,[10-14] proliferating pannus,[27-29] and cartilage cells.[30-31] Stimulation of enzyme production by fibroblasts and related mesenchymal cells may furthermore be influenced by soluble factors produced by macrophages and synovial tissue. The result is elaboration of enzymes such as cathepsin G and D, collagenase, elastase, and metalloproteinases which destroy connective tissue.[32-34]

The complexity of this destructive mechanism suggests a multiple approach to prevent enzyme-induced connective tissue destruction. However, the literature is bereft of reference to suitable agents which specifically inhibit mediator cell or effector cell secretions. The application of enzyme inhibitors as agents to prevent connective tissue degradation is a relatively new approach. Nonetheless, reports indicate that numerous drugs used clinically to treat RA and other related diseases provide varying degrees of inhibition against production, secretion and/or activity of enzymes involved in breakdown of connective tissue components.

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Specific Enzyme Inhibitors - Many specific inhibitors have been derived from both natural and synthetic sources. An excellent review published in this series in 1979 described these substances.[35] In general, use of these direct inhibitors has been very limited.

This review will describe agents which demonstrate potential therapeutic value in preventing connective tissue degradation by inhibition of enzyme production and secretion, enzyme activity, or mediator cell production of substances which act to stimulate other cells to produce enzymes.

Agents with Potential Therapeutic Value by Inhibition of Connective Tissue Degradation

Nonsteroidal Antiinflammatory Drugs (NSAID's)-Most NSAID's show little to no direct effect on release, synthesis, or activity of enzymes involved in connective tissue degradation. Drugs which do exhibit varying degrees of inhibitor activity include flufenamic acid,[36] phenylbutazone,[37] indomethacin,[38-39] and fenoprofen.[40] In some studies, aspirin at 10^{-4}M and 10^{-5}M has been shown to be effective in inhibiting collagenase release from chondrocytes activated by macrophage secreted factors.[21]

Steroidal Agents - Steroidal agents exhibit the most consistent inhibitory effects on enzyme production and release.[41-43] Exposure to dexamethasone at concentrations as low as 10^{-10}M produced significant inhibition in the synthesis and release of cartilage proteoglycan-degrading enzyme.[36] Dexamethasone, hydrocortisone and prednisone consistently inhibit enzyme release from macrophages,[36] cartilage-synovial cell cocultures,[38] and from human PMN's.[39]

Antirheumatic Agents - The effects of therapeutic antirheumatic agents on enzyme synthesis, release, and activity have also been inconsistent. Gold sodium thiomalate at 10^{-3}M inhibited release or synthesis of cartilage-proteoglycan degrading enzymes from macrophages[36] but failed to significantly reduce proteoglycan degradation in cartilage-synovial cell co-cultures.[42] This compound also was effective in reducing collagenase production from adherent synovial cells isolated from patients under treatment.[44]

Chloroquine at 10^{-5}M exhibited inhibitory activity against secretion of cartilage-proteoglycan degrading enzyme produced by macrophages.[36]

D-Penicillamine was ineffective against release of cartilage-proteoglycan degrading enzyme from macrophages[36] and minimally effective against collagenase and neutral protease release from macrophage activated chondrocytes. This compound was shown to increase collagenase production by adherent synovial cells from RA patients,[44] but reduced elevated levels of collagenase produced by skin specimen of RA patients.[45]

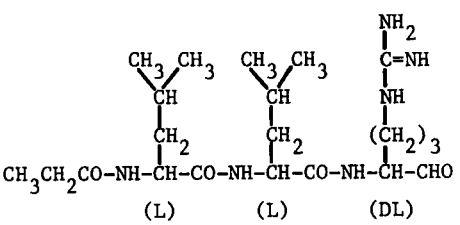
Levamisole at 1μM to 100μM had no effect on β-glucuronidase or cathepsin D release from macrophages up to 22 hours but did reduce enzyme release after 3 days.[46] Other studies report stimulated enzyme release.[36]

Arteparon® - Arteparon® is a glycosaminoglycan polysulfate derivative containing galactosamine, glucosamine, glucuronic acid and sulfate that has been reported to be effective in the treatment of osteoarthritis.[47] After intramusaular injection this material is transported to the joints and can be found associated with articular cartilage. Evaluations in vitro show that arteparon® is an effective inhibitor of human lysosomal elastase [37,48] and human cathepsin G.[38] Concentrations as low as 1μg/ml are effective in inhibiting elastase.[48]

Arumalon - Arumalon is a cartilage and bone marrow extract that has been used to treat osteoarthritis for nearly two decades.[49] Recent reports indicate that Arumalon effectively inhibits human lysosomal elastase particularly if both cartilage and bone marrow extracts are used in combination.[50]

Diphenylhydantoin - Diphenylhydantoin is an anticonvulsant compound that has recently been examined for potential anticollagenolysis properties. Retardation of collagen degradation by diphenylhydantoin (sodium) has been suggested from in vitro studies using cat palatal mucosa cultured in the presence of this drug.[51] More recently, diphenylhydantoin was shown to inhibit collagen breakdown in gingival tissue of patients treated with this drug.[52]

Antibiotics - Leupeptin (1) and antipain (2) are microbial inhibitors to proteolytic activity.[53] These agents were reported to prevent the onset of muscular dystrophy in experimental mice and chickens and have potential to prevent protein degradation in arthritis.[54-55]



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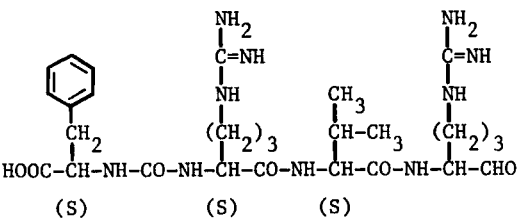

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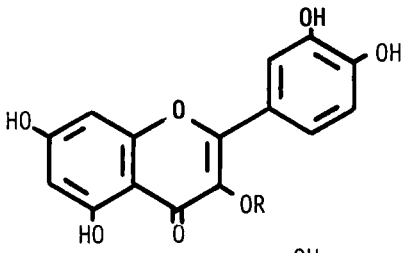
Retinoids - Retinoids are known to exert a variety of effects on epithelial cells and on cells of mesenchymal origin. Treatment of adherent synovial cells with all-trans-retinoic acid inhibited production of collagenase by these cells.[56] At 10^{-6}M, this retinoic acid inhibited collagenase production from rabbit synovial fibroblasts by 60 to 70%. Combining prednisone with retinoic acid reduced collagenase more effectively than either drug alone.

Flavinoids - Flavinoids, such as rutin (3), (+) catechin (4) and dihydroquercetin (5) have a high affinity for connective tissue, particularly the collagen component.[57] (+) Catechin was shown to increase collagen stability and has been used to treat collagen related diseases.[58] This flavinoid apparently complexes with collagen, changing its conformation and increasing its resistance to mammalian collagenase.[57] In addition,

(+) catechin directly inhibits collagenase.[59] These preliminary results indicate that (+) catechin or other flavinoids might be useful as therapeutic agents to stabilize collagen and reduce excessive enzyme degradation.



R=Rutinose

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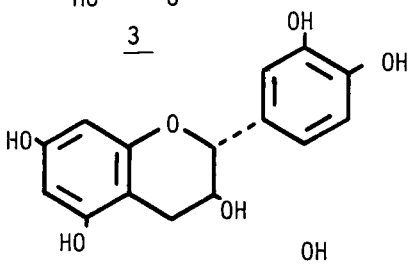

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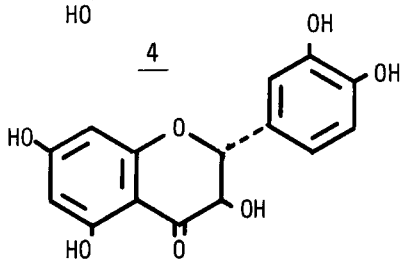

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Miscellaneous Agents - Numerous additional substances have been implicated to possess potential therapeutic value in preventing connective tissue degradation. These include oleic acid, shown to effectively inhibit activity of human lysosomal elastase and cathepsin G;[37] ε-amino-caproic acid n-hexyl ester, shown to inhibit collagen breakdown by inhibiting latent collagenase-activating proteinases;[60] vitamin E, shown to increase the stability of cartilage proteoglycans;[61] and theophylline, shown to inhibit collagenase production by human skin explants.[62]

Conclusion - The pathogenesis of rheumatoid arthritis and other proliferation diseases clearly indicates the role of extracellular enzymes in mediating the destruction of connective tissue macromolecules. This suggests that inhibitors of enzyme synthesis, release, or activity might be useful in preventing or treating such proliferative diseases. The development of selective inhibitors offers a relatively new approach to control the connective tissue destruction associated with these diseases. This approach to therapy has not directly been applied. Nevertheless, numerous therapeutic agents discussed in this review could offer some degree of value in preventing connective tissue degradation.

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Chapter 19. Leukocyte Motility

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Introduction - We have reviewed the recent literature for the medicinal chemist interested in tempering the motile leukocyte's response to chemotactic stimuli as a means to the therapeutic control of inflammatory events or to the treatment of compromised patients rendered unduly susceptible to infectious agents due to subnormal chemotactic responses. We have stressed those reports dealing with the neutrophilic leukocyte (PMN) due to its major role in acute inflammatory processes.[1-4] It is not the purpose of this review to discuss the complex cellular events intimately associated with the stimulated motile responses of the leukocyte but rather to identify chemical agents that modulate induced translocation in an in vitro or in vivo setting. Several current excellent reviews are recommended for a detailed discourse on the cell biology associated with locomotor responses of the leukocyte.[5-8] Of special interest is a review of the burgeoning literature concerning oxygenation products of arachidonic acid and their critical role in leukocyte physiology.[9] When appropriate we have stated the purported mechanism(s) of action of the therapeutic agent. We have adhered to current definitions of chemotaxis, chemokinesis, and spontaneous migration[10,11] (SPM) and have indicated the methodology utilized in the assessment of each compound or class of compounds. Whenever chemotaxis and chemokinesis have not been clearly distinguished, the term stimulated migration (STM) is used. Generally, the techniques utilized in the study of STM include modifications of the micropore filter assay (MFA)[12-19] originally described by Boyden,[20] the under agarose technique (UAT),[21,22] and direct visual assessment of cell orientation towards a gradient of chemoattractant.[23] The most widely used chemotactic factors include: 1) the cleavage product derived from the fifth component of complement (C5a), either purified or residing in plasma or serum via activation with zymosan (zymosan activated serum, ZAS), endotoxin (endotoxin activated serum, EAS) or immune complexes, 2) the formylated peptides described by Schiffmann et al.[24] which are related to chemoattractants derived from Escherichia coli (bacterial derived chemotactic factor, BCF)[25] and stimulate the neutrophil through the formylated peptide receptor,[26] 3) Leukotriene B_4 (LTB_4),[9] and 4) casein.

Cyclic Nucleotides - Intracellular biochemical reactions involving the cyclic nucleotides are clearly coupled to activation of the leukocyte by chemotactic factors. As reviewed by Gallin[5] and Hill,[6] cyclic AMP (cAMP) and cyclic GMP (cGMP) play a modulatory role in chemotaxis. Accordingly, agents that cause an increase in intracellular cAMP, such as reserpine, caffein, serotonin, theophylline and epinephrine, decrease the stimulated motile response of the neutrophil. The inverse effect on neutrophil motility occurs with those agents causing decreased levels of cAMP. The reader is referred to more detailed discussions on this subject.[5,6] The β-receptor blocking agent propranalol has recently been shown to increase neutrophil chemotoxis, chemokinesis, and SPM at 10^{-5}M by utilization of a MFA-"checkerboard"[17] assay with EAS, C5a, and casein.[27] This agent may be enhancing STM by increasing intracellular levels of cGMP.

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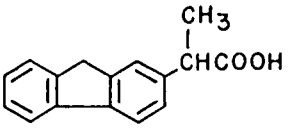
Anti-inflammatory Agents - Phenylbutazone has been reported to suppress STM of human, rat, rabbit[28] and guinea pig[29] neutrophils at 10^{-5}M, 8 x 10^{-5}M, 3 x 10^{-7}M, and 9 x 10^{-7}M, respectively (MFA), while also inhibiting SPM.[29] Indomethacin (6 x 10^{-5}M) suppressed guinea pig[29] but not rat or rabbit[28] neutrophil STM via MFA. Halcinonide, cyclophosphamide, isothioprine, naproxen, cicloprofen (1) and ibuprofen were reported to diminish (IC_{50}) STM in the 10^{-5}-10^{-6}M range.[28] Niflumic acid was inhibitory (STM) at 4 x 10^{-7}M.[28] The inhibitory activity of niflumic acid, phenylbutazone, and naproxen on rabbit neutrophil STM with BCF was antagonized by bovine serum albumin (BSA) binding of these drugs.[30] High concentrations of aspirin (10^{-2}M) and aminopyrine (10^{-3}M) suppressed guinea pig neutrophil STM.[29]



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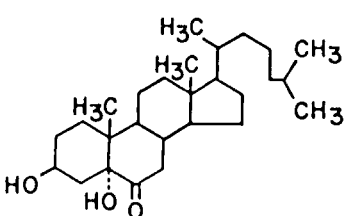
Several anti-inflammatory and anti-rheumatic drugs were tested for their effect on the STM of rat pleural exudate PMN and mononuclear cells via MFA subsequent to in vivo administration. The effect of in vivo drug treatment on the chemotactic activity of the cell free exudate was also assessed with both cell types. Indomethacin (5 mg/kg), naproxen (30 mg/kg), and dexamethasone (0.5 mg/kg) were found to be significantly inhibitory utilizing both methods with mononuclear cells and PMN. Colchicine (1.0 mg/kg) was shown to be significantly suppressive to only PMN.[31]

Human eosinophil STM was found to be suppressed (MFA) after ingestion of 60 mg prednisone,[32] without similarly affecting neutrophil motility. Hirata et al.[33] reported that fluocinolone acetonide, dexamethasone, hydrocortisone, and cortisone down-regulated rabbit PMN STM to f-Met-Leu-Phe with decreasing potency in the order listed. The author associated inhibition by these agents with induced inhibition of intracellular phospholipase A_2 activity. Gordon et al.[34] assessed the effect of several oxygenated sterols on human neutrophil STM via UAT with f-Met-Phe. The most potent inhibitor in the series was 5-hydroxy-6-ketocholestanol (2). Hydrocortisone and 6α-methylprednisolone were reported to suppress eosinophil STM (MFA)[35] at 50 µg/ml and 10 µg/ml respectively (IC_{50}). Moreover, intravenous administration of 300 mg hydrocortisone resulting in serum levels of 3.23 µg/ml suppressed in vitro eosinophil STM. Hydrocortisone succinate inhibited (IC_{50}) rabbit, rat, and human neutrophil STM in the 2-5 x 10^{-6}M range,[28] and 6α-methylprednisolone, prednisolone, and triamcinolone acetonide were found to be equally potent. Using autologous, ^{51}Cr labeled rat neutrophils, Perper et al.[36] showed that adoptively transferred cells were inhibited (50%) from accumulating at the inflamed site in carrageenin-induced lesions due to in vitro treatment of 5 x 10^{-4}M 6α-methylprednisolone hemisuccinate. The same cell population was suppressed 63% in MFA. Using this same experimental design, indomethacin (5 mg/kg), phenylbutazone (100 mg/kg) and mefenamic acid (300 mg/kg) were shown to suppress mononuclear cell migrations, but not PMN migrations. Betamethasone suppressed (0.01-0.10 µg/ml) human neutrophil STM (MFA) to aggregated gammaglobulin-activated plasma.[37]



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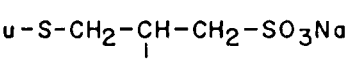
Sodium aurothiopropanol sulfonate (3) diminished the human PMN STM in a dose-response fashion from 3-100 µg/ml (MFA).[37] Gold sodium thioma-



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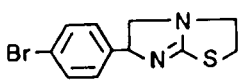
late produced dose-related reductions in PMN STM in patients who responded clinically to chrysotherapy as well as in normal subjects (MFA).[38] Penicillamine therapy gave equivocal results in this study although in vitro investigations have shown inhibition of STM.[38,39]

Agents That Enhance Stimulated Leukocyte Locomotion - Levamisole enhances human monocyte STM to a variety of chemoattractants at 10^{-5}-10^{-3}M.[40,41] Para-bromotetramisole (4), but not the dextroisomer also possessed enhancing properties. Evidence was presented indicating binding of levamisole to monocytes. The mechanism of action for levamisole may include inhibition of cellular oxidative mechanisms[42] and increases in intracellular levels of cGMP.[43] Levamisole was reported to correct anergic patients' depressed PMN STM in vitro at 10^{-4}M.[44]



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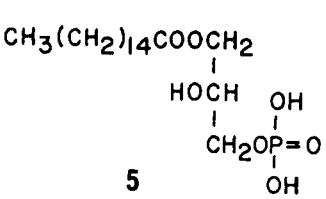
Ascorbic acid (5 x 10^{-3}M) has recently been shown to enhance neutrophil chemotaxis and SPM by utilizing the checkerboard MFA.[45] The up-regulation of ascorbate may be due to prevention of "chemotactic deactivation".[46] Patients with defective PMN chemotaxis and recurrent bacterial infections showed clinical improvement during ascorbate therapy. This improvement was associated with enhanced STM of the isolated neutrophils.[47,48] One mechanism of action of ascorbate enhancement may be related to the enhanced formation of polymerized tubulin since the same concentration (5 x 10^{-3}M) that enhances polymerization of purified bovine brain tubulin in vitro also enhances human PMN STM (MFA).[49]

The lysophosphatidic acid, 1-palmitoyllysophosphatidic acid (5) at 0.24 mM was demonstrated to enhance PMN STM to f-Met-Phe (MFA).[50] This agent may exert its enhancing effect by acting as a calcium ionophore. Inosiplex caused enhanced SPM and chemotaxis at 500 µg/ml.[51] Dapsone (0.1 mM) enhanced STM to EAS of neutrophils from lepromatous leprosy patients and normal individuals (MFA).[52] One 100 mg dose of dapsone resulted in increased STM of isolated leukocytes.



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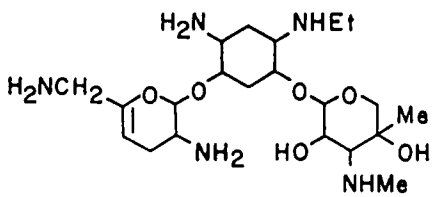
The myeloperoxidase catalyzed oxidation of chloride by the H_2O_2 system (MPO-H_2O_2-Cl^-) generated from activated PMNs has been shown in vitro to inactivate the methionine-containing chemotactic factors C5a, f-Met-Leu-Phe and f-Met-Leu-Phe-Lys, by oxidation to their respective sulfoxides.[53,54] The antioxidants methionine, 2-mercaptoethanol, and ascorbic acid inhibit this inactivation in vitro as does KCN, NaN_3, and catalase, but not superoxide-dismutase. Three β-blockers, metoprolol, sotalol, and propranolol caused increased SPM and chemotaxis of human PMN, possibly by their inhibitory effect on several cellular oxidative mechanisms, including superoxide[27] and H_2O_2 production.[55] The antioxidant properties of 2,3-dihydroxybenzoic acid[56] and thiamine[57] may contribute to their enhancement of PMN STM (MFA) in vitro; however, superoxide dismutase (2000 units) did not increase PMN responsiveness to chemoattractants.[57]

Paradoxically, superoxide has been reported to generate a chemotactic substance from lipids bound to serum albumin in plasma.[58] Superoxide-dismutase (10-25 µg/ml), but not catalase, inhibited formation of

this chemotactic factor in vitro. Superoxide-dismutase inhibited both the edema and PMN involvement in the reverse passive Arthus and carrageenin edema reactions in rats.

Physiologically attainable concentrations of Li^{+} (0.8mM) corrected the abnormal PMN STM in vitro and in vivo of a patient with recurrent infections. This response was attributed to the patient's elevated cAMP levels being normalized during Li_2CO_3 therapy, apparently due to an effect on cAMP formation.[59,60] Acrodermatitis enteropathica (AE) patients have low Zn^{++} levels and abnormally low monocyte and PMN STM. Normal STM was obtained in vitro by adding Zn^{++} to AE patients' monocytes and PMN. Disease symptoms were reversed, Zn^{++} levels increased, and monocyte and PMN STM normalized by treatment of these patients with oral $ZnSO_4$.[61]

Antibiotics and Antifungals - The immunologic importance of assuring uncompromised PMN motility during bacterial infection has stimulated the study of antibiotic effects on PMN STM. Several tetracyclines, tetracycline (0.01-100 µg/ml), chlortetracycline (10-100 µg/ml, demeclocycline (10-100 µg/ml), doxycycline (10-100 µg/ml), methacycline (10-100 µg/ml), minocycline (10-100 µg/ml), oxytetracycline (10-100 µg/ml), rolitetracycline (10-100 µg/ml), and lymecycline (25-100 µg/ml), have been shown to inhibit human PMN STM via MFA or UAT to a variety of chemotactic factors, including BCF.[62-67] A conflicting report that tetracycline did not inhibit PMN STM to ZAS or f-Met-Leu-Phe with UAT has appeared.[68] Volunteers treated with tetracycline, doxycycline, or lymecycline showed depression of PMN STM.[62,65] Speculation has been presented that tetracycline inhibition of PMN STM is related to the effectiveness of these drugs in skin disease (acne vulgaris).[62] The aminoglycosides, sisomicin (10-30 µg/ml), gentamicin (0.5-50 µg/ml), tobramicin (0.5-50 µg/ml), amikacin (2-32 µg/ml), kanamycin (2-32 µg/ml) and netilmicin (6) (0.5-8 µg/ml), inhibit human PMN STM (MFA),[69,70] and gentamicin has been reported to cause a transient decrease of PMN STM in humans.[71] Other investigators have reported no inhibition of PMN STM in vitro for gentamicin at high (100 µg/ml) concentrations.[64,67] Erythromycin (0.1-10 µg/ml) and clindamycin (0.2-200 µg/ml) apparently inhibited human PMN STM to ZAS with the MFA[63,67] but not the UAT.[64,68] Chloramphenicol at 1-100 µg/ml has been reported to enhance human PMN STM (MFA) toward casein in serum,[67] while at 2.5-100 µg/ml inhibition of STM (UAT) was noted.[64] Relevant therapeutic levels of fusidic acid (0.5-100 µg/ml) inhibited human PMN STM with the UAT.[64] Amphotericin B at 1-5 µg/ml was the only one of five antifungal drugs studied that inhibited human PMN STM with MFA and using BCF.[72]



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Miscellaneous Agents That Inhibit Leukocyte Locomotion - The importance of transmembrane Ca^{++} influx during locomotion of human neutrophils was demonstrated in vitro by the concentration-dependent inhibitory effect of La^{+++} (0.1-1.0 mM) on both PMN STM and intracellular Ca^{++} accumulation.[73] Heparin (12.5-200 units/ml) inhibited STM of human PMN and eosinophils to eosinophil chemotactic factor, BCF, and ZAS by direct interaction with the chemoattractant. Chondroitin sulfate A and hyaluronic acid were reported to inhibit PMN STM in a similar fashion.[74]

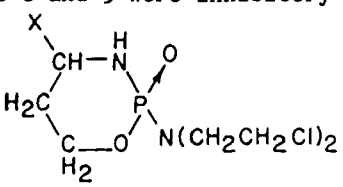
N-Acetyl-L-tyrosine ethyl ester (0.5-2.5 mM), N-benzoyl-L-arginine ethyl ester (5-80 mM), benzamidine (1-8mM), and p-nitrophenyl p'-guanidinobenzoate (0.1-0.5 mM) suppress human PMN STM (MFA).[75] This class of inhibitors may interact with a cell associated serine proteinase.[76]

4-Imidazole acetic acid ($<10^{-8}$M) inhibited PMN STM (MFA) with ZAS.[77] This concentration of imidazole acetic acid also inhibited C3b-induced histaminase release, but not A23187-induced release, from PMN, thus suggesting a regulatory role of histamine in complement mediated PMN functions.

Unlike human monocytes and guinea pig macrophages, human PMN STM (MFA, ZAS) showed only slight inhibition by the phospholipid and cholesterol synthesis inhibitor, clofibrate (10mM), and moderate inhibition by the cholesterol synthesis inhibitor, triparanol (10-30 μM), indicating less dependence on new membrane lipid synthesis in the neutrophil.[78]

Several tricyclic tranquilizers and antidepressants, prochlorperazine, perphenazine, thioridazine, amitriptyline, and imipramine, inhibited human PMN STM (MFA, BCF) at .06-.15 mM. The most potent compound, chlorpromazine, inhibited STM 63% at .014 mM, which was 2-10X higher than plasma levels found in treated patients.[79]

A study of cyclophosphamide (7) and its metabolites, 4-hydroperoxy- (8), 4-hydroxy- (9), and 4-ketocyclophosphamide on human PMN STM (MFA, EAS) indicated that 8 and 9 were inhibitory at 0.2 mM.[80]



7 , X = H

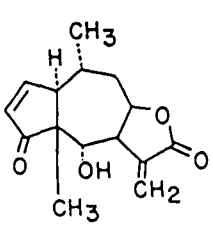
8 , X = OOH

9 , X = OH

Thalidomide (1-100 μg/ml) has been shown to inhibit PMN STM in a non-dose related fashion (MFA, ZAS).[81]

Psoratic patient enhanced PMN STM was reduced to normal levels by the orally administered phosphodiesterase inhibitor diphylline.[82] This reduction in PMN STM was associated with clinical improvement and may have been caused by drug related alterations in cAMP/cGMP ratios.

Eight of twenty sesquiterpene lactones that were investigated as antiinflammatory agents exhibited 100% inhibition of human PMN STM (UAT, BCF) at 0.05 mM. The four most potent compounds, helenalin (10), 2,3-epoxyisotenulin (11), deoxyelephantopin (12) and eupatolide (13), were 40-60% inhibitory at 1 μm. This activity appeared to be unrelated to effects in carrageenin-induced edema, prostaglandin synthesis inhibition, or adjuvant arthritis assays.[83]



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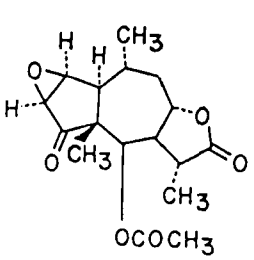

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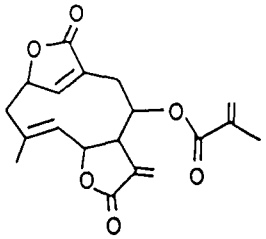

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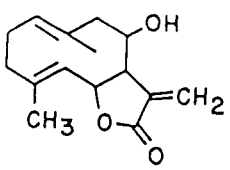

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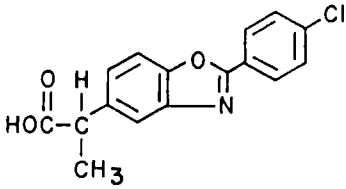
Inhibitors of Arachidonate Lipoxygenase - LTB_4 has been shown to be a chemotactic factor for PMN equipotent to C5a and f-Met-Leu-Phe, and other lipoxygenase products, including 5-HETE, can also promote PMN chemotaxis.[84] In contrast the cyclooxygenase products PGE_1 and PGA_1 inhibit PMN STM.[85] A central role for lipoxygenase products in PMN STM has been indicated because nordihydroquiaretic acid (NDGA), a selective lipoxygenase inhibitor, at 10^{-5}M, inhibits PMN STM with either C5a or f-Met-Leu-Phe.[86] Three other less selective lipoxygenase inhibitors, 5,8,11,14-eicosatetraynoic acid (ETYA) (10^{-5}M),[87] indomethacin (10^{-4}M)[87] and benoxaprofen (14) (30 μg/ml)[88] also suppressed PMN STM to C5a or f-Met-Leu-Phe. Enhanced PMN STM was reported at 10^{-7}M indomethacin, possibly due to selective inhibition of prostaglandin synthesis.[87]



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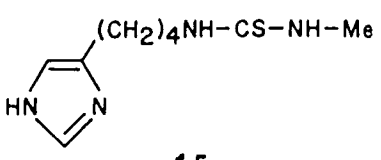
Receptor Specific Drug Interactions - Several structurally dissimilar competitive inhibitors of the formylated peptide receptor have been discovered. Phenylbutazone (0.5 μM) and sulfinpyrazone (0.5 μM) inhibited chemotaxis of human PMN (MFA) toward f-Met-Leu-Phe but not C5a and antagonized f-Met-Leu-Phe induced granulocytopenia in rabbits.[89] Evidence was presented that these observations were not caused by effects on prostaglandin synthesis. Other in vitro studies confirmed that phenylbutazone competitively inhibited the formylated peptide receptor.[90] Potent antagonists (5 x 10^{-4}M) of f-Met-Leu-Phe STM were obtained by replacing the formyl group of f-Met-Leu-Phe with either a trimethylacetyl, adamantyloxycarbonyl, adamantylcarbonyl, or adamantylsulfinyl group.[91] A series of tert-butoxycarbonyl peptides (2.6-8.0 x 10^{-4}mM) (Boc-Phe-Leu-Phe, Boc-Leu-Phe-Leu-Phe, Boc-Phe-Leu-Phe-Leu-Phe) have also been reported to competitively inhibit f-Met-Leu-Phe STM.[92,93] Diazoacetylnorleucine methyl ester (1.0 mM), in the presence of 0.01 mM Cu^{++}, inhibited PMN STM (UAT) pepstatin-stimulated lysosomal enzyme secretion. This agent also inhibited binding of radiolabeled f-Met-Leu-Phe to neutrophils and interfered with pepstatin-induced chemotactic deactivation. The authors speculated that cathepsin D is the neutrophil formylated peptide receptor.[94]

The relevance of enhanced PMN responsiveness to histamine in atopic and hyperimmunoglobulinemia E patients has been investigated. The compromised chemotactic response of these patients' PMN was corrected in vitro (MFA) after treatment with the H_2 histamine receptor blockers, cimetidine[95] and burimamide (15).[96] Cimetidine therapy normalized PMN STM in a hyperimmunoglobulinemia E patient.[97] Similarly, the histamine induced inhibition of normal PMN STM was overcome by cimetidine[98] or 15.[96] Treatment in vitro with the H_1 histamine antagonist promethazine did not correct atopic patients abnormal PMN STM.[95]



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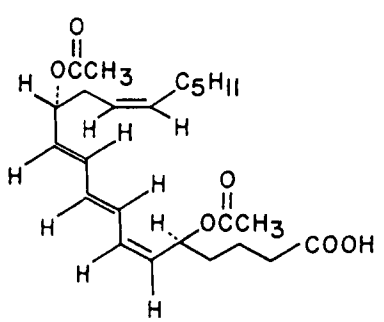

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Concanavalin A (Con A) apparently has a specific receptor on macrophages and at 1-4 μg/ml induces STM of rat peritoneal macrophages and human blood monocytes (MFA). The interaction of Con A with its receptor and the STM caused by Con A were both inhibited by 50 mM α-methylmannoside.[99]

Diacetyl LTB_4 (**16**) was shown to be a potent competitive inhibitor of LTB_4 STM in human neutrophils.[100] An equal molar mixture of **16** and LTB_4 resulted in 50% suppression of LTB_4 STM (MFA).

It is noteworthy that in recent years specific receptors on inflammatory leukocytes have been identified (e.g., C5a, f-Met-Leu-Phe) and a model system with in vivo relevance has been reported for the formylated peptide receptor. Antagonists of these receptors may provide a basis for identification of novel anti-inflammatory agents.

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Chapter 20. Therapeutic Modulation of Cellular Mediated Immunity

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Cell mediated immunity (CMI) is thought to play a crucial role in chronic infection from fungi, bacteria, viruses and parasites, in immunity to soluble proteins, in reactions against tumors and transplants, in contact sensitivity and in some autoimmune diseases.[1] There has been considerable recent interest in immunotherapy which, unlike chemotherapy, attempts to modify the immune mechanism of the host. Earlier chapters in this series have reviewed adjuvants to the immune system,[2] immunosuppressive and immunostimulant agents in rheumatoid arthritis[3] and immunostimulants in general.[4] The terms immunostimulant, immunopotentiator and immunoenhancer have been used often interchangeably but perhaps immunomodulator, immunoregulant or biological response modifier are more appropriate since immune active agents are often capable of stimulating and depressing immune functions. Immunosuppressants, such as antimetabolites, alkylating agents and glucocorticoids, may also be covered by the latter nomenclature since they have recently been shown to produce condition-dependent immunostimulation.[5,6]

The immune system in part consists of a complex interacting network of functionally distinct thymus-derived lymphocytes (T cells) that include helper (T_h), several types of suppressor (T_s), cytotoxic (T_c) and delayed hypersensitivity (T_d) cells that contribute to CMI. There is evidence that immune response genes control the magnitude of responses mediated by T cells; furthermore, B lymphocytes, macrophages, killer cells, natural killer (NK) cells, mast cells and basophils often play an integral part in these responses. T cells also regulate the B cells that are responsible for immunoglobulin production, or humoral immunity. The immunoregulatory mediators of CMI, namely lymphokines (including interferon) and monokines, have been classified into factors that influence cell proliferation, cell motility and cell activation.[7] Research on these soluble products has been reviewed extensively.[8,9] The identification of T cell subsets by means of expression of cell surface antigens, and their role in health and disease, has attracted much recent attention. Imbalances in the number and/or function of these T cell populations may have important implications in the pathogenesis of immunological diseases.[11,12,13,14]

The complexity of the immune system presents an opportunity for a large number of points for therapeutic intervention. Most immunomodulators of current interest influence the function/activity of at least one of the cell types involved in CMI and many also affect humoral immunity. It is important to establish the specificity of such agents and consequently screening batteries are necessary for development of new immunomodulators. There is no consensus as to which specific models should be used, although assays include experimentally-induced infections, neoplasms, tissue transplants, autoimmune models and direct immunological parameters (e.g., delayed hypersensitivity (DH), antigen and mitogen proliferation, antibody production, cell cytotoxicity).[15-19]

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Unfortunately, the immunopharmacologic evaluation of these agents is fraught with problems and is dependent on a multitude of factors, such as the species, age, sex, strain (genetics), biorhythm, nutritional status, environmental conditions and concurrent infection of the animal used, as well as the dose, route, duration, and time of administration of the agent. Where tumor, pathogen or antigen is involved in the response, the immunogenicity, dose, and site of administration are also important. Potency determinations are often hampered by biphasic responses produced by the same compound and consequently assessments such as ED50 or EC50 are rarely described in immunopharmacologic literature.

None of the existing classifications for immunomodulators is entirely satisfactory, since we do not yet possess agents that exert an action on a restricted number of immune functions. Their chemical nature and pharmacologic characteristics vary considerably, so the simplest division appears to be into biologically-derived and synthetic substances. It would be an immense task to review all of these agents, so we intend to review recent developments with an emphasis on selected synthetic substances with therapeutic potential. Nevertheless, it is prudent to mention some recent reviews that have appeared on the many bacterial and fungal preparations that are the subject of extensive interest. New data and reviews [20,21] have been reported on the peptidoglycans and the β-1,3 polysaccharides, including lentinan, glucan, Krestin (PSK), schizophyllan (SPG), mannan, mannosyn, and the β-2,6 fructan, levan.

Peptides The recent interest in modulating the immune response with biologically active, small molecular weight peptides continues. Reviews on the immunomodulating properties of muramyl dipeptides (MDP). bestatin, thymic peptides, cyclosporin A (CSA) and tuftsin have been published.[22-31] MDP and its derivatives are a series of synthetic glycopeptides capable of stimulating CMI, antibody production and non-specific resistance to infection. An extensive structure-activity relationship was discussed in a earlier chapter.[3] The importance of the carbohydrate moiety was recently explored.[32] Only D-mannosamine and D-galactosamine analogs of MDP were as active in inducing DH to azobenzenearsonate-N-acetyltyrosine in guinea pigs as MDP, a derivative of D-glucose.

A new tetrapeptide, isolated from crude immunostimulatory extracts of a Streptomyces strain, was chemically conjugated with lauric acid (N2-[N-(N-lauroyl L-alanyl)-γ - D-glutamyl]N^6-(glycl)-DD,LL2,6-diaminopimalic acid; LTP) and exhibited immunostimulatory activities similar to those of MDP.[33] This indicates that such activities are not restricted to glycopeptides and that the presence of muramic acid is not essential. LTP enhances phagocytosis, exerts a slight mitogen-like effect in mouse splenocytes, enhances DH responses and increases resistance to Listeria monocytogenes. In addition to LTP, 30 different lipopeptides have been synthesized varying peptide and fatty acid composition.[33] A lipodipeptide ([palmitoyl]L-Ala-D-Glu) was the smallest substance with immunomodulatory effects. Greater activities were seen with lipotetrapeptides particularly those containing diaminopimelic acid.

Bestatin, an aminopeptidase inhibitor, binds to immunocompetent cells and subsequently enhances rosette forming cells,[34] NK activity, migration and phagocytosis of granulocytes,[25] and decreases immunoglobulin (Ig) secretion[36] in vitro. These effects may not be due to enzyme inhibition.[37] Bestatin is orally active against several forms of cancer in man.[25] Amanstatin, forphenicine and esterastin are related to bestatin and also possess immunomodulatory activity.[26]

The tetrapeptide tuftsin (Thr-Lys-Pro-Arg) resembles an integral component of IgG and is released physiologically as the free peptide fragment after enzymatic cleavage.[33] Tuftsin analogs can be identified in the IgE and HLA-B7 molecules.[38] Tuftsin stimulates phagocytosis[39], chemotaxis,[40] anti-bacterial activity of macrophages[41] and NK cell activity.[42]

A number of thymic peptides have been chemically defined;[43-46] N-desacetylthymosin α_1 was synthesized using recombinant DNA technology.[47] Thymic peptides delay the appearance of specific autoantibodies,[48] enhance NK cell activity,[49] graft versus host reactions,[50] and differentiation and functional activity of T_h, T_s and K cells.[51] Thymic peptides have been used successfully to treat primary immunodeficiencies[52] and autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis.[53] Thymosin α_1 also augments specific T cell functions in immunodepressed cancer patients.[54]

CSA is a cyclic polypeptide, containing a novel C_9 amino acid designated C_9 ene.[29] The immunosuppressive actions of CSA are either due to the inhibition of T_h cells or to accelerating the appearance of T_s cells.[30] Its actions are reversible, since treated lymphocytes can proliferate when washed free of CSA.[56] The early stage of the immune response is especially sensitive to CSA[57-61] and, contrary to earlier dogma, the humoral arm of the immune response can be suppressed by CSA, depending on the antigen.[62] Clinical trials have been held with CSA to treat graft rejection[63] and rheumatoid arthritis.[64] So far, the results have been encouraging, although side effects, such as nephrotoxicity and lymphoma-induction, have been reported.[65,66]

Neuroendocrine Agents - Several reviews have analyzed the involvement of both the central and autonomic nervous systems in the immune response.[67-69] A recent book has been published on this subject.[70] Cholinergic,[71] adrenergic,[72] dopaminergic[73] and opiate receptors[74] are present on lymphocytes. No difference has been observed in β-receptor number on T- and B-cells.[75] Beta-blockers have been shown to inhibit lymphocyte capping and transformation,[76] and depletion of synaptic catecholamines have been correlated with decreased plaque forming cells (PFC).[77] Treatment of T cells with morphine also leads to a reduction of PFC.[78]

H_2-Antagonists - Histamine H_2 receptors are present on lymphocytes and may be involved in T and B cell activation.[79,80] Cimetidine and metiamide, two H_2-antagonists, increase lymphocyte proliferation,[81] Ig production,[82] and abrogate both T_s cells[83] and macrophage function.[84] In vivo studies indicate that depending on the time and dose of both antigen and metiamide administration, suppression or enhancement of DH is observed. Cimetidine protects animals from lethal tumor challenge;[86] its immunorestorative effect is more pronounced among cancer patients with increased T_s function.[87] Increased T_s action is believed to be mediated by the release of a histamine-induced suppressor factor; the release of this factor is inhibited by cimetidine.[88] Clinically, cimetidine has been shown to alleviate rheumatoid arthritis[89] and mucocutaneous candidiasis,[90] although it may also induce bacterial infections[91] and arthritis.[92]

Antimicrobial agents - Several different classes of antimicrobials have immunomodulating effects on CMI and humoral immunity.[93,94] For example, amphotericin B enhanced phagocytosis and microbicidal activity of macrophages and promoted selective toxicity for thymocytes and T_s cells.[93,95-97] It also inhibits PHA blastogenesis of lymphocytes.[93] The cephalosporin, ceftriaxone, inhibited PHA-induced human blastogenesis, while other cephalosporins were inhibitory only in combination with gentamicin.[98] Other antibiotics (penicillin, chloramphenicol, carbenicillin, gentamicin, kanamycin, nalidixic acid and streptomycin) did not influence lymphocyte blastogenesis.[93] Rifampicin also suppresses phytohemagglutinin-A (PHA) blastogenesis of human lymphocytes, lymphokine (MIF) secretion and DH to dinitrochlorobenzene (DNCB) in guinea pigs; it also prolongs skin allograft survival in rabbits.[93] A number of tetracyclines, including doxycycline, suppress PHA-induced blastogenesis and also inhibit DH to sheep red blood cells (SRBC) in mice.[99] Leukocyte migration and phagocytosis are also impaired by tetracyclines.[93]

Sex hormones - The higher incidence of certain autoimmune diseases including rheumatoid arthritis and SLE in females suggest an important regulatory role for sex hormones. Nandrolone, testosterone and 5α-dihydrotestosterone(DHT) increase survival time and reduce anti-DNA antibodies in the murine lupus model, using castrated and noncastrated NZB/W-F_1 mice and noncastrated MRL/1 mice.[100-102] Testosterone has been shown to stimulate PHA-induced blastogenesis, using rabbit peripheral T cells, although PHA or concanavalin A (ConA) responses of splenocytes from NZB/W-F_1 mice treated with androgens were unaltered.[103] In contrast, 17-β-estradiol (E 2) decreased survival time, increased anti-DNA antibodies and decreased splenocyte response to PHA and ConA in the latter model.[100] The section of the androgens and estrogen in murine lupus is associated with release of an immunoregulatory factor (s) from the thymus or perhaps an influence on the thymic epithelium or thymocytes.[100,104] Sex hormones also influence phagocytic function, i.e., DHT stimulates and E2 inhibits.[100]

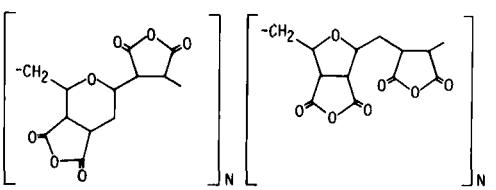
Lynestrenol, a progesterone-like drug, enhanced PHA-induced blastogenesis, mixed lymphocyte culture, skin testing and T cell rosetting, in both normals and cancer patients.[105,106] It also reduces human monocyte phagocytosis.[105]

Retinoids - The immunologic and biologic activities of retinoic acid and its analogs have been reviewed.[107-109] Retinoids have antitumor properties[110,111] and are being used to treat skin disorders, such as psoriasis and acne diseases due to Vitamin A deficiency.[111] Currently, two oral aromatic retinoids (13-cis-retinoic acid and trimethylmethoxyethyl retinoate, Ro 10-9359) are undergoing intensive clinical trials.[107] Retinoids augment lymphocyte proliferation,[112] suppress F_c receptor expression on macrophages[113] and enhance antibody production.[114]

Modulators of Arachidonic Acid Metabolism - Prostaglandins (PGs) possess immunoregulatory activities but are generally thought to be immunosuppressive.[115-120] It is suggested that during lymphocyte activation endogenous PGE_2 acts as an inhibitory agent, while the generation of TxA_2 is stimulatory.[121] The role of the recently discovered leukotrienes and lipoxygenase products in the immune response is not yet apparent, although it has been shown that BW755C (3-amino-1-trifluoromethyl-phenylpyrazol-2-ine), an irreversible cyclooxygenase and lipoxygenase inhibitor, caused an inhibition of ConA-induced T_s-cell activation.[122] The potential use of indomethacin as an immunoadjuvant has been reviewed.[123] Indomethacin inhibited macrophage-mediated but not T_s cell-mediated suppression.[124]

Although selective TxA_2 inhibitors of the imidazole family decrease lymphocyte proliferation, their mode of action via TxA_2 inhibition has been questioned.[125,126] Other antiinflammatory agents such as aspirin and salicylic acid were effective in enhancing macrophage-mediated cytotoxicity but the more potent prostaglandin synthetase inhibitor, indomethacin, had no effect.[127] Similarly, in vivo DH responses using normal antigen sensitization were inhibited by indomethacin and aspirin, although neither drug affected DH responses from mice sensitized subliminally to antigen.[128]

Polymers - In attempts to improve the therapeutic index of pyran copolymer (NSC-46015), a synthetic polymer with a wide spectrum of effects on the immune response, maleic vinyl ethers were prepared.[129] These are several controlled Mol. Wt. (range 12,500 - 52,600) polymers with narrow polydisposition. The lower Mol. Wt. fractions are less toxic than the high Mol. Wt. forms and yet are biologically effective.[130,131]



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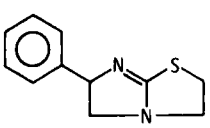
The pharmacology, distribution and toxicity of MV-2 (Mol. Wt. 15,500; 1 N = 56) has been extensively examined and it possesses antitumor, antiviral and host immune stimulatory actions.[130,131] The presence of a furan or a pyran is debatable and at present it is believed an equal mixture of the two exists. MV-2 appears to activate macrophages and the reticuloendothelial system.[132]

Levamisole And Related Compounds - Several reviews describing the immunopharmacology of levamisole (2) have appeared recently.[133,135] The compound influences several fundamental aspects of CMI and may be useful in the treatment of rheumatoid arthritis, ankylosing spondylitis, Reiter's syndrome and SLE. Several other heterocyclic compounds having the cyclic isothioureido moiety incorporated into a fused ring system possess immunological activity. Wy-13,876 (3) inhibited Lewis lung tumor and increased T cell rosetting,[136] but possessed thyrotoxicity in dogs and rats. The toxicity was attributed to a metabolite, 2-mercapto benzimidazole.[137] Wy-18,251 (4), an analog of Wy-13,876, was neither metabolized to 2-mercapto benzimidazole nor was thyrotoxic but did possess antimetastatic activity and stimulated DH reactions in mice and guinea pigs.[138,139] Unlike most immunomodulators, Wy-18,251 also possesses antiinflammatory activity and is twice as potent as aspirin in inhibiting PGE_2 biosynthesis in sheep seminal vesicles.[139]

Compounds 5 and 6 were the most potent of a series of hexahydronaphthimidazothiazoles that enhanced PFC and SRBC-induced DH in mice.[140] Compounds with trans-fusion of rings A and B appeared more potent stimulators of the PFC response than those with cis-fusion.

Wy-40,453 (7) was one of a series of thiazolobenzothiazoles shown to inhibit PFC in mice.[141,142] It also augmented DH induced by methylated bovine serum albumin (MBSA) and oxazolone in mice, although it reduced purified protein derivative (PPD) responses in guinea pigs. It exacerbated adjuvant arthritis, and possessed antitumor activity (Lewis lung) at low doses but was prometastatic at high doses. Its mechanism of action is not known, although it may suppress the reticuloendothelial system. TEI-3096 (8) inhibits ConA and lipopolysaccharide (LPS) lymphocyte blastogenesis, reduces PFC and enhances DH to SRBC in mice and suppresses adjuvant arthritis.[143]



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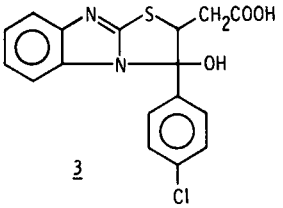

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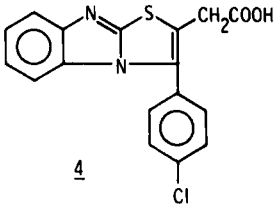

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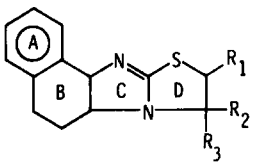
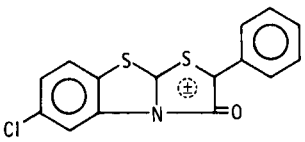


5 $R_1 = R_2 = R_3 = H$

6 $R_1 = Me, R_2 = OH, R_3 = Ph$



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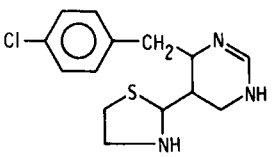
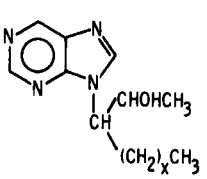


8

N,N-diethyldithiocarbamate (DTC) enhances cytotoxic, DH and T_s cell activity, and stimulates B cells to secrete IgG antibodies.[144] It promotes synthesis of hepatosin, a prothymocyte inducer released from the liver.[145] It is not effective in vitro and unlike many immunomodulators it is equieffective after either chronic administration or single treatments. DTC restores abnormal T cell mitogen responses and prevents immunodepression associated with surgery, anesthesia and aged cancer patients.[146] The effect of D-penicillamine on the immune system, as well as its pharmacokinetics and toxicity have been reviewed.[147-149] It appears to both stimulate and inhibit mouse T cell responses, depending on cell source examined (splenocyte and lymph node cells, respectively),[150] but does not influence monocyte function.[151] D-penicillamine is established as one of the few remission-inducing drugs in the treatment of rheumatoid arthritis, although side effects prohibit its widespread usage.

Isoprinosine And Related Compounds - Isoprinosine (inosiplex) is formed from inosine and the p-acetamidobenzoate salt of N,N-dimethylamino-2-propanol (DIP-PAcBA) in a 1:3 molar ratio. Its immunopharmacologic profile has been recently reviewed.[152,153] It is capable of modulating T cell proliferation, T cell secretion, cytotoxicity, T_s, T_h and macrophage function.[153,154] It reduces virally-induced clinical symptoms concomitant with reversal of virus-induced immunosuppression.[155] It has also been considered for use in cancer in conjunction with chemotherapy and has clinical benefits in rheumatoid arthritis. NPT 15392 (9) is a more potent inducer of T cells, than is isoprinosine, but, unlike isoprinosine, it augments ConA produced suppression.[156] Modification of the hydroxyalkyl side chain alters the activity, e.g., NPT 15465 (10) inhibited the immune response, whereas NPT 15461 (11) was a more potent stimulator than NPT 15392.[157]



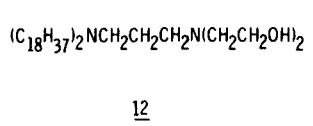
9 x = 4

10 x = 5

11 x = 6

The addition of DIP-PAcBA significantly potentiates the antiviral and immunomodulating properties of NPT 15461.[157]

Lymphokines And Their Inducers - Lymphokines devoid of interferon activity are presently being prepared from a human, non-leukemic, lymphoblastic cell line (RPMI 1788).[158] They cause primary tumor regression and prevent metastatic[158] spread possibly by enhancement of NK activity.[159] Interferons, which modulate many lymphocyte and macrophage functions, are being produced in large quantities using genetically engineered recombinant organisms. They are considered to be antineoplastic, antiviral and immunomodulating agents.[160,161] Numerous chemical and microbial agents have been described, which act as interferon inducers, yet many of these agents (e.g. poly I:C, tilorone) possess undesirable toxicity.[162,163] CP-20,961 (12)[164] and CP-28,888 (13),[165] two compounds from a series of lipoidal amines, were shown to act as interferon inducers. CP-20,961 also activates macrophages, stimulates humoral immunity and CMI, and possesses antitumor activity. Another compound within this series, CP-46,665 (14), is not an interferon inducer, yet possesses antitumor activity.[166] It appears that synthetic modification of lipoidal amines may produce selective interferon inducers, antitumor agents and/or adjuvants.[167]



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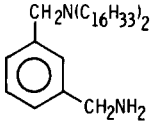

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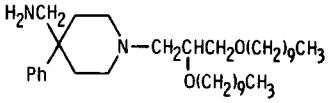

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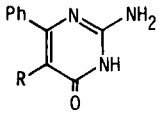
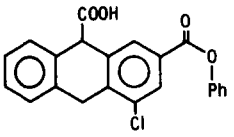


15; R= Br
16; R= I

In a series of 5-halo 6-aryl-4-pyrimidinones, there was no correlation between agents stimulating interferon production and their antiviral, antitumor or immunomodulating properties.[168,169] For example, 15 is a potent interferon inducer, whereas substitution of I for Br (16) drastically reduced interferon induction. Both compounds, however, had similar immunostimulatory effects.

Metals - Considerable interest has been aroused in the role of trace elements and metal complexes in immunocompetence, inflammation and cancer chemotherapy.[170,171] Zinc deprivation appears to inhibit immune function and is also associated with some immunodeficiency syndromes, malignancies and infectious disease.[172] The controversial use of zinc sulfate in rheumatoid arthritis was reviewed.[173] Zinc supplementation in old people improves immune function, as measured by circulating T cells and DH reactions.[174] The inhibition of T_h cell function by thiols, such as D-penicillamine, appears to require copper.[151] Other drugs may also owe a part of their *in vivo* immunopharmacologic activity to interaction with metal ions, such as copper.[175] Gold compounds, including sodium aurothiomalate and auranofin, appear to possess immunomodulatory activities *in vitro* and *in vivo* partly attributable to their inhibitory effects on macrophages.[176-178] Several platinum amines, including cis diamminodichloroplatinum (cisplatin), have been shown to possess stimulatory and suppressant immune effects.[179] Cisplatin is a selective anticancer agent and possesses prophylactic antiarthritic activity in rats.[175]

Ge-132 (carboxyethylgermanium sesquioxide), an organogermanium compound under consideration as an antirheumatic and antineoplastic agent, induces interferon and augments NK activity in man.[180] In man, mitogen responses were enhanced after lithium therapy, and production of migration stimulatory factor, a product of T_S cells, was inhibited.[181] It is proposed that lithium abrogates T_S cells[181,182] and stimulates T_h activity.[183]



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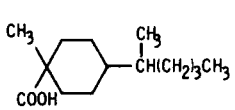

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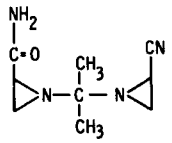

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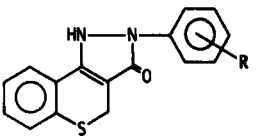

20

Miscellaneous - CCA (17) is antiarthritic in rats, inhibits proteinuria in NZB/W-F_1 mice and is immunomodulatory, especially in immunodepressed animals.[184,185] It may assist maturation of T cells and induce the function of these cells.[186] Preliminary clinical studies indicate that CCA normalized imbalances in T cell subsets in rheumatoid arthritis during a 16 week open trial, although it was not therapeutically beneficial.[187]

IG-10 (18), a compound corresponding to the E ring of glycyrrhizin inhibited experimental DH in guinea pigs and mice and IgE-mediated hypersensitivity.[188] IG-10 also inhibits MIF and macrophage chemotactic factor and is clinically effective in asthma and atopic dermatitis.[189]

The immunomodulating activities of azimexon (19) have been recently reviewed.[190-192] It stimulated both DH and humoral immunity to SRBC in mice when administered before SRBC administration; however, suppression of antibody production was observed when it was administered after antigen. Preliminary studies in man indicate that azimexon ameliorates immunodepression in cancer after chemotherapy and irradiation.[190]

A number of benzothiopyrano-4,3-c-pyrazol-3-ones were shown to be potent inhibitors of both humoral and cellular immune responses developed in mice in response to EL_4 tumor cells.[195] CP-17,193 (20, R=Cl) was chosen for further studies and inhibited humoral and cellular responses to SRBC, yet stimulated lymphocyte proliferation.[196] The mechanism may involve an effect on the formation of T cell blasts.[197]

Monoclonal Antibodies (McAbs) - In addition to their immunodiagnostic utility. McAbs and their fragments may provide potent and specific therapy of use in autoimmune diseases, as well as in transplantation and cancer.[198-200] In this regard, they offer advantages over currently available immunosuppressants and heteroantisera, and they may also be useful as delivery systems for immunomodulating drugs. Already, murine OKT3 McAb has been shown to stimulate human T cell proliferation and production of immune interferon and to inhibit mitogenic responses of human lymphocytes.[201] OKT4 is immunosuppressive when given to monkeys and prolongs kidney graft survival.[202]

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Chapter 21. Lipoxygenase and the Related Arachidonic Acid Metabolites

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Introduction - Interest in and investigation of mammalian lipoxygenase systems and their products has continued to contribute to the rapidly expanding bibliography of this area. This review will deal largely with citations in 1981 but will include some work of early 1982.

Reviews devoted to arachidonate lipoxygenase,[1,2] and the subject of leukotrienes (LTs) as mediators of inflammation and hypersensitivity,[3-6] have appeared. Structure, biosynthesis and pharmacological actions of prostaglandins (PGs) and leukotrienes have been reviewed,[7] as have those of the slow-reacting substances (SRSs).[8,9] Nutritional factors in eicosanoid biology have also been considered.[10] A symposium on LTs held in September of 1980 has been reviewed;[11] and the proceedings have been published in book form.[12] A report on the 1981 international LT conference in Florence has also appeared.[13] The relationship of lipoxygenase products to immune mechanisms in immediate hypersensitivity was included in a symposium in print on clinical allergy.[14] Leukotriene B_4 (LTB_4) was the topic of a minireview.[15]

Numerous chemical syntheses of LTs,[16-21] hydroxy- and dihydroxy-eicosatetraenoic acids (HETEs and DHETEs),[22-24] as well as derivatives and isomers[25-28] and versatile intermediates,[29-31] have been published. These and others are reviewed in detail elsewhere in this volume.[32]

Cascade - Figure 1 depicts the major cascade products from the lipoxygenase oxidation of arachidonic acid (AA). Incubations of human leukocytes have yielded two new LTs, 8,15-LTB_4 and 14,15-LTB_4 (each a mixture of two isomers) that arise through initial oxidation of AA at C-15.[33] The intermediacy of the 15-oxygenated AA is supported by the fact that these same metabolites are formed if 15-HPETE (from the action of soybean lipoxygenase on AA) is added to leukocyte preparations.[34] Incubations of porcine polymorphonuclear leukocytes (PMNL) with AA also resulted in products arising from 15S oxygenation of the substrate.[35] These materials were identified as diastereomeric 8,(15S)-dihydroxy-5,9,11,13-eicosatetraenoic acids and two erythro-14,15-dihydroxy-5,8,10,12-eicosatetraenoic acids.

In rat thyroid incubated with AA, at least two new metabolites have been tentatively identified as arising through lipoxygenase reactions - a diketomonohydroxyeicosatrienoic acid and a monoketodihydroxyeicosatrienoic acid.[36] Reciprocal effects of iodide and a peroxidase inhibitor in this system suggest the first example of a functional link between the metabolism of AA and the metabolism of iodide by the thyroid. The metabolism of AA in this tissue is stimulated by 5,8,11,14-eicosatetraynoic acid (ETYA), an anomaly that must be reconciled before final identification of these metabolites as lipoxygenase products can be made.

Human PMNL have provided a rich source of new AA metabolites. An ω-hydroxylated metabolite of LTB_4 has been identified.[37] It and the corre-

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sponding ω-carboxylic acid isolated from the same incubation were 10X as potent as histamine in producing contraction of guinea pig lung strips. Omega hydroxylation of a novel dihydroxy acid, 5S,12S-dihydroxy-6-trans-8-cis,10-trans,14-cis-eicosatetraenoic acid (5S,12S-DHETE), had previously been noted in the same system.[38] In the latter studies it had been concluded that these materials had been formed from double and triple oxidation of AA and had not arisen through LTA_4. They are not, therefore, regarded as LTs.

Details of the formation of 11-trans-LTC_4 from LTC_4 were studied in rat basophilic leukemia, (RBL-1) cells.[39] It was concluded that LTC_4 isomerizes via thiyl radical catalysis.

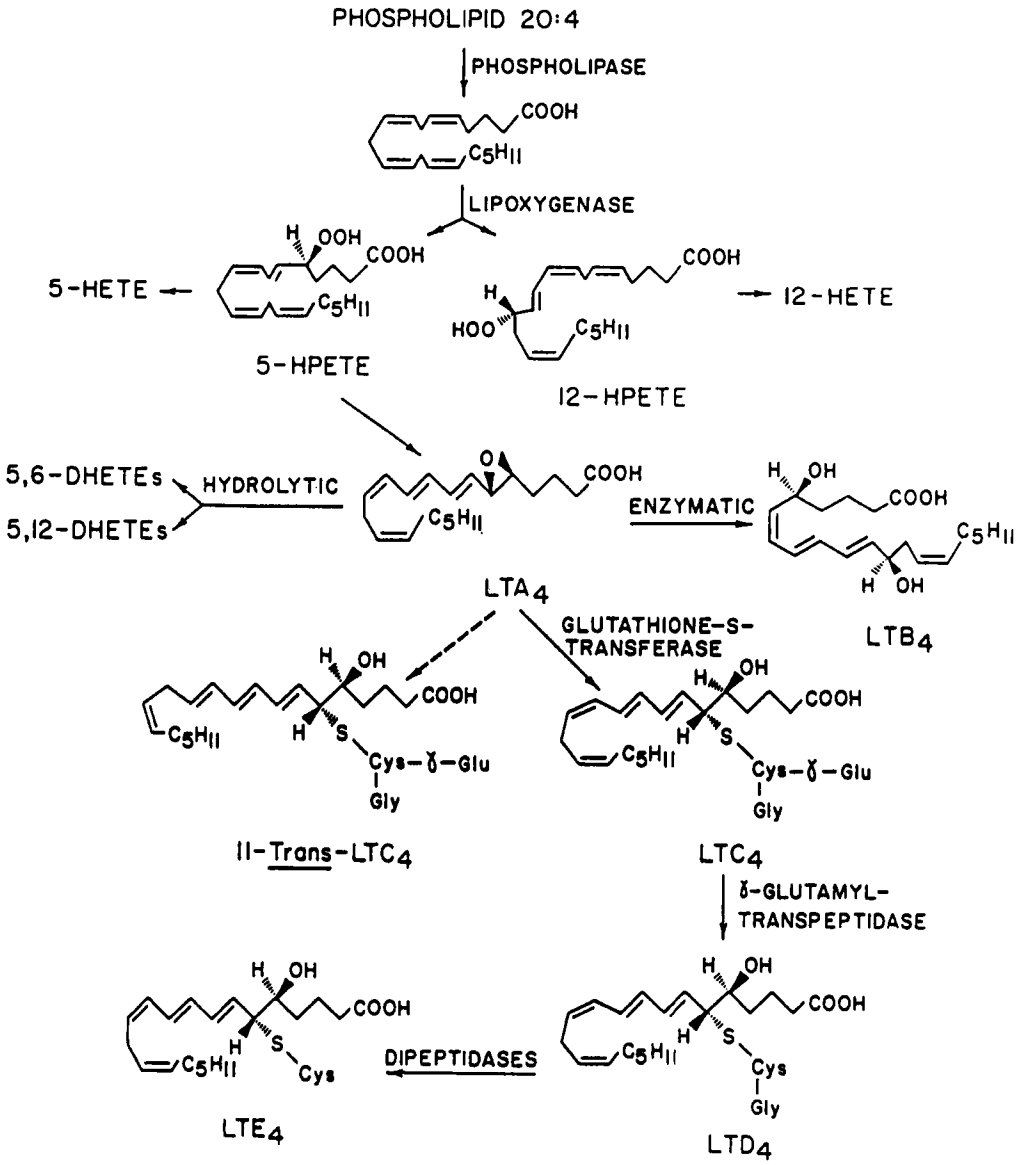


FIGURE I

The enzymatic inactivation of LTC_4, LTD_4 and LTE_4 by peptidases and arylsulfatases has been further examined. Purified arylsulfatase B from human eosinophils was competitively inhibited by synthetic LTC_4, LTD_4 and LTE_4 but the contractile activity of these LTs on guinea pig ileum was not inhibited by the enzyme.[40] Purified arylsulfatase from limpets or guinea pig lung was ineffective in cleaving the cys-gly bond of LTD_4 (guinea pig ileum assay).[41] Peptidases in preparations of these arylsulfatases and those from RBL-1 cells and peritoneal eosinophils inactivated LTD_4 but not LTE_4. Incubation of LTD_4 and 11-trans-LTD_4 with porcine kidney preparations enriched in a dipeptidase resulted in the formation of LTE_4 and 11-trans-LTE_4. The latter two metabolites were equieffective in contracting guinea pig ileum at a potency level 8 - 12X less than LTC_4.[42]

Lipoxygenase does not show substrate specificity for AA and the action of this enzyme on other polyunsaturated fatty acids has been extensively examined. Mouse mastocytoma cells incubated with 5,8,11-eicosatrienoic acid and stimulated with ionophore A23187 produced LTC_3 and 11-trans-LTC_3.[43] Treatment of these isomers with kidney γ-glutamyl transpeptidase produced LTD_3 and 11-trans-LTD_3. These metabolites were bioactively equivalent to their LTC_4 and LTD_4 analogs on guinea pig ileum. Further treatment of LTD_3 with dipeptidases produced LTE_3.[42] Labeled LTC_3 given intravenously to mice resulted in significant concentration of it and its metabolite LTD_3 in liver, bile and small intestine.[44] Radioactivity in lung consisted predominantly of LTE_3. In monkeys, LTC_3 was rapidly converted to LTD_3 and LTE_3.[45] Guinea pig liver and lung homogenates partly converted LTD_3 back to LTC_3, presumably by γ-glutamyl transpeptidase using glutathione as a glutamyl donor.[46] Dihomo-γ-linolenic acid was similarly converted by mouse mastocytoma cells to 8,9-LTC_3.[47] Preparations of RBL-1 cell converted 5,8,11,14,17-eicosapentaenoic acid into the previously reported[48] LTC_5 and its metabolites LTD_5 and LTE_5.[49] Labeled studies with this substrate have been performed with mouse mastocytoma cell suspensions.[50] In mice fed a fish oil diet rich in eicosapentaenoic acid, a maintained neoplastic mast cell tumor line showed cell levels of AA and eicosapentaenoic acid of 3.9 and 4.5 mg%, respectively, and developed similar levels of LTB_4 and LTB_5. In contrast, the ratio of LTC_4 to LTC_5 was 10:1, suggesting a strong dependence on structure for the reaction of glutathione with the LTA_5.[51]

Enzyme Location and Characterization - Neoplastic cells continue to be of interest as sources of lipoxygenase enzymes.[41,43,48,50,51,53] Both interferon-resistant and interferon-sensitive clones of L1210 mouse leukemia cells demonstrated lipoxygenase activity; cyclooxygenase activity was absent in the former.[54] The human promyelocytic leukemia cell line HL60 produced largely 5-HETE and LTB_4, along with 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT).[55,56] It was noted, however, that blood cyclooxygenase and lipoxygenase activities were reduced from normal in patients with chronic myelogenous leukemia.[57]

Rat alveolar macrophages stimulated with ionophore A23187 released material with SRS-like activity on guinea pig ileum.[58] Incubation of these cells in the presence of aspirin produced 12-HETE as the only detectable lipoxygenase product.[59] SRS-like activity has also been observed with rabbit alveolar macrophages.[60] Peritoneal tissue from this species yields largely 15-HETE.[61] A lipoxygenase product other than a HETE is proposed as a stimulant of insulin release in the rat pancreatic islet cell.[62] Rat neutrophils from 3-hour carrageenan pleural exudate metabolize exogenous AA to HHT, 11-HETE and 15-HETE, but under stimulation by ionophone A23187 also produce 5-HETE and LTB_4.[62] Two-day old mouse keratinocytes also

respond to ionophore stimulation in the presence of added AA to give the same products (LTB_4 inferred by detection of LTD_4).[63]

Studies with rabbit kidney suggest the presence of lipoxygenase activity in papillary and cortical microsomal fractions.[64,65] Later studies indicate that while renal medullae produced 12-HETE and 15-HETE, the cortex did not metabolize AA via lipoxygenase pathways.[66] Lipoxygenase products were identified in human gastrointestinal tissues,[72] synovial fibroblasts[73] and neutrophils.[74,75] Involvement of the lipoxygenase system in mononuclear leukocyte-induced suppression of lymphocyte responses in vitro could not be demonstrated.[76] Other cell and tissue types shown to possess lipoxygenase activity were horse eosinophils,[77] chicken thrombocytes,[78] pig lung[79] and dog spleen.[80] Several papers have described reticulocyte lipoxygenase systems acting directly on mitochondriol phospholipids.[81-84,85] A quasi-lipoxygenase reaction was reported to be catalyzed by haemoglobin.[86]

Arachidonate 15-lipoxygenase from rabbit PMNL was partially purified and characterized.[87] It had an apparent molecular weight of 61,000 daltons and was sensitive to ETYA and BW755C but not indomethacin. Both calcium-dependent and calcium-independent isozymes of a soybean lipoxygenase have been isolated and their properties examined.[88] The use of mass spectrometry for biomedical problems, including structural elucidation, has been reviewed.[89] Reports of deuterium nuclear magnetic resonance and electron paramagnetic resonance spectroscopy studies on soybean lipoxygenase have appeared.[91] The use of high performance liquid chromatography continues to be of value in the separation of lipoxygenase products.[92-94] Methods reported for quantification of lipoxygenase products included measurement of chemiluminescence,[95] radioimmunoassays[96] and various procedures depending on thiol reactivity of products.[97-100]

Actions of Cascade Products - Historically, SRS-A, LTC_4 and LTD_4 have been demonstrated to characteristically contract guinea pig ileum smooth muscle preparations; likewise, it has been shown that LTE_4 contracts this tissue.[101] The contraction due to LTC_4 was weaker and of shorter duration than that due to LTD_4.[102] The most active LTD_4 configuration was 9-cis-5(S),6(R); 9-cis-5(R),6(S) LTD_4 was only 1/100 as active.[103] Additionally, the NH_2-terminal amino, free glycine carboxyl and 5-OH were essential for activity while the free eicosanoid carboxyl was not.[104,105] Histamine induced the early phase, and LTD_4 the late phase of antigen-stimulated contractions.[106] The SRS-A and LTD_4-induced ileum smooth muscle contraction involved opening of calcium channels as the initial event.[107] LTA_4 did not induce ileum contraction,[108] while LTB_4 has been reported to induce weak contraction,[109] none at all[108] or increase spontaneous contraction.[110] LTB_4 did contract guinea pig duodenum[109] and lung parenchyma.[108,109,111,112] Both naturally derived and synthetically prepared LTB_4 were active.[111] Only the naturally occurring 5(S)12(R) LTB_4 6,14 cis, 8,10-trans isomer retained contractile activity.[112,113] Lung parenchymal strip responses to LTD_4 and SRS-A were blocked by indomethacin, suggesting the involvement of thromboxane (TX) and possibly other cyclooxygenase products.[102,114] SRS-A, LTC_4, LTD_4 and RBL-1 cell-derived SRS contracted guinea pig lung strips to a much greater degree than the tissues of rabbit or rat.[114] Lipoxygenase products of AA metabolism contracted guinea pig lung parenchymal strips but did not affect basal tone.[115]

LTC_4 contraction of guinea pig trachea and bronchus was biphasic, while that of parenchymal strip was sigmoidal.[116] LTC_4 and LTD_4 equally contracted guinea pig trachea; neither contracted rat trachea and isomers

of LTD_4 (7,9-trans,11,14-cis and 7,9,11-trans, 14-cis) were much less active.[117] Both LTC and LTD contracted human lung smooth muscle bronchus, pulmonary artery, pulmonary veins and parenchymal strips.[118,119] Rabbit pulmonary artery and veins and portal veins were less responsive to LTC_4 and LTD_4 than were coronary arteries. Renal artery and veins, mesenteric artery and thoracic aorta were unresponsive.[120] SRS-A induced contraction of isolated guinea pig stomach preparations[121] and was apparently involved in C_{5a}-induced guinea pig tracheal contraction.[122] Lipoxygenase products were involved in the endothelial-dependent inhibition of acetylcholine relaxation of femoral artery.[123] The canine basilar artery isolated from brain was contracted by 13-HPETE.[124]

Aerosol LTC_4 was bronchoconstrictive in man,[125] monkeys,[126] and guinea pigs.[127] In the latter, the reaction was independent of cyclooxygenase inhibition while i.v. LTC_4 effects were blocked by indomethacin.[128] LTE_4 given i.v. induced bronchoconstriction in guinea pigs.[129] Lipoxygenase products were responsible for hyperactivity of guinea pig pulmonary responsiveness to aerosol histamine.[130] A model of SRS-A component bronchoconstriction in guinea pigs has been described.[131]

SRS-A impairs pulmonary mucus transport in asthmatic patients.[132] Mucus secretion was enhanced by HETE and LT synthesis in human bronchiol tissue.[133] It has been demonstrated that 5-HETE,[134-136] as well as 8-,11- and 12-HETE[134] increased mucus release. The actions of 5-HETE were blocked by dexamethasone pretreatment.[135]

Parasympathetic stimulation of nasal mucosa induced the release of SRS with increased mucosal blood flow in cats.[137] LTD_4 increased the vasopermeability of the pulmonary vasculature in isolated rabbit lung preparations.[138] Both LTC_4 and LTD_4 increased vasopermeability in guinea pig skin, but were much less active in rat and rabbit tissue.[139] LTE_4 increased vascular permeability in rat skin.[129] Injected LTB_4 increases vascular permeability in the skin of rabbits,[140] humans and monkeys.[141] The effects of both LTB_4 and LTD_4 in the latter two species were enhanced by PGD_2. PGE_2 was required for the action of LTB_4 in rabbit[140,142,143] and guinea pig[142,143] skin. LTC_4 was a more potent vasoconstrictor than LTD_4.[127,144] The action of LTC_4 was augmented by PGE_1 and that of LTD_4 by PGE_2.[144] Topically applied LTC_4 and LTD_4, but not LTB_4, induced transient vasoconstriction with plasma leakage.[145] Both LTC_4 and LTD_4 constricted rat cerebral arteries in vivo. LTB_4 and several HPETEs enhanced bradykinins induced plasma exudation.[143,146]

Vasodilatation of rat and rabbit gastric circulation by lipoxygenase derived hydroperoxides was of the potency order: 12-HPETE > 11-HPETE > 5-HPETE > 15-HPETE, while 15-HPETE blocked vasodilation by PGH_2 leading to the speculation that these lipoxygenase products modulate gastric mucosa circulation.[147,148] Both 5-HETE and 12-HETE had little effect on rabbit or rat gastric vascular resistance.

Transient pulmonary and systemic hypertension followed by prolonged hypotension were seen after LTC_4-induced bronchoconstriction in monkeys[126] and guinea pigs.[128] Intravenously administered LTC_4 caused an increase in blood pressure in rats and in vitro caused contraction of distal, but not proximal pulmonary artery.[149] LTC_4, and to a lesser effect LTD_4, decreased coronary flow in guinea pig heart.[150-152] Both positive[152] and negative[150] inotropic effects have been reported. A triphasic response of hypotension with bradycardia, hypertension with tachcardia and hypotension with bradycardia has been reported in SHR rats[153] but not in other

strains.[151,153]

The chemotactic activity of LTB_4 has been reviewed.[154] It is apparently equipotent to C_{5a}[177,155] and formyl-met-leu-phe.[177] Both naturally occuring and synthetically prepared LTB_4, were the most active of several isomers for leukocyte chemotaxis,[111,112,155] chemokinesis[156,157] and aggregation.[156-158] 5-HETE and 12-HETE, but not 8-,9-,11- or 15-HETE, aggregate human leukocytes. The action of these compounds was blocked by indomethacin.[163] LTB_4 also stimulated endothelial adherence of PMNLs in rabbits[159] hamsters[145,160] and humans.[161] LTB_4 desensitized PMNLs to further compound-induced aggregation.[158] Likewise, both LTB_4 and 5-HETE deactived cells to compound-specific chemotaxis while the effects of 5-HPETE were non-specific.[162] LTB_4 was also chemotactic in vitro for guinea pig eosinophils, human monocytes and rat macrophages, but not in vivo for monocytes in the hamster.[160] LTB_4 caused leukocyte infiltration in the skin of monkeys[111,141] and humans.[125,141] A less completely described 5,12-DHETE increased PMNL infiltration in rabbit skin. Lipoxygenase products[164] and specifically LTB_4[165,166] caused infiltration of leukocytes into the aqueous humor of the rabbit eye without increasing occular pressure.

Eosinophil chemotactic factor, a lipoxygenase product initially obtained from neutrophils, was chemotactic for eosinophils.[167] It has been additionally isolated from mast cells.[168-171]

Conflicting reports concerned degranulation and enzyme release. There were reports that 5-HETE[172,173] and 5,12-DHETE[174] induced degranulation of human PMNLs. Another report stated that while 5,12- and 5,6-DHETE had no effect, LTB_4 and 5-HETE released lysozyme but not β-glucuronidase.[175] Yet another report stated that LTB_4 released both enzymes from cytochalasin B-treated PMNLs.[176] Similarly treated cells have been reported to release lysosomal enzymes (not further identified) in response to LTB_4.[177,178] This latter activity apparently involved both microtubules and microfilaments.[179]

Increased membrane permeability to calcium has been observed for LTB_4,[180-183] 5-, 11-, and 12-HETE, 5- and 11-HPETE and formyl-met-leu-phe, but not 15-HETE or 15-HPETE.[182] 5-HETE also stimulated hexose uptake by human PMNLs.[183]

Platelets from asthmatics[184] and patients with chronic idiopathic thrombocytopenia purpura[185] synthesized increased amounts of 12-HETE and less TX and HHT than those from normal individuals. 12-HPETE and 15-HPETE inhibited both AA-induced platelet aggregation and thrombus-induced TXB_2 formation in platelets, while 12-HETE and 15-HETE were inactive.[186,187] AA-induced platelet aggregation and serotonin release were dependent upon both cyclooxygenase and lipoxygenase activity.[188] The lipoxygenase product of 5,8,11 eicosatrienoic acid was reesterified in the membrane of platelets and thereby increased platelet susceptability to thrombin-induced aggregation.[189] LTB_4 infusion in rabbits had no effect on platelet counts.[160]

In guinea pig lung, SRS-A stimulated the release of both lipoxygenase and cyclooxygenase activity, while histamine stimulated only cyclooxygenase.[190] Specifically, both TXA_2 and 12-HETE syntheses were elevated.[191] SRS-A did not stimulate TXA_2 from guinea pig heart.[192] LTC_4 induced release of TXA_2 from perfused guinea pig lung[193] and elevated both TXA_2 and PGI_2 in vivo in guinea pigs.[194] Both LTC_4 and LTD_4 induced

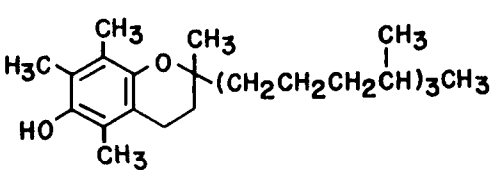
release of TXA_2, PGE_2 and 6-keto $PGF_{1\alpha}$ from rat peritoneal macrophages.[195-197] LTD_4 alone inhibited SRS-A release from monkey lung tissue.[198] With respect to other lipoxygenase products, 15-HETE blocked AA metabolism to 12-HETE in mouse thyroid homogenate. Further, 12-HETE reduced PG synthesis.[199]

The anaphylactic release of SRS-A from guinea pig lung tissue was accelerated by $PGF_{2\alpha}$, $PGF_{1\beta}$ and inhibited by PGI_2, $PGF_{1\alpha}$, PGE_1 and PGA_1.[200] Porcine alveolar macrophages inactivated and somewhat inhibited the release of SRS-A from lung tissue.[201] Both lipoxygenase and cyclooxygenase products appeared to augment histamine release.[202] In this respect, 5-HPETE was approximately 3-10 times more active than 5-HETE.[203] LTC_4 by itself stimulated histamine release from rat peritoneal cells and augmented 48/80-induced release, while LTD_4 inhibited 48/80-induced release and had no direct effect on secretion.[204]

Regarding other actions, lipoxygenase products mediated the increase in cGMP levels following phytohemagglutinin stimulation of lymphocytes.[205] More specifically, 5-,8-,9-,11- and 12-HETE all stimulated amylase release from guinea pig pancreatic acini.[206] LTD_4, but not LTB_4, induced prolonged excitation of rat cerebella Purkinje cells.[207]

Lipoxygenase, but not cyclooxygenase, appeared to be involved in the incorporation of phosphatidic acid into mast cell membrane phospholipids.[208] Lipoxygenase peroxidation of mitochondiral membrane lipids of retoculocytes appeared to be involved in the process of inactivation and degranulation of cell organelles.[209]

Modulation of Synthesis and Actions - Although no "pure" inhibitor of mammalian lipoxygenase has appeared, much work has been dedicated to the clarification of actions of the myriad non-specific effectors of this enzyme system. Antioxidants such as α-tocopherol (**1**) (vitamin E), butylated hydroxytoluene and butylhydroxyanisole (BHA) have been examined *in vitro*.[210] In these studies, BHA at low concentrations was shown to have differential effects on cyclooxygenase and lipoxygenase activity, inhibiting the former and stimulating the latter in suspended human platelets. Using vitamin E and the chromanecarboxylic acid (2) Wolf *et. al.* have examined AA-induced pulmonary vascular resistance in isolated rabbit lung and have concluded that the phytol side chain contributes to the lipoxygenase-induced permeability increases while this effect is inhibited by the chromane part structure.[211] *In vivo* effects of vitamin E and another antioxidant, propylgallate, have been briefly reviewed.[212] The highly oxidizable fatty acids in fish oils are reputed to have beneficial effects due to their slowing of LT production.[213]



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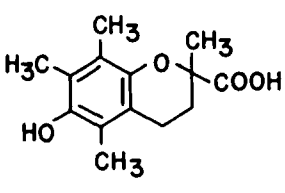

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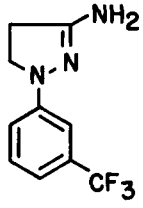


3

An interactive study of sulfhydryl reagents and lipoxygenase inhibitors on soybean lipoxygenase produced the interesting finding that mercuric chloride inhibition of the enzyme can be completely reversed by thiol compounds, phenidone inhibition can be inhibited but not reversed, and dihydroxynaphthalene, nordihydroguaiaretic acid and acetone phenylhy-

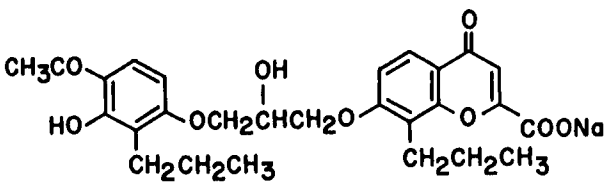
drazone inhibition is not affected.[214] The modification of a few "essential" enzyme sulfhydryl groups was held responsible for AA release in human platelet membranes.[215]

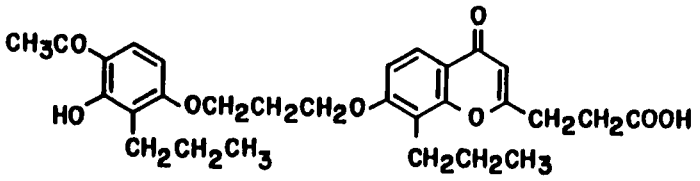
Numerous papers have appeared describing the action of ETYA and other acetylenic acids on lipoxygenase systems. In ionophore A23187-stimulated guinea pig peritoneal PMNL, ETYA enhanced the formation of 5-HETE but markedly inhibited the formation of DHETEs.[216] The use of ETYA, as well as nordihydroguaiaretic acid and phenidone as antagonists in low, medium and high substrate AA-induced contraction of guinea pig lung strips, suggested that cyclooxygenase products are predominantly involved in the early phase of tissue contraction and lipoxygenase products are predominantly involved in the late phases.[217]

In another study, ETYA and phenidone each inhibited antigen-induced contraction of human bronchi and guinea pig trachea without inhibiting the antigen-responsiveness to histamine.[218] In canine neutrophils, ETYA but not aspirin or indomethacin blocked the AA-induced hexose monophosphate shunt.[219] Aerosolized ETYA is effective in inhibiting responses to threshold and standard antigen challenge in the rhesus monkey (ascaris) model of asthma.[220,221] The acetylenic acid is the first agent, other than β-agonists or cromolyn, to have shown this action. In this same model BW755C (3) blocked the antigen-induced increase in pulmonary resistance and decrease in dynamic compliance but was ineffective in amelorating the abnormalities in respiratory frequency, peak expiratory flow and tidal volume.[222] Given subconjunctivally in the rabbit eye, ETYA was able to block the inflammatory infiltration of polymorphonuclear leukocytes induced by the C5a component of complement and other pro-inflammatory agents, but the target enzyme was not identified.[223]

Three acetylenic acids (4,7,10-hexadecatryynoic acid, 4,7,10,13-nonadecatetraynoic acid and 4,7,10,13-heneicosatetraynoic acid) inhibited SRS activity in RBL-1 cells but appeared to act on glutothione transferase rather than on 5-lipoxygenase itself.[52] By contrast, 4,7,10,13-icosatetraynoic acid (as well as 5,8,11,14-henicosatetraynoic acid) were reported as highly selective inhibitors of 12-lipoxygenase in human platelets.[224,225] In a series of diynoic acids, peak inhibition of sheep seminal vesicle cyclooxygenase was reported with the C_{18} acid while peak inhibition of human platelet lipoxygenase occurred with the C_{21} acid.

The SRS antagonist FPL 55712 (4) and the related FPL-59257 (5) have been used clinically in two subjects in an attempt to prevent LTC_4 and LTD_4-induced bronchoconstriction.[228] In these studies, aerosolized FPL-59257 provided partial protection, while FPL55712 clearly inhibited the bronchoconstrictor response. In a short open study in four chronic asthmatics, FPL55712 gave equivocal results.[229] Krell, et. al. have reported that FPL55712 showed a biphasic effect on LTD_4-induced contractions in guinea pig parenchymal lung strips and was ineffective in antagonizing the actions of LTC_4 in this tissue.[230] Anomalous results were obtained with FPL55712 and LTC_4 on guinea pig trachea. In RBL-1 cells, FPL55712 blocks the formation of 5-HETE and 5,12-DHETE.[53]





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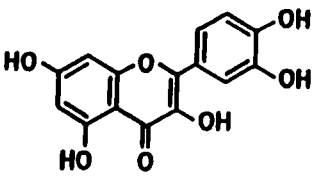
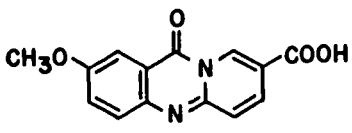


6

Intravenous aspirin but not salicylate or cromolyn prevented LTC_4 and LTD_4-induced bronchoconstriction and hypotension in guinea pigs.[231] In human platelets stimulated with collagen, aspirin inhibited the formation of lipoxygenase products, as well as those of cyclooxygenase with the action on the former enzyme being manifested primarily by suppression of HETE formation.[232]

Numerous in vitro studies with BW755C (3) have been published. Examples of tissues and cells examined were chopped human[233] and guinea pig[234] lung, human leukocytes[235] and rat peritoneal cells.[236]



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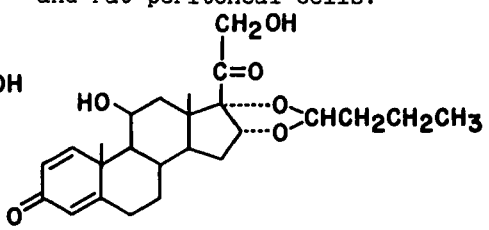

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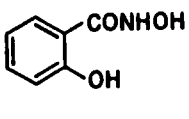
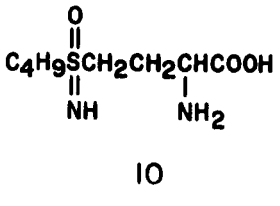


9

Quercitin (6) was shown to inhibit the 12-lipoxygenase from human platelets[237] but estradiol apparently stimulates this same enzyme.[238,239] The antiallergy agent RO21-7634 (7) inhibited the release of SRS-A from rat peritoneal cells and guinea pig lung tissue.[240] In the latter preparation, the effect on histamine release was considerably less than that on SRS-A. In a study using glucocorticoids, a similar differential effect on antigen-induced SRS-A and histamine release from sensitized guinea pig lung was noted with beclomethasone dipropionate and budesonide (8).[241] A direct inhibtion by glucocorticoids of LTC_4 and LTD_4-induced vascular permeability in rat skin has been demonstrated.[242] The ATP-dependent proteolysis of mitochondria that occurs in rabbit reticulocytes is attenuated by salicylhydroxamate (9).[85] This effect is attributed to inhibition of cytosolic lypoxygenase by the drug. In preparations of mouse peritoneal macrophages, buthionine sulfoximine (10) a γ-glutamylcysteine synthetase inhibitor, produced a time-of-exposure-dependent decrease in zymosan-stimulated release of LTC_4.[243] The decreased production of LTC_4 was accompanied by compensatory increases in the levels of HETEs. The release of SRS and platelet activating factor (PAF) from human PMNL was inhibited in vitro in a concentration-dependent manner by the calcium antagonist nifedipine (11).[244] At concentrations above 10^{-6}M, the effect of 11 on PAF was less than that on SRS. Although the chlormethyl ketone, 12, inhibited the formation of 12-HETE in human platelets, the drug's effect on TXB_2 formation was much greater.[245] A large number of arylhydrazine derivatives, among the most potent of which was 13, were examined for their ability to inhibit human platelet and soybean lipoxygenase and sheep seminal vesicle cyclodioxygenase.[246] One to two orders of magnitude higher concentrations were required to inhibit the cyclodioxygenase than the lipoxygenase. Sulfasalazine (14), a drug used in ulcerative colitis, and one of its metabolites, 5-aminosalicyclic acid, have been found to block the synthesis of 5,12-DHETE in human neutrophils.[247] The parent drug additionally blocks the synthesis of 5-HETE in the same preparation.



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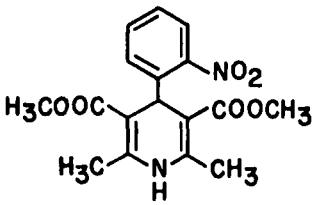

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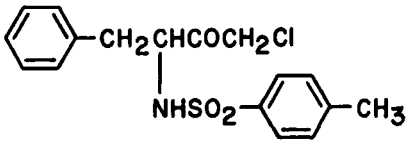

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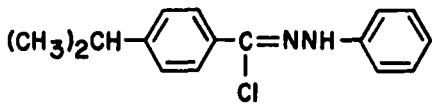

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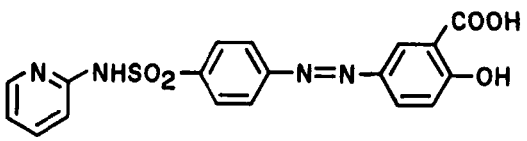
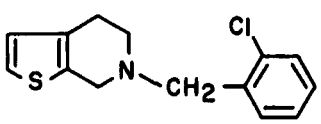


14

Inhibitors of cyclic nucleotide phosphodiesterases completely blocked the zymosan-stimulated formation of LTB_4 in human PMNL, and this action was attributed to the inhibition of synthesis of LTB_4 by elevated levels of cyclic AMP.[248] In sensitized and normal guinea pig trachea, isoprenaline, aminophylline and dibutryl cAMP antagonized LTD_4 contractions.[249] A feedback inhibition by 15-HETE on the production of 5-HETE and LTB_4 by human T-lymphocytes has been noted.[250]

Adolapin, a peptide from bee venom, was reported to be a lipoxygenase inhibitor.[251]

When ticlopidine (**15**) is added to cultures of mouse peritoneal macrophages, there is an increased production of 12-HPETE and 12-HETE.[252] Paradoxically, prostacyclin production is not inhibited in this preparation.



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Section V - Topics in Biology

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Chapter 22. Protein Growth Factors

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Introduction - Growth factors are a class of naturally occurring hormone-like proteins that cause cells to increase in size or number. These factors are important not only for embryonic development and subsequent growth but also for the maintenance of viability and differentiated characteristics by their target cells. In this context, they may facilitate both the normal cell replacement and the responses to wound healing in adults. Aberrant control by growth factors appears to be involved in a variety of disease states.

From this perspective, the four types of growth factors that have been purified and characterized will be reviewed. These are nerve growth factor (NGF), epidermal growth factor (EGF), the somatomedins (SM) and platelet derived growth factor (PDGF). Recent individual reviews of NGF[1-5], EGF[6,7], SM[8-10] and PDGF[11] have appeared. General background material that is included here but is covered in more detail in these previous reviews is usually not referenced.

Nerve Growth Factor - The first protein growth factor to be purified, NGF, causes neurite growth from sympathetic and embryonic sensory neurons. In the developing animal the sympathetic neurons require a continual supply of NGF to survive since antibodies to NGF destroy them by a complement-independent mechanism.[12] Adult animals also have been shown to require the presence of NGF for the maintenance of differentiated characteristics and target cell survival.[13]

The physiologically important sources of NGF are thought to be the non-neuronal cells that form synapses with the responsive neurons. These are the end-organ cells of sympathetic innervation. It appears, therefore, that the innervated end-organs support the innervating neurons. The chemotactic activity of NGF for neurites of responsive neurons may help direct them to their appropriate target.[14] Those sympathetic neurons that are unable to synapse with NGF-secreting cells are assumed to die. This type of selective survival may explain, in part, the extensive neuronal cell death observed during embryonic development. Prior to synapse formation, the supply of NGF to the immature neurons may be supplemented from the placenta.[15]

As expected from the large number of targets of sympathetic innervation, a variety of organs and cells produce and secrete NGF in culture. Nevertheless, in some animals high concentrations are found in specific tissues or secretions. Elevated NGF levels were originally observed

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in the venom of poisonous land snakes. Similarly high concentrations have been found in the male mouse submaxillary gland (2% of the soluble protein) and saliva. These levels are only high in the male and are under testosterone control. Recently, the prostates and/or semen of guinea pig[16], bull, sheep and goat[17] also have been reported to have high NGF levels. In spite of the uncertain significance of these locally high concentrations, they have provided sufficient material for purification and characterization.

The most extensively studied NGF is that from the male mouse submaxillary gland. The growth factor is stored in intracellular granules and secreted into saliva as a multisubunit complex of 140,000 molecular weight composed of two α-subunits (MW ≈ 26,500 each), a stable β-dimer (MW = 13,250 per monomer) and two γ-subunits (MW = 28,000 each). The entire complex is substantially stabilized by 1 or 2 tightly bound zinc ions. The function of the α-subunits is unknown. The stable β-dimer, formed from two identical subunits, can independently elicit neurite outgrowth *in vitro*. The γ-subunit, a serine protease of arginine specificity, can cleave pro-NGF, a 22,000 dalton protein, to the physiologically relevant 13,250 dalton form found in the high molecular weight complex. The 233 residue γ-subunit recently has been sequenced and shown to be a glycoprotein having about 40% sequence identity with trypsin.[18]

The active 118 residue β-subunit from mouse (pI = 9.3) has been sequenced and shown to be distantly related to insulin. The single chain proinsulin molecule is known to be converted to insulin by removal of an internal polypeptide (C-chain) of about 35 residues in length depending on its species origin. The resulting pair of disulfide-bonded polypeptide chains comprising insulin has 25% sequence identity with NGF, including a common location for one of the three disulfide bonds in each protein. The larger NGF molecule retains a 35 residue region corresponding to the insulin C-chain in addition to a 35 residue extention on the carboxyl-terminus. The insulin C-chain and the equivalent region of NGF show no homology but insulins from different species have little or no similarity in this poorly conserved area.

The responsive neurons have been shown to have specific high affinity receptors (K_D = 10^{-10} - 10^{-11}M) for the neurite-outgrowth-promoting β-subunit on the cell surface plasma membrane, probably predominantly at the tips of the neurites. Both the NGF receptor and those for other growth factors require detergents for solubilization indicating that they are integral membrane proteins. By affinity labeling with radiolabeled NGF, the molecular mass of the receptor polypeptide chain is estimated to be about 140,000 daltons.[19]

Within minutes after NGF binding, protein phosphorylation is observed.[20] Membrane containing NGF in complex with its receptor invaginates and forms internal cytoplasmic vesicles, a process referred to as endocytosis. Radiolabeled NGF, presumably bound to its receptor, is transported from the neurite tip to the neuronal cell body over a period of many hours.[21] This may be the source of the NGF receptors located on[22] or in[23] the nucleus, partially localized at the nucleolus.[24] Correlated with the appearance of labeled NGF in the cell body is the induction of synthesis of the enzymes needed to increase neurotransmitter production such as tyrosine hydroxylase and dopamine β-hydroxylase, rate limiting enzymes in norepinephrine biosynthesis. Late synthesis of a large number of unidentified proteins has also been observed in NGF responsive cell lines.[25]

Introduction of NGF directly into the interior of a responsive cell has been reported not to lead to neurite outgrowth. Furthermore, the direct introduction of antibodies to NGF into NGF stimulated cells did not block the induction of neurites by externally administered NGF. From these data it can be inferred that either NGF must be in a unique and protected internal environment, such as an endocytotic vesicle, or NGF induces a second messenger that mediates its activity.[26]

NGF activity is implicated in facilitating the development of hypertension in spontaneously hypertensive rats. By destroying the sympathetic nervous system with a combination of guanethidine and antibodies to NGF, the appearance of hypertension in these animals is prevented.[27] The viability of the sympathetic nervous system can also be diminished or destroyed by either 6-hydroxydopamine or vinblastine, compounds that prevent NGF from being transported from the synapse to the neuronal cell body.[28]

Human melanoma cells both secrete NGF and recognize it by specific cell surface receptors.[29] This may be an example of a type of autostimulation in transformed cells by which the cell makes self-stimulating growth factors and thereby escapes normal control mechanisms.[30] The untransformed counterpart, the melanocyte, is derived from the same embryonic structure, the neural crest, as the target neurons. The activity of NGF on untransformed melanocytes has not been determined.

Epidermal Growth Factor - The second growth factor to be purified, EGF, was originally recognized by its ability to induce precocious eyelid opening and incisor eruption in newborn mice. These effects, in part, may be the result of the stimulation both of the growth of epidermal cells and of keratinization. Like NGF, EGF has been reported to have chemotactic activity.[31] Unlike NGF, however, induction of cell division, or mitosis, is characteristic of growth induced by EGF.

Mitogenic responses to EGF are reported for fibroblasts, chondrocytes, glia, mammary epithelium, granulosa and both corneal and vascular endothelial cells in culture. During fetal development, induction of EGF receptors have been followed by radioreceptor assays. Based on parallel radioimmunoassay, however, it appears that more than one fetal protein may bind to the EGF receptor.[32] The presence of EGF in mouse milk (300 ng/ml) may to some extent support tissue growth since oral administration of the acid stable EGF causes early eyelid opening in newborn mice. Human milk EGF, also found in substantial concentration (80 ng/ml), by analogy could be active in human infants.[33]

As is the case for NGF, EGF occurs in large quantity in the adult male mouse submaxillary gland where its synthesis is also under testosterone control. Material purified from this source has been most extensively characterized. The sequenced 53 residue mouse mitogen contains three stabilizing disulfide bonds and has an isoelectric point of 4.6. As with NGF, it is found in complex with a unique binding protein, a specific trypsin-like protease that can cleave a pro-EGF polypeptide chain of 9,000 daltons to the well characterized 6,000 dalton protein and subsequently form a stable complex with the processed mitogen.[34] The molecular weight of the complex, composed of two mitogenic EGF subunits and two binding protein protease subunits, is 74,000.

Most responsive cell types have about 40,000 to 100,000 high affinity (K_D = 2-4 x 10^{-10}M) receptor binding sites per cell. The glycoprotein receptor mass has been determined to be from 125,000 to 180,000

daltons, depending on the cell source and the means of identification. The 180,000 molecular weight human placental receptor can be degraded by proteases through a series of successively smaller sizes down to 25,000 molecular weight, while retaining binding to both the membrane and the mitogen.[35] It has been reported that the conversion of the EGF receptor from 140,000 to 125,000 daltons by trypsin requires binding by EGF.[36] Therefore, the range in reported size may reflect, in part, limited proteolysis. These degradation studies indicate that the receptor is probably composed of multiple stably folded 3-dimensional domains, a feature common to other large globular proteins.[37]

EGF binding to the cell membrane receptor triggers a multitude of effects including increased transport of ions and precursors for macromolecular synthesis. Ultimately, increased production of polysaccharides, RNA, proteins and, within about 24 hours, DNA is observed. The mechanism by which the mitogen stimulates these responses is unknown. Almost immediately after binding, an increased receptor associated kinase activity has been identified that stimulates the incorporation of radioactive phosphate into tyrosine side chains of multiple proteins[38] including the EGF receptor.[39] This phosphorylation does not appear to be sufficient to stimulate all of the subsequent steps required for cell division since a CNBr-modified EGF (hydrolyzed between methionine 21 and histidine 22) binds to the EGF receptor, induces phosphorylation, but has little or no mitogenic activity.[40] Within a few minutes after binding of fluorescently labeled EGF to its fibroblast receptor, the mitogen-receptor complexes are observed to aggregate in the membrane to form patches.[41] Upon binding of the inactive CNBr-modified EGF, however, the receptor-EGF complexes do not aggregate in the membrane. Addition of divalent anti-EGF antibodies mediates aggregation of these complexes in the membrane and restores mitogenic activity.[42] The clustering of the receptor-EGF complexes, therefore, appears to be correlated with subsequent mitogenesis.

Evidence recently has been presented that the EGF receptor is directly involved in the induction of EGF-activated responses. Specific monoclonal IgM antibodies to EGF receptors that prevent EGF binding not only mediate early EGF-induced effects, such as increased phosphorylation, but also lead to the characteristic late mitogenic response of DNA synthesis. Similarly, insulin-specific activities (glucose uptake and oxidation) can be induced by certain anti-insulin receptor antibody populations. The antibody binding to EGF receptors may mimic the mitogen by facilitating receptor clustering and, perhaps, inducing other EGF-mediated perturbations of the receptor. From the antibody activity it appears that many or all of the functions of EGF at the target cell membrane are triggered by an "activated" receptor.[43]

By about 30 minutes after initial binding, the labeled growth factor is found in endocytotic vesicles within the cell. These internalized vesicles migrate through the cytoplasm to the edge of the nucleus.[44] The internalized mitogen is ultimately degraded by lysosomal enzymes. Since removal of uninternalized EGF hours after exposure to a cell is reported to substantially inhibit the ultimate mitogenic response,[45] the significance of mitogen internalization remains to be determined.

Cytoplasmic extracts from EGF-treated cells are observed to stimulate DNA synthesis in isolated frog nuclei. Direct addition of EGF, however, has no effect on this increased synthetic rate. Three peaks of activity from the extracts can be observed with molecular weights, roughly estimated from sedimentation in density gradients, to be 46,000, 110,000

and 270,000. As inferred from the receptor antibody results, EGF-induced DNA synthesis seems to be mediated through a second messenger molecule.[46]

The EGF mitogen may have possible therapeutic effects. In fetal and neonatal lambs it enhances the rate of maturation of the lungs with resulting prevention of hyaline membrane disease,[47] a condition that significantly contributes to the increased mortality of premature human infants. In ointment form, EGF has been reported to produce faster and better healing in a rabbit ear wound model.[48] Human urogastrone, a gastrointestinal hormone that inhibits gastric acid secretion, is very similar to mouse EGF. Of the 53 common residues in the two amino acid sequences, 37 are identical. In the typical bioassays for either urogastrone or EGF, both molecules are active.[49] It has not been reported if EGF has the anti-ulceration activity attributed to urogastrone.

In many types of tumor cells, there appears to be a decreased requirement for EGF.[50] This may result from tumor produced transforming growth factors (TGF's) that have been shown to bind to the tumor cell EGF receptors[51,52] and lead to phosphorylation of specific tyrosine side chains of some proteins including the EGF receptor, in a manner similar to that caused by EGF.[53] The ability of TGF's to induce cells to grow in soft agar, a marker for transformation, may be mediated by additional TGF receptors that are not recognized by EGF.[54] The capacity of transformed cells to synthesize, secrete and subsequently respond to a mitogenic growth factor could be the basis for their loss of growth control. The TGF family of proteins, ranging from 6,000 to at least 24,000 daltons, does not immunologically cross-react with antibodies to EGF.[51,52,55,56] TGF-like mitogens may be made in small amounts in some normal adult animal cells.[57] The relationship between TGF's and the previously mentioned unidentified embryonic EGF receptor binding molecules has not been determined.

Somatomedins - Although pituitary growth hormone stimulates the growth of a wide variety of tissues, it was observed that the hormone did not act directly on at least one of its targets, cartilage, but rather stimulated its growth indirectly through a mediator. The growth hormone mediators for cartilage, termed somatomedins, have subsequently been recognized to have four characteristics: (1) regulation of their concentration in serum by growth hormone, (2) stimulation of sulfate incorporation into the cartilage proteoglycan chondroitin sulfate, (3) insulin-like effects on both adipose and muscle tissue, and (4) mitogenicity for fibroblasts.

Somatomedins are synthesized in liver, pituitary, various regions of the brain[58] and in fetal intestine, heart, brain, kidney and lung.[59] In the fetus and neonate, their synthesis may be induced by placental lactogen prior to development of growth hormone control.[60] The somatomedins may also influence the level of their inducer, growth hormone, through a negative feedback loop by stimulating the release of somatostatin, a small polypeptide from the hypothalamus that subsequently inhibits growth hormone release from the pituitary.[61]

Although the somatomedins have insulin-like activity it is at levels low enough to question its physiological significance. In general, the stimulation of the conversion of glucose to CO_2 in adipocytes by somatomedins is only about 1-2% that of insulin, whereas the mitogenic activity is approximately 50-100 times greater than that of pure insulin. The insulin-like effects of somatomedins may be the result of their approximately 100-fold weaker binding to the insulin receptor.[62] In contrast, the mitogenic activity of somatomedins for cultured chondrocytes, the cell

type found in cartilage, presumably reflects its direct growth promoting effects on cartilage *in vivo*.[62,63]

The somatomedins comprise a family of similar small proteins, most of which have molecular weights of about 7,500. At least five categories of somatomedins have been described. These are somatomedins A and C (SMA, SMC), insulin-like growth factors I and II (IGF-I, IGF-II), all purified from human plasma, and multiplication stimulating activity (MSA) purified from rat liver cell cultures. An additional mitogen, somatomedin B, is apparently a misnomer since the protein does not appear to have sulfation activity. Although reported to be purified, the active species may be an EGF contaminant of the purified product.[64]

Both IGF-I and IGF-II have been completely sequenced. These proteins are single polypeptide chains that have extensive sequence homology with insulin. IGF-I is a 70 residue chain containing a 12 residue polypeptide segment in a position equivalent to the longer insulin C-peptide connecting regions homologous to the insulin amino terminal B chain and carboxyl terminal A chain. The IGF-I sequence has an 8 residue extension beyond the insulin carboxyl terminal location.[65] Only 2 of the 19 residues found to be invariant among all sequenced insulins are substituted in IGF-I. The 3 disulfides and most of the non-polar hydrophobic core residues are conserved clearly indicating a very similar tertiary structure to that determined for insulin.[66] IGF-II has been shown to be a 67 residue protein with 62% of the sequence identical to IGF-I including the location of the 3 disulfide bonds. In IGF-II, the region equivalent to the insulin C-peptide is shortened to only 8 residues and the carboxyl terminal extension is only 6 residues long.[67] Although modest differences in some biochemical effects can be demonstrated between IGF-I and II *in vitro*, their *in vivo* physiological significance is uncertain.

Partial sequence information for SMC reveals that it is very similar to IGF-I. Of the 25 residues assigned from preliminary sequence results of homologous peptides, 22 are identical in these two growth factors.[68] Striking homology is also observed between a rat somatomedin and IGF-I based on partial sequence data.[69]

MSA is a composite of at least 7 molecular species, most of which are very similar and may be partial degradation products of a single mitogen.[70] One homogeneous form of rat MSA has been sequenced and shown to contain 67 residues having 93% homology with human IGF-II.[71] The relationship of SMA and some of the other MSA forms to the better characterized somatomedins remains to be elucidated.

The number of unique types of receptors for somatomedins has not been unambiguously determined. ^{125}I-MSA has been shown to cross-link to a 255,000 molecular weight plasma membrane receptor protein. IGF-I competes with this form of MSA for the binding site.[72] Nevertheless, separate receptors for IGF-I/SMC and IGF-II/MSA may exist, perhaps with some level of cross-binding reflecting the similarity of the somatomedin structures.[73]

Although the low molecular weight somatomedins are relatively well characterized, they circulate in plasma as large (150,000-200,000 dalton) complexes formed by association with other subunits.[8,74] Two types of binding proteins, each of about 30,000 daltons, are described that complex with MSA to form a 60,000-75,000 MW form.[75] This may be a partially reconstituted form of the larger complex. No proteolytic activity is reported for either binding protein. The presence of these additional

subunits are also under growth hormone control. Although they inhibit the activity of somatomedins in vitro, their only known function in vivo is to increase the half-life of plasma somatomedin from minutes to hours.[76] The large size of the complex may prevent this substantial reservoir of mitogenic stimulatory activity from rapidly diffusing out of the circulation to reach its target cells except in situations involving vascular damage.[8]

Both normal growth and growth abnormalities are correlated with circulating somatomedin levels. Controlled infusion of either growth hormone or IGF-I into rats who have had their pituitaries removed shows a dose-dependent increase in both cartilage size and DNA synthesis in addition to an overall gain in body weight.[77] Measurements of the levels of both IGF-I and IGF-II in human serum indicates that the ratio between these two somatomedins is not constant. The serum levels of IGF-I continue to increase from birth (50 ng/ml) to puberty (400 ng/ml) and then decrease to the normal adult level (200 ng/ml). In contrast, by the end of the first year of life IGF-II appears to increase to its adult level, about 650 ng/ml. As expected, the growth hormone induced IGF-I is elevated in acromegalic patients to approximately 700 ng/ml and decreased in growth hormone deficient patients to only 25 ng/ml. The level of IGF-II, however, may be maximally expressed in healthy adults since it is not increased in acromegalic individuals. These levels are decreased in growth hormone deficient patients, but only to about 250 ng/ml.[78] A primary IGF-I lesion may exist in pygmies, who despite normal growth hormone and IGF-II levels, have substantially reduced IGF-I levels (70 ng/ml).[79] Finally, a human fibrosarcoma cell line is reported to produce MSA-related peptides, a finding at least consistent with the autostimulatory theory of tumor cell escape from growth control.[80]

Platelet Derived Growth Factor - Somatomedins are not only under the control of growth hormone for their synthesis and/or release but also under the influence of a second growth factor, PDGF, for their expression by Balb/c-3T3 fibroblast target cells. Brief exposure to PDGF renders these cells competent for several hours to respond to mitogens such as SMC. The somatomedin, in turn, mediates the progression of the cells into the S, or DNA synthesis, phase of the cell cycle. Neither PDGF nor SMC alone have a substantial mitogenic effect on these common target cells.[81]

Although whole blood serum contains ample PDGF (770 pg/mg protein), the blood plasma contains significantly lower levels (112 pg/mg protein).[82] The high concentrations found in whole blood serum are the result of the release of PDGF from the α-granules of the blood platelets upon coagulation. In vivo, the platelets are small unnucleated cells that bud off from large multinucleated bone marrow progenitor cells, the megakaryocytes. The platelets are induced to clump and release their α-granule contents on exposure to collagen, thrombin or arachidonic acid.[83] During injury platelets would be exposed to one or more of these agents. In this regard, PDGF may be considered to be a mediator of wound healing.

The protein has a molecular weight of about 30,000 with an isoelectric point of 10.2. The extreme heat stability of this mitogen may be generated, in part, by the large number of disulfide bonds that presumably constrain the molecule from irreversible denaturation. Two closely related forms of PDGF have been separated and appear to differ only in their carbohydrate content.[84] If purified in the absence of certain protease inhibitors, the polypeptide chain appears to be susceptible to limited proteolysis so that upon reduction of the disulfide bonds under

denaturing conditions polypeptide chains of about 14,000 to 18,000 daltons are observed.[85,86] A structurally and functionally similar mitogen, fibroblast derived growth factor, can be expressed by baby hamster kidney cells that have been transformed with simian virus 40.[87]

The mechanism of action of PDGF is unknown. From 40 to 90 minutes after exposure of Balb/c-3T3 target cells to PDGF several new proteins are generated in the cytoplasm. Their appearance is sensitive to RNA synthesis inhibitors, indicating de novo synthesis. They are not induced by plasma, EGF or insulin but their level follows the same dose-response as DNA synthesis to PDGF. A mutant PDGF-independent cell type constitutively makes these proteins.[88] In addition to the cytoplasmic proteins, PDGF has been reported to increase the number of cellular receptors for SMC.[89] Evidence has recently been reported that PDGF generates a second messenger within the cell. The cytoplasm from PDGF-treated cells can transfer competence to untreated cells. If RNA synthesis is inhibited during PDGF treatment, however, then the competence is not transferred.[90]

In blood vessels of animals that are spontaneously or artifically damaged, atherosclerotic plaque formation often results. Once the endothelial cell monolayer is removed from the inside of the vessel, the underlying collagenous substratum, upon which these cells anchor, is exposed. Platelets adhere to such sites and would be expected to degranulate, releasing the PDGF and thereby initiating smooth muscle cell mitogenesis. The resulting proliferation of these underlying cells, and their possible migration toward the vessel lumen in chemotactic response to PDGF,[91] may be one of the initial events in plaque formation. In support of this hypothesis is the observation that either inherited or artifically induced decrease in platelet numbers or function is correlated with diminished susceptibility to vascular atherosclerotic lesions. Similarily, removal of the pituitary with the accompanied decrease in growth hormone-induced somatomedins results in less smooth muscle cell proliferation in vivo after endothelial cell damage (reviewed in ref. 92).

It has been claimed that metastatic tumors are a source of PDGF-independent cells whereas nonmetastatic tumors contained only PDGF-dependent cells.[93] To date, however, there is no report of the ability of transformed cells both to make and to recognize their own PDGF in an autostimulatory fashion.

Conclusions - The functions and significance of the growth factors described in this review are probably only incompletely recognized. The mechanism of action is, at best, only partially determined for any of these factors. It is likely that the four described examples only represent a small fraction of the set of such proteins utilized by an organism to control and coordinate development and homeostasis. An increasing number of poorly defined growth factor activities are being discovered for a variety of types of differentiated cells. Our recognition of the influence of these proteins on health and disease will undoubtedly increase as our knowledge of them expands.

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Chapter 23. A Review of the Basic Elements of Recombinant DNA Research

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Introduction - The last 10 years have seen remarkable advances in our understanding of gene structure and function. Today it is possible for molecular biologists to isolate individual structural genes, manipulate the DNA, and reintroduce it into cells, often crossing species barriers. Understanding of the detailed structural organization of many eucaryotic genes is now within reach. We are at the start of a new era of molecular genetics. Recombinant DNA technology stands at the center of this new methodology. The purpose of this chapter is to update and acquaint the reader with some of the concepts and techniques used today in recombinant DNA research.

The seeds for the beginning of this methodology go back over a decade. The discovery by Mandel and Higa that E. coli could be made competent to take up both linear and circular DNA by treatment with calcium ions was an early and important observation that is still exploited today.[1] The demonstration by Cohen et. al. of E. coli transformation with $CaCl_2$ treatment and purified R-factor plasmid DNA, leading to antibiotic resistance, marked the beginnings of plasmid transformations in vitro in E. coli.[2] The covalent joining of SV40 DNA and λ phage DNA by Jackson et. al. marked the beginning of the construction of DNA chimeras in vitro.[3] This was quickly followed by the construction of a biologically active plasmid chimera that could replicate in vivo.[4] However, the above developments themselves were a direct result of basic discoveries made in the late nineteen sixties. The isolation of mutant E. coli strains unable to degrade foreign DNA laid the groundwork for developing recipient strains.[5] The discovery of site-specific restriction endonucleases[6-9] may be the single most important factor for the rapid advancement of the field. Enzymes such as T4-DNA ligase, which was shown by Sgaremella and Khorana[10] to catalyze the joining of fully base-paired double-stranded DNA (now known as blunt-end ligation), the enzyme terminal transferase, characterized by Bollum and co-workers[11] and used to link one DNA to another via synthetic complementary tails,[12] and EcoRl, [13-15] the first enzyme capable of creating self-complementary cohesive termini on DNA were all landmark observations in the development of recombinant DNA as we know it today.

The basic elements of recombinant DNA technology today can be partitioned into five major methodological components:

1) DNA vehicles which can replicate with foreign DNA inserted into them;
2) methods to incorporate foreign DNA into the above vehicles;
3) a means for introducing the vehicle (carrying the DNA sequence to be cloned) into a host organism;
4) a means for screening those cells that have the desired recombinant molecule;
5) methods to alter the cloned DNA sequence.

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The current state of the art of each of the above facets of recombinant DNA research is briefly summarized below.

1) Cloning Vehicles: Presently, these consist of five basic types: plasmids, cosmids, M13 derivatives, phage λ, and animal or plant viral DNA derivatives. We shall consider each separately.

A) Plasmids - Many bacterial plasmids have been developed as cloning vehicles. Indeed, most of the work to date has been carried out with E. coli plasmids. So far, they constitute the most versatile type of host/vector system for DNA cloning. Plasmid vectors for cloning DNA into Bacillus subtilis have also been developed.[16]

The two bacterial plasmids used in most of the initial DNA cloning experiments were pSC101[2] and Col E1.[17] However, these plasmids had serious disadvantages. The pSC101 plasmid (which conferred tetracycline resistance (Tc^r) to E. coli) could not be amplified within the E. coli cell in vivo, while the assay for Col E1 positive cells had high background values and was less than convenient to carry out. The pioneering work of Boyer's group[18] brought about the construction of a hybrid plasmid pMB9 that was immune to Colicin E1, exhibited a Col E1 mode of DNA replication in terms of copy number and replication in the presence of chloramphenicol, and conferred Tc^r to the host cell. Although pMB9 was a successful cloning vector it did suffer from the fact that there was only one (EcoR1) unique restriction site that could be used to clone foreign DNA fragments without the loss of antibiotic resistance. Boyer and his colleagues then went on to construct the plasmid pBR322.[19] This plasmid in many ways has come to represent what we expect a bacterial cloning vector to be. In addition to the above properties of pMB9, it has a number of unique restriction sites that can be used for cloning. These are HindIII, BamHl, Sall, Sphl and Clal in the Tc^r gene, and Pstl and PvuI sites in the ampicillin resistance (Ap^r) gene. DNA cloned into any one of these sites can conveniently be detected by observing the sensitivity of the bacteria to one antibiotic and their resistance to another. This is because only one of the two genes for Ap^r and Tc^r will be inactivated by the inserted foreign DNA. The unique PstI and SphI sites provide two advantages for molecular cloning of DNA by means of homopolymeric DNA extension techniques. First the sites provide a protruding 3'OH which is a good substrate for terminal transferase. Secondly, by extending the PstI site with guanosine residues (G) (and the sphl site with C's) it is possible to regenerate the sites after annealing complementary tailed foreign DNA.[20,21] While improved derivatives of pBR322 have been made,[22,23] pBR322 is still the most widely used E. coli cloning vehicle.

B) Lambda - The other major vehicles used to clone foreign DNA in E. coli are derivatives of the well characterized phage λ. Before wild type strains could be used to clone foreign DNA, they had to be modified to remove the numerous restriction sites that occur in such a large piece of DNA. An example of early work along these lines is that of Murray and Murray.[24] Wild type λ contains 5 EcoRl sites. They constructed derivatives that had only one or two EcoRl sites. These λ species, they demonstrated, could be used as cloning vehicles. Another early λ cloning vehicle (λgt.λC) was constructed by Thomas et. al.[25] It contained two EcoRl restriction enzyme sites between which EcoRl-generated DNA fragments of 100 to 14,000 base pairs could be inserted. Leder and coworkers[26] improved this cloning vehicle with their "WES" series of λ phages. To conform with the need, at that time, for strict safety features to be incorporated into recombinant DNA cloning vehicles, they included three

amber mutations (Wam 403, Eam 1100 and Sam 100) into λgt.λC. In this way the phage could grow only in hosts carrying the appropriate supressors. In addition, they further modified the resulting phage (λgt.WES λC) by substituting an inert fragment of λ DNA in between the two EcoRl sites of λgt-WES.λC, yielding the currently popular λ cloning vehicle known as λgt WES.λB.

Another group of popular λ cloning vehicles are the "Charon phages". Starting with wild type λ, Blattner et. al.[27] substituted into the wild type λ genome DNA from other lambdoid phages that did not have EcoRl restriction sites in those regions. They constructed a diverse range of λ cloning vehicles that could be used to clone EcoRl-cut DNA fragments (and in a few cases HindIII-or SstI-cut DNA fragments). As we shall see below, these λ vehicles are useful for cloning large fragments of DNA.

C) Cosmids - In vitro λ packaging systems can be used for the efficient cloning of DNA sequences. The in vitro packaging system is insensitive to most of the DNA sequences inserted internally in λ DNA. The only requirements appear to be a minimum and maximum DNA size and the presence of a λ sequence known as the "cos" site. A number of new types of plasmid vectors known as "cosmids" have been constructed where the λ cos site is incorporated into the vector.[41] While these vehicles behave like plasmids once inside the cell, they can function like λ as far as packaging techniques are concerned. This yields a very high cloning efficiency. Since most of the λ genome is removed (only the λ cos site remains), large fragments of DNA can be packaged and cloned.

D) M13 and its derivatives - M13, a filamentous, male-specific coliphage, contains a single stranded DNA (ss-DNA) that is 6,407 nucleotides in length and shares extensive homology with the other filamentous phages, fd and fl. These filamentous phages do not lyse their host; rather, they are released from infected cells as the cells continue to grow and divide. However, because infected cells grow with an increased generation time, the phage infection results in plaque formation. During infection of a suitable E. coli host, the infecting ss-DNA (+ strand) of the phage is converted and amplified into double strand replicative forms (approximately 100 molecules per cell) which serve as intermediates in the production of progeny (+ strand) ss-DNA. These ss-DNA molecules are packaged into a protein coat and extruded from the cell. Because M13 is a filamentous phage, there is no size constraint on the packaging reaction and double-length phage particles are often seen in wild-type infections. This length flexibility is an important requirement of a cloning vehicle, as it permits foreign DNA of various sizes to be packaged. However, because all M13 gene functions are required for replication, the wild-type M13 has been extensively modified to provide usable cloning sites.

Messing and co-workers have constructed a number of M13 derivatives in which a promoter-operator region is inserted into the intergenic space (507 base pairs (bp) of nonprotein-coding DNA) between gene IV and II of the phage.[46-49] The lac promoter-operator insert codes for the first 145 amino acid residues of the β-galactosidase gene of E. coli. Following the infection of certain cell lines (e.g., E. coli K12 JM101 and JM103) this information complements deletion mutants of the β-galactosidase gene and restores β-galactosidase activity (α-complementation). The utility of such lac^+ M13 derivatives was limited due to the presence of only a small number of cloning sites in the lac promoter-operator region. To deal with this shortcoming, Messing et. al. used elegant recombinant DNA techniques to insert an digonucleotide stretch of 17 bases into the lac region of a lac^+ M13 derivative. The result was a 48 bp region of DNA

which contained restriction enzyme recognition/cleavage sites for EcoRl, Sall, Accl, BamHl, HincII and Pstl, and thus functions as a multipurpose cloning site. This bacteriophage strain, M13 mp 7, retained the ability to mediate α-complementation.

Although M13 mp 7 is a ssDNA phage, its intracellular replicative form (RF) is a double-stranded supercoiled DNA, and resembles a plasmid vehicle. Thus, DNA fragments are inserted into the M13 mp 7 RF in a manner analogous to that with pBR322. However, as we shall see later, the main use of M13 as a cloning vehicle is for DNA sequencing and not for initial cloning experiments.

E) Animal Virus DNA Derivatives - Vectors that contain DNA sequences that are able to multiply in animal cells are now numerous. Unlike those used for E. coli, there is little consensus for a universal cloning vehicle. Simian virus 40 (SV40) was an early choice for a cloning vehicle. Ganem et. al. [28] demonstrated in 1976 that a 520 base pair fragment of λ DNA could be inserted into a derivative of SV40 and propagated in monkey cells (in the presence of wild-type SV40 helper). The structure of the λ-DNA segment after serial passage in monkey cells was well preserved. Similar work at this time was also done by Goff and Berg [29] with a 1.5 kb section of λ containing the origin of λ DNA replication and the two structural genes CII and cro. The DNA was inserted into the late region of the SV40 genome. Again it was clear that virus chimeras could be propagated in vivo. Extending the construction of eucaryotic viral chimeras one step further, Mulligan et. al.[30] inserted a rabbit β-globin cDNA sequence into SV40 DNA at the location in the SV40 genome that codes for the major capsid protein, VPI. They demonstrated not only that they could propagate the resulting SV40/cDNA chimera in CVl monkey cells but that cells containing the viral sequence produced substantial quantitites of rabbit β-globin polypeptide sequences. The important innovation in these experiment was to leave intact the regions in SV40 implicated in SV40 late mRNA processing. These include the late mRNA leader sequence, the region in which the leader is spliced during maturation of late mRNA's, and the region in which late transcripts are terminated and polyadenylated. Similiar work was also carried out by Hamer et. al.[31] and Hamer and Leder.[32,33] They introduced a fragment of chromosomal mouse DNA containing the β-globin gene into SV40. However in this case the intervening sequence and poly(A) addition site was derived from the mouse gene. The β-globin gene was in fact inserted into SV40 in two orientations relative to the SV40 late region promoter. This showed that the fragment is transcribed regardless of orientation. However, the RNA splice signal and poly(A) addition site are utilized only when the fragment is inserted in the "sense" orientation. One problem with these experiments was that the recombinant SV40 genomes replicate in the virus' permissive host. The cell is killed during the course of infection, thereby precluding the opportunity to monitor expression of the transduced genes in continuously multiplying cells. In 1980 Berg's group set about constructing transducing SV40-derived vectors that could be introduced into a variety of cells.[34] They used the bacterial gene of E. coli xanthine-guanine phosphoribosyltransferase (XGPRT) (inserted into an SV40 vector) as a marker to select for human Lesch-Nyhan tissue culture cells that had been transfected with this SV40 chimera. They demonstrated that the E. coli XGPRT could overcome the physiological defect of Lesch-Nyhan cells in purine nucleotide synthesis. A later demonstration by Mulligan and Berg that the XGPRT gene could be used as a dominant selective marker for "normal" cultured mammalian cells, because of the efficient utilization of xanthine for GMP during the growth

of vector transformed cells, was a more useful application of this methodology.[35]

Progress with other virus vector systems is still not as yet well developed. However, well-characterized viral chimera for Papilloma,[36] Retrovirus,[37] Adenovirus[38] and Herpes virus[39] exist.

Vectors for cloning in yeast are somewhat of a special case in that they do not fall in the class of procaryotic vectors described above nor are they virus derived as is the case for most eucaryotic vectors. A summary of the characteristics of many of these vectors was presented by Botstein et. al.[40] They usually are constructed with a bacterial replicon and a characteristic marker which makes possible their amplification and selection in E. coli. Examples are vectors containing drug-resistance genes derived from pBR322 or yeast DNA which can be expressed in E. coli K12 mutants. Finally, all vectors carry yeast DNA fragments that provide for the maintenance of the plasmid in yeast. They can either integrate into the yeast genome or exist as episomes.[42] In almost all instances where integration into the host chromosome occurs there is strong homology between at least some sequences on the vector chimera and the genomic DNA.

F) Plant Vectors - Potential vectors for the plant kingdom are now only starting to appear. The Ti-plasmids [43-45] are perhaps the best characterized to date in this area. These plasmids are responsible for the synthesis of opines and a tumerous growth called grown gall disease seen in most dicotyledonous plants. In vivo the plasmid is harbored by the gram negative bacterium Agrobacterium tumefaciens. The genome size of these plasmids is large (usually >23kb). This makes it difficult to form chimera by the usual methods involving cutting the plasmid at a unique site. Instead one may have to rely upon E. coli transposon-induced recombinations between two plasmids in the same cell to get viable chimeras.[44]

2) Methods to Incorporate Foreign DNA Into the Above Vehicles: An important decision in any recombinant DNA cloning experiment is how to generate the DNA chimera. Methods to join DNA to that of the cloning vehicle invariably start with the process of cutting the vehicle with one or more restriction enzymes. In the case of plasmid, M13 and SV40, this will generate linear molecules. In the case of λ phage, the DNA will be fragmented into one or more pieces. This step can be easily monitored by gel electrophoresis. Methods to join DNA to the cut vector fall into four classes:

A) Restriction Enzymes - This is perhaps the simplest approach. The procedure is easy, fast and often efficient. Essentially, one cuts foreign DNA with a restriction enzyme that has a sequence homology at its ends with those of the vector DNA. The vector and fragment of DNA to be cloned do not have to be cut by the same restriction enzyme. The only requirement is that the extended "sticky ends" be homologus. Such ends can then be joined together with T4 DNA ligase[3] to yield viable chimeras that can be cloned. A more recent variation of this approach is where both DNAs are cut with restriction enzymes that generate "blunt ends" (for example SmaI), i.e., the double strand DNA is cut straight across both strands. T4 ligase can also ligate (join) such fragments to form viable chimeras. Because blunt ends can be generated by cutting DNA with any restriction enzyme followed by digestion with S1 nuclease (to remove any overhanging single-stranded tails), the blunt end ligation procedure has found numerous uses in cloning DNA fragments.

The disadvantage of using this procedure is that ligation reactions as described above can often join more than one DNA fragment and the vector DNA into the final chimera. Also, sequence specificity often determines which fragments can be cloned. If blunt end ligation is used, nucleotides may be lost during the S1 step leading to an interrupted coding sequence and the loss of flanking restriction enzyme sites.

B) Terminal Transferase Tailing - This is usually the method of choice for cDNA cloning. This was the first method used to construct chimera for cloning. The enzyme terminal deoxynucleotidyl transferase is used to add a single-stranded homopolymer tail to the 3' termini of the cloning vehicle. The same enzyme is used to add a complementary tail to the DNA species to be cloned. A simple annealing step is all that is then required to obtain chimeras suitable for cloning. If pBR322 is used with "GC" homopolymer tailing at the PstI site of this plasmid, the cloned DNA can be excised from the plasmid by redigestion, with PstI.[50] One problem with the procedure is that with tailing reactions, it is difficult to get homopolymer tails of the desired length.[51]

C) Linkers - Linkers are an answer to the problem of blunt end ligation described above where the restriction site at the site of ligation is not regenerated. In order to regenerate such sites or indeed insert another restriction site at that point, short oligonucleotide double stranded molecules are first ligated onto the end of the DNA to be cloned. These oligonucleotides contain sequences recognized by a restriction enzyme. Because usually more than one oligonucleotide will be attached to the DNA during the ligation step, afterwards it is usually necessary to treat the DNA with a restriction enzyme to trim it back to one oligonucleotide per DNA. The DNA, now with sticky ends, can be ligated to the plasmid vehicle as described above for the restriction enzyme method (A). Unfortunately, in many cases one must protect internal restriction enzyme sites in the DNA to be cloned before adding the linker. In the case of EcoR1 linkers, for example, this can be done with EcoR1 methylase.[52] However, in many cases, a methylase such as this is not commercially available.

D) Adaptors - These are an improvement of the linker method. They are synthetic oligonucleotides with sticky ends that can be conveniently ligated to DNA cut with the appropriate restriction enzymes. Often the site used to cut the DNA can be regenerated. The oligonucleotide is selected to contain within its sequence another restriction site that can be used to ligate the DNA into an appropriate cloning vehicle. In effect, DNA cut with one enzyme is "converted" into DNA cut by a different enzyme. Because the ligation reactions involve DNA with sticky ends (rather than blunt ends as for linkers), the ligation process is more efficient. Unfortunately, to date, such adaptors are in rather limited commerical supply, although this will probably only be a temporary situation.

3) Introducing Vehicles Into a Host Organism:

A) Bacteria - Having constructed plasmid or λ chimeras, the next step is to introduce the DNA into a cell. This involves the process of transformation for plasmids and transfection for phage λ. The frequency of transformation is determined by the uptake of DNA molecules and by the restriction system of the recipient cell. The restriction systems of different strains of E. coli have been studied in great detail; however, very little is known about the mechanism of DNA uptake through the

bacterial cell wall. Several bacterial species have developed the ability to transport DNA into the cell. In Bacillus subtilis and Diplococcus pneumoniae the uptake of DNA is nonspecific, whereas DNA uptake in Haemophilus influenzae has been demonstrated to be highly specific.[53-55] Most bacteria, however, have not developed the ability to take up DNA (i.e., exhibit "natural" competence). To obtain competence in these bacterial species, special procedures have to be used. Mandel and Higa demonstrated transformation in $CaCl_2$-treated E. coli K12 cells.[1] Later, Cohen et. al.[2] described a procedure that yielded a high frequency of transformation of calcinated E. coli with covalently closed circular DNA. Recently, further improvements in the methods of transformation using $CaCl_2$-treated E. coli cells have been described.[56,57] A major disadvantage of the calcinated cell system is that many bacterial species are insensitive to calcium treatment. A variety of spheroplast systems have also been used in transfection experiments. Spheroplasts obtained by treatment with lysozyme can give very high transfection frequencies up to 5×10^9 infective centers/μg of φX174 RFI DNA, approximately 500 times higher than that obtained with calcinated cells. However, most spheroplasts from wild-type bacterial cells cannot be converted back to viable cells, and therefore, are unsuitable for transformation with plasmid DNA. Suzuki and Szalay have described temperature-sensitive mutants deficient in peptidogycan synthesis that can be used as spheroplasts to take up DNA.[58] However, for plasmid DNA the transformation efficiency was similar to that of calcium-treated cells. At least in the case of E. coli, the state of the cells (with respect to their growth conditions) before Ca^{++} treatment has an important effect on the transformation efficiency.[59] Also, different strains of E. coli exhibit marked differences in their transformation efficiency.[51]

One of the attractive features of bacteriophage λ vectors is the existance of in vitro packaging procedures. The method was initially developed by Becker and Gold.[60] It has since been modified so that only exogenous DNA could be efficiently incorporated into infectious λ particles.[61,62] The essence of the procedure is that a cell extract from one mutant of E. coli containing empty phage heads is mixed with extracts from one or more other cell types that contain the necessary factors to force exogenous λ chimeric DNA into the empty heads. While this process is complex, involving a number of steps, the availability of E. coli mutants carrying the necessary factors makes the procedure simple and efficient to use.[62] In most cases in vitro packaging of λ DNA chimera is the method of choice for cloning of eucaryotic cell genomic DNAs.

B) Eucaryotic Cells - A more diverse range of methods exist to introduce recombinant DNA chimeras into eucaryotic cells. There are three major problems facing the investigator in this area. One is that transformation is a relatively rare event. Therefore, many cells are needed, making detection of the transformed cell difficult. Another problem is that very often the foreign DNA is not retained by the cells for a reasonable length of time. Finally, it is clear that different cell types do not undergo transformation with the same frequency; some cells such as murine LTK^- cells can be transformed with an efficiency of 1 in 10,000 cells. Others, such as hamster cells are transformed with an efficiency of one or two orders of magnitude less. The introduction of DNA into eucaryotic cells is usually achieved by one of three methods - The calcium phosphate technique which Graham and Von der Eb[63,64] developed is possibly the most used technique. With this method the DNA is added to a buffered phosphate solution which is then mixed with a solution of

$CaCl_2$. The resulting precipitate (which includes the DNA) is added directly to cultured cells. The procedure has been used to transfer many genomic DNA fragments into eucaryotic cells.[70] DNA vectors, as described in the previous sections of this article, behave in most respects like genomic DNA as far as DNA uptake into eucaryotic cells is concerned, so the Von der Eb method can be used directly as described above. Lowy et. al.,[65] for example, used pBR322 as a marker to clone a mammalian gene. They ligated the plasmid to hamster DNA and used the ligated DNA mixture to transform APRT-deficient mouse cells. From $APRT^+$ transformants they isolated and cloned the genome in phage λ. They then used pBR322 as a marker to identify and isolate the Chinese hamster APRT gene. DEAE-dextran can also be used to introduce DNA into eucaryotic cells.[66,67] This method is often used for transfection by small viral DNAs such as SV40 and polyoma (and their cloning vehicle derivatives). Unlike the calcium phosphate-mediated transfection procedure, which yields many stably transformed cell lines, DEAE-dextran-mediated transfection with bacterial plasmid DNA containing eucaryotic genes yield primarily cells with transiently expressed DNA. Although the expression of DEAE-dextran transfected DNA appears to be transient, the high efficiency and speed of detection together provide a valuable alternative to permanent transformation for the study of transfective cloned gene fractions. The third way of introducing DNA into eucaryotic cells is directly by microinjection.[65] More recently the procedure has been refined to include injection into cell nuclei. Anderson et. al injected a mixture of two plasmids containing the HSV TK gene or human β-globin gene into L cells that were TK^-.[68] Approximately 50% of injected cells gave rise to TK^+ colonies in HAT selection, and all of these contained the co-injected β-globin sequences as well. Capecchi found that 20% of L cells injected with a plasmid containing the HSV TK gene and the SV40 origin of DNA replication gave rise to TK^+ colonies in HAT selective medium.[69] Introduction of DNA directly into the nucleus should allow transfer of genes into cells that normally are refractory to transformation. Additionally, the high frequencies of transformation observed may allow the transfer of genes into wild-type cells where selection has not been possible.

4) Screening Cells Clones for Recombinant DNA: Having succeeded in geting a recombinant DNA molecule into a cell, the next step is to isolate that cell. This is often the most difficult part of a cloning project. Unless it is well thought out beforehand, the whole project can fail. There are six principal methods presently used to select for recombinant clones. Let us now look at each in turn:

A) Genetic Selection - Struhl et. al. were the first to use this method to select eucaryotic genes cloned in E. coli.[71] They cloned a segment of DNA from Saccharomyces cervisiae in phage λ DNA. Using a histidine auxotroph of E. coli, they selected for a yeast DNA fragment that allowed the E. coli mutant to grow in the absence of histidine. Such functional genetic expression of a fragment of yeast genomic DNA in E. coli, however, will probably be the exception rather than the rule because of the occurrence of introns within the genes of most eucaryotes. E. coli clearly does not have the required facilities to process mRNA as do eucaryotes. Cloned cDNAs of mRNAs should not present such difficulties. For example, Chang et. al. could demonstrate the phenotypic expression in E. coli of mouse dihydrofolate reductase (DHFR) using DHFR cDNA.[72] They took advantage of the fact that mammalian DHFR has a much lower affinity for the antimetabolic drug trimethoprim than does the

bacterial enzyme. Thus, bacteria which biologically express mammalian DHFR activity are resistant to levels of trimethoprim that normally inhibit growth.

B) Nucleic Acid Hybridization - This is often the method of choice for many cloning experiments. Grunstein and Hogness first demonstrated this procedure when cloning Drosophila melanogaster 18 and 28s rRNA in E. coli.[73] With this method very large numbers of colonies of E. coli carrying different hybrid plasmids can be rapidly screened to determine which hybrid plasmids contain a specified DNA sequence or gene. The colonies to be screened are transferred to nitrocellulose filters, and, after a reference set of these colonies has been prepared by replica plating, they are lysed and their DNA is denatured and fixed to the filter in situ. The resulting DNA-prints of the colonies are then hybridized to a radioactive RNA (or cDNA) that defines the sequence or gene of interest, and the result of this hybridization is assayed by autoradiography. Colonies whose DNA-prints exhibit hybridization can then be picked from the reference plate. There are now numerous examples in the literature of this technique.[74-76] A more recent extension of the method has been the use of short synthetic oligonucleotide probes to pick out clones containing a particular short nucleotide stretch.[77,78] There are many cases where the mRNA for a protein cannot be isolated. However, sufficient protein is available to get a partial or complete amino acid sequence. Following careful examination of the amino acid sequence, suitable oligonucleotide probes can often be constructed that will allow the detection of clones that contain that sequence. These clones can then be further analyzed by the other techniques described here to obtain the desired clone.[79]

C) Translational Analysis - Paterson et. al. presented a simple method for directly correlating structural gene sequences in DNA with their corresponding mRNAs.[80] This is based upon the fact that mRNA hybridized with its complementary DNA will not direct the cell-free synthesis of a complete polypeptide. Full translational activity of the mRNA is recovered upon thermal melting of the hybrid. Utilizing a rabbit β-globin clone (pβG1) they demonstrated the application of hybrid-arrested translation for the identification of structural gene sequences within recombinant DNA molecules. This method, often called "hybrid-arrested cell free translation" has its complement known as "positive-selection-translation". In the latter case, the cloned plasmid or λ DNA is bound to a nitrocellulose membrane. Messenger RNA is hybridized to that DNA and irrelevant mRNAs are then removed. The bound hybridized mRNA is eluted and analyzed by in vitro translation. As an example of this method, Parnes et. al. isolated three cDNA clones for β_2-microglobulin, the small subunit of the major histocompatibility antigens.[81] β_2-Microglobulin makes up less than 0.1% of mouse liver protein, and its mRNA is approximately 0.03% of liver poly(A)$^+$ mRNA. Yet, a number of cDNA clones were identified by screening 1400 cDNA clones made from 9-10S mouse liver poly(A)$^+$ mRNA using this method.

D) Immunological Screens - It is sometimes possible to detect small amounts of proteins made in bacterial clones by immunological methods. If a gene fragment is inserted within a bacterial protein in the correct RNA polymerase reading phase, antigenic determinants on the higher cell protein can be synthesized and detected. Two methods have been used to date. One depends on the precipitation of the antigen by antibodies included in the agar on the plate or in an agarose overlay;[82,83] the second uses plastic sheets coated with antibody.[84-86] These sheets are exposed to

lysed bacteria so that released antigen can bind. The immobilized antigen is then labeled with radioiodinated antibody. Autoradiography identifies the clones that are producing the antigenic sequences. Using this method, for example, Chang et. al. observed translation of dihydrofolate reductase when its cDNA was inserted out of phase into the β-lactamase gene of pBR322.[72] Translation of hemagglutinin could be detected by this method even when the gene was inserted in the wrong orientation into the equivalent HindIII site of a tryptophan expression plasmid,[87] and it was necessary in this case to invoke both internal initiation of translation and transcription from a previously unrecognized promoter.

E) Antibiotic Selection - This method is in most cases only useful for the construction of new cloning vehicles or for estimating the number of background clones that do not contain any inserted DNA sequences. Essentially it involves plating the transformed bacteria on agar that contains an appropriate antibiotic. Should the plasmid contain an intact gene for this antibiotic, the cells will survive; otherwise they die. The construction of pBR322 itself is a classic example of the use of this method.[88]

F) Characterization by Size - Often the desired plasmid (or λ phage) can be selected simply by looking at the size of the chimeric DNA in each clone. Such an assay, when carried out by electrophoresis on mini gels, require little starting material. The popular procedure of Birnboim and Doly allows one to rapidly assay individual plasmid clones for this purpose.[89]

Methods to detect recombinant clones in eucaryotic cells are quite analogous to their counterparts for procaryotes. Two commonly used methods exist.

A) Villarreal and Berg were the first to describe the adoption of the Grunstein and Hogness procedure in situ hybridization for eucaryotic cells.[90] They described a simple procedure that distinguishes plaques containing SV40 genomes carrying specific segments of non-viral DNA from those infected with SV40 alone. They did this by transferring the cell monolayers and their plaques to nitrocellulose disks and treating the imprinted dishes with alkali to denature and immoblize the cell and viral DNA. Hybridization with appropriate highly labeled nucleic acid probes and subsequent radioautography allowed them to identify the plaques containing DNA homologous to the probes. Virus contained in the plaques could be recovered from the corresponding region of the agar overlay which was removed from the cell layer prior to the imprinting step. With this procedure, λ-SV40 hybrid viral genomes could be detected and recovered even when they are present in an SV40 preparation at a frequency of about 10^{-5}. More recently, this procedure has been improved upon by Hayday et. al.[91] Their procedure facilitates the screening of mixed cell populations and the distinction of those clones containing multiple copies of a particular DNA.

B) Genetic selection is also rapidly becoming a powerful tool with which to screen for recombinant eucaryotic cell clones. There are numerous examples where viral thymidine kinase genes have been used as a selectable marker to isolate cell lines that contain other cloned DNA sequences.[92,93] There are other examples of mutant cell lines lacking a particular enzyme,

which are being used to select for recombinants. For example, hypoxanthine phosphoribosyltransferase deficient cells transfected with appropriate vectors and grown on the appropriate medium offers a very sensitive method to detect the desired recombinant clones.[34,35] Indeed, by utilizing the fact that a cell infected and transformed by some SV40 vectors can utilize xanthine in the medium, a very general way to detect positive clones in eucaryotes has been designed.[35]

5) Alteration of Cloned DNA Sequences: The ability to alter cloned DNA sequences in a specific manner has many potential applications in recombinant DNA technology. Long-term goals of almost any gene cloning project can now include a study of how altered gene function behaves in vivo in a cell. A number of methods are now available to alter cloned DNA fragments at precise regions. Shartle and Nathans[94] demonstrated the use of sodium bisulfite to cause local mutagenesis in SV40 in a manner that is now of general use for recombinant DNA. SV40 DNA was prepared for localized mutagenesis by nicking the molecule specifically at a site with a restriction endonuclease that recognizes a single site in the SV40 DNA, and then extending the nick enzymatically to expose a short, single-stranded segment of DNA. The "gapped" DNA was then treated with a single-strand-specific mutagen, sodium bisulfite, which converts cytosine to uracil. After mutagenesis, the gap was repaired with DNA polymerase, generating molecules resistant to the restriction enzyme used to make the initial nick. From cells infected with DNA thus modified, SV40 mutants could be isolated. Shartle et. al. improved upon this idea with the use of a homologous ss-DNA fragment to direct the nicking of circular duplex DNA within a segment defined by the DNA fragment in a two-step reaction.[95] First, E. coli recA protein was used to catalyze assimilation of the homologous single-stranded DNA, producing a displacement loop ("D-loop") in the circular DNA. Second, a small amount of the single-strand-specific S1 nuclease was used to nick the displaced DNA. The segment-directed nicks were converted to small gaps, which could be mutagenized specifically with sodium bisulfite.

The single stranded DNA phage M13 lends itself well to site-directed mutagenesis. Using oligonucleotide primers with specific base sequences and E. coli polymerase I to make a double-stranded DNA vehicle, very precise gene alterations can be made.[96] D-loop structures can also be used to get targeted deletions in cloned DNA sequences. Green and Tibbetts observed that the site of D-loop formation can be directed by using ss-DNA derived from a selected restriction fragment.[97] Circular DNA containing a D loop can then be linearized by cleavage with endonuclease S1. This cleavage appears to remove a limited number of nucleotides from each strand of the circular DNA substrate. Incubation with polynucleotide ligase followed by propagation in vivo leads to circular DNA molecules that bear small, single deletions in the region of the single-stranded DNA sequence chosen for the formation of the D loops. Green and Tibbetts utilized these manipulations of DNA to construct tetracycline-sensitive deletion mutants of plasmid pBR322. The level of mutagenesis obtained by the procedure is sufficiently high that selective growth and screening procedures are not necessary for the isolation or identification of mutants.

Procedures also exist for generating insertions in cloned DNA sequence sequences. For example, Heffron et al. have described a method for mutagenizing circular DNA molecules that involves synthetic oligodeoxynucleotide restriction sites as mutagens.[98] A single synthetic restriction

site is introduced at random by cleaving circular DNA with a nonspecific double-strand endonuclease. The restriction site is then ligated to the ends and the molecule is subsequently recircularized. These small additions to the genome are then mapped by digestion with the appropriate restriction enzyme. Rearrangements such as duplications and deletions can be engineered at will by using the added restriction sites. This technique has been used by Heffron et. al. to produce a fine-structure map of RSF1050,a ColE1 derivative, 60% of which is a transposable DNA sequence encoding the TEM β-lactamase (Tn3).[98]

Conclusion - Recombinant DNA research is perhaps the most active area of scientific research we have at present. New ideas and techniques are appearing weekly. At this rate of progress it should be possible within a few years to obtain any information desired about a particular gene, to alter that gene, supplement its function in organisms where it is lacking (including man), and study how it is expressed in vivo.

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Chapter 24. Platelet Activating Factor (PAF), A Novel Type of Phospholipid with Diverse Biological Properties

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Introduction - In the Fall of 1979, three separate research groups independently described the partial synthesis of a new type of biologically active phospholipid that possessed potent platelet aggregating[1,2] antihypertensive,[3] and allergic (see Anaphylaxis) properties. The structure of this unique phospholipid is 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (alkylacetyl-GPC). Each group prepared the active phospholipid by organic synthetic modifications of a naturally occurring plasmalogen from bovine heart. Structural proof for the cellular occurrence of alkylacetyl-GPC came later when platelet-activating factor (PAF), isolated from stimulated rabbit basophils[4] and hog leukocytes,[5] was shown to have an identical chemical structure; identification was based on the formation of derivatives formed by chemical and lipase reactions, thin-layer and gas-liquid chromatography, high performance liquid chromatography, and mass spectrometry. In addition, Clark et al.[6] have shown that the PAF secreted by stimulated neutrophils and monocytes from humans and the PAF produced by stimulated neutrophils and basophils from rabbits are identical in terms of their chromatographic and chemical behavior. The minute amount of alkylacetyl-GPC produced by cells makes it difficult to conduct definitive chemical structural studies, since large quantities of cells are required for the isolation of alkylacetyl-GPC for analytical studies. However, the capacity of mammalian tissues to synthesize[7-9] and catabolize[10,11] alkylacetyl-GPC by highly specific enzymes has been documented in rat tissues and in human blood cells;[12] assays of these enzymes should be useful in assessing the role of alkylacetyl-GPC in different cell types and in evaluating the regulatory factors involved. Several articles are available that have reviewed both the earlier and more recent developments of research on PAF.[13-16] Studies of an antihypertensive polar renomedullary lipid (which appears to have similar biological properties to alkylacetyl-GPC and could be identical) have also been summarized.[17]

Organic Synthesis of Alkylacetyl-GPC and Related Analogs — Initial studies that showed alkylacetyl-GPC possessed biological activities used choline plasmalogens[1,3] or lysoethanolamine plasmalogens[2] as the starting material for the synthesis of the bioactive phospholipid class. Choline plasmalogens, which are uniquely high in heart tissue,[18] were hydrogenated, deacylated, and acetylated to form alkylacetyl-GPC.[1,3] The lysoethanolamine plasmalogens were converted to the active molecule by successive methylation, hydrogenation, and acetylation.[2] The complete organic synthesis of the natural isomer, 1-octadecyl-2-acetyl-GPC, has also been accomplished; it requires ten reaction steps beginning with D-mannitol.[19] Another method has used 1-alkyl-2-acyl-sn-glycerols (isolated after lipase treatment of rat fish liver oil); the latter are then phosphorylated and aminated and after partial hydrolysis to 1-alkyl-2-lyso-GPC, an acetylation reaction produces the natural isomer, alkylacetyl-GPC.[20] Recently, alkylacetyl-GPC, 3-alkyl-2-acetyl-GPC, and the racemic mixture

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of alkylacetyl-GPC were obtained by acetylating 1-alkyl-2-lyso-GPC derived from 1-alkyl-sn-glycerol (prepared from D-mannitol); the synthesis of the lysophospholipid provides better yields and a more versatile derivative for the subsequent synthesis of analogs[21] than when the acetate is added at an earlier stage.[19] The synthetic routes for a number of specific analogs of alkylacetyl-GPC have been described in conjunction with the biological testing of alkyl phospholipid analogs.[22-25] Alkylacetyl-GPC itself is available commercially.

Structural-Activity Relationships — The synthesis and testing of various analogs of alkylacetyl-GPC have produced important information on the structural features of the phospholipid that are required for biological activity. The most potent activity, with respect to both platelet aggregation and hypotensive activity, is obtained with alkylacetyl-GPC or alkylpropionyl-GPC. However, substitution of the dimethylethanolamine[22] at the sn-3 position or an ethoxy[25] group at the sn-2 position produces analogs that also have significant biological activity. Some short chain acyl substituents at the sn-2 position appear to alter the potency of specific biological activities, e.g., the 2-maleyl and 2-succinyl analogs cause neutrophils to have greater adherence, whereas the 2-acetyl lipid causes greater secretory activity.[26]

It was originally reported that the unnatural enantiomer, i.e., 3-alkyl-2-acetyl-GPC, also showed significant activity in stimulating the release of serotonin from rabbit platelets,[23] but it now appears that the preparation used was contaminated with the sn-1 enantiomer.[24] Thus, current evidence indicates that only the naturally occurring isomeric form of the phospholipid possesses the biological activities and that a stereospecific receptor is involved.[21,24] Long chain acyl groups and the lyso form at the sn-2 position have very low or no biological activity and relatively small modifications of the polar head group cause decreases and ultimate loss of biological activity. The O-alkyl chain at the sn-1 position seems to be absolutely essential for optimum activity. Recently, two fractions of alkylacetyl-GPC were prepared in which one was enriched (95 mol %) in the 16:0 alkyl species and the other in the 18:0 alkyl species;[27] the O-hexadecyl preparation was approximately 3-fold more effective than the O-octadecyl preparation in inducing an equivalent release of serotonin from rabbit platelets. Similar results were obtained for the 16:0 vs. 18:0 alkyl species of 1-alkyl-2-lyso-sn-glycero-3-ethanolamine except much higher levels (3.7×10^{-7} M) of the phosphoethanolamine analog were required for the serotonin release.[27]

The relative potencies of the phospholipid analogs so far tested in relation to alkylacetyl-GPC appear to show responses of similar magnitude with respect to platelet aggregation and blood pressure. However, since different species of animals have generally been used for the platelet and blood pressure studies, it is difficult to generalize the relationships of the various biological activities.

Metabolism

1. In vivo Studies: A number of articles have been published on the biological effects of alkylacetyl-GPC when administered in vivo (see Anaphylaxis), but only one report has appeared on its in vivo metabolism.[28] Clearance curves for 1-[1′,2′-^{3}H]alkylacetyl-GPC from blood after an intravenous injection in normotensive male rats ($T_{1/2}$ ≈30-120 s) closely resemble the hypotensive response curves. The highest quantities of tritium were found in lung, liver, spleen, and kidney, both as metabolites

(primarily the sn-2 lyso and sn-2 long chain acyl analogs) as well as unaltered alkylacetyl-GPC. The results of these experiments indicate that deacetylation and reacylation reactions involving long chain acyl moieties are the primary metabolic pathways involved in the inactivation of alkylacetyl-GPC in vivo.

2. Cellular Metabolism: Only limited studies have been published on the metabolism of alkylacetyl-GPC in homogeneous populations of intact cells. Chap et al.[29] reported that when platelets are stimulated by ionophore A23187, labeled acetate is rapidly incorporated into alkylacetyl-GPC; the results imply that 1-alkyl-2-lyso-GPC can serve as a precursor of alkylacetyl-GPC. Studies conducted with L-M fibroblasts grown in monolayer cultures[30] have shown that 1-[1′,2′-^{3}H]alkylacetyl-GPC is metabolized to sn-2 lyso and sn-2 long chain acyl products. These latter results support the whole animal studies, which suggest that a deacetylation-reacylation reaction is the common pathway for the inactivation of alkylacetyl-GPC.

3. Biosynthetic Enzymes: The first step in the formation of the O-alkyl linkage in glycerolipids involves a unique reaction catalyzed by alkyl-DHAP synthase, a microsomal enzyme.

$$\text{Acyl-DHAP} + \text{ROH} \longrightarrow \text{Alkyl-DHAP} + \text{RCOOH}$$

Acyl-DHAP, a substrate for alkyl-DHAP synthase, represents an important branchpoint in lipid metabolism since it can be utilized as a precursor for the synthesis of both ester and ether phospholipids.[31] The precursor of the O-alkyl linkage, fatty alcohols, are derived from acyl-CoAs via acyl-CoA reductase. Details of these key enzymic reactions and others involved in the biosynthetic ether lipid pathway have been reviewed.[31] Most recent advances in this field have dealt with the mechanism of action of alkyl-DHAP synthase.[32-35] Stimulated neutrophils and eosinophils contain significant alkyl-DHAP synthase activities and it is this enzyme that would provide the O-alkyl skeleton structure for subsequent reaction steps that lead to the formation of alkylacetyl-GPC.[12]

Acetyltransferase (Fig. 1) catalyzes a highly specific transfer of acetate from acetyl-CoA to 1-alkyl-2-lyso-GPC.[7] Acetyltransferase activity is present in many rat tissues, but it is highest in kidney, spleen, lung, lymph nodes, and thymus. The apparent K_m for acetyl-CoA was 67 μM in kidney cortex. Results obtained with inhibitors and substrate competition experiments indicate that the properties of acetyltransferase are distinct from long chain acyltransferases. The activation and inactivation of the alkyl phospholipid through acetylation and deacetylation would appear to be governed by acetyltransferase and acetylhydrolase (discussed below); however, phospholipase A_2 and acyltransferase are also undoubtedly involved (Fig. 1). It is noteworthy that sizable quantities of alkylacyl-GPC has been found in target cells, i.e., rabbit polymorphonuclear neutrophils.[36]

Another biosynthetic pathway that has been described for the synthesis of alkylacetyl-GPC is the following reaction catalyzed by a cholinephosphotransferase:[8]

$$\text{1-Alkyl-2-acetyl-}sn\text{-glycerol} + \text{CDP-choline} \longrightarrow \text{alkylacetyl-GPC} + \text{CMP}$$

The fact that cholinephosphotransferase activity was stabilized by dithiothreitol when 1-alkyl-2-acetyl-sn-glycerol was the substrate and inhibited when 1,2-diacyl-sn-glycerol was the substrate, combined with substrate competition results and other data with inhibitors, suggest that two

different cholinephosphotransferases are involved in the conversion of 1-alkyl-2-acetyl-sn-glycerol and diacylglycerol to their respective phospholipid products.

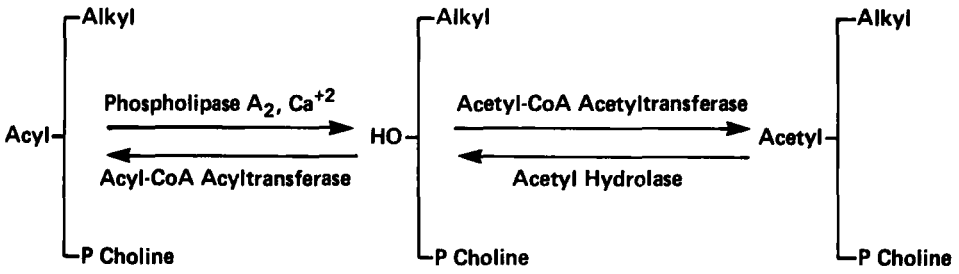

Fig. 1

4. Catabolic Enzymes: Alkylacetyl-GPC can be inactivated by acetylhydrolase (Fig. 1), a cytosolic enzyme that hydrolyzes the acetate group; other phosphatides with a sn-2 acetyl group also appear to serve as substrates, whereas 1-alkyl-2,3-diacetyl-sn-glycerol did not.[10] Acetylhydrolase is found in most tissues but its activity is especially high in kidney, lung, and brain; in kidney cortex the K_m was 3.1 μM and the V_{max} was 17.8 nmol/min/mg protein when alkylacetyl-GPC was the substrate. Effects of calcium, magnesium, EDTA, dithiothreitol, deoxycholate, and diisopropylfluorophosphate on acetylhydrolase activity differed from those reported for phospholipase A_2. Moreover, the addition of egg phosphatidylcholine to the incubation system had no effect on the reaction kinetics. These results indicate acetylhydrolase has completely different properties than the known activities of phospholipase A_2 that hydrolyze the long chain acyl moieties at the sn-2 position of phospholipids.

An acid labile factor that can inactivate alkylacetyl-GPC has been reported to be present in rabbit[37] and human[38] sera. Since the thermolability of this factor was not tested, it is not clear whether the inactivation of alkylacetyl-GPC was due to an enzymatic activity or some other component. The factor appears to be a lipoprotein or at least very closely associated with lipoproteins.[38] It is possible that the serum factor is acetylhydrolase, since it is known that plasma contains acetylhydrolase.[10]

Characteristics of a Pte•H_4-dependent monooxygenase in rat liver that cleaves the O-alkyl linkage in glycerolipids[39] have been described in considerable detail.[40] Cleavage activity is most prominent in liver and appears to bear an inverse relationship to the tissue level of alkylglycerolipids.[41] The same monooxygenase also cleaves the ether linkage in 1-alkyl-2-lyso-GPC. Earlier studies of substrate specificity[42] had shown that the alkyl cleavage enzyme can cleave the ether chain of a number of glycerolipids, providing the sn-2 position is lyso. Thus, it was not too surprising that 1-alkyl-2-lyso-GPC served as a substrate for the cleavage enzyme and that cleavage activity was absent when the acetate was at the sn-2 position.[11] Thermal inactivation, catalase treatment, and the Pte•H_4 requirement for the reaction indicate that the monooxygenase that utilizes 1-alkyl-2-lyso-GPC is identical to the alkyl cleavage enzyme that cleaves the O-alkyl linkage in other lipids. It appears then that the first step in the catabolism of alkylacetyl-GPC is catalyzed by acetylhydrolase and that either the Pte•H_4-alkyl monooxygenase can then remove the lyso product from the lipid pool by cleavage of the O-alkyl linkage or an acyltransferase can acylate it.

Biochemical Properties

1. Calcium Involvement: It is well established that calcium ionophore A23187 stimulates the release of alkylacetyl-GPC from alveolar macrophages derived from rabbits, rats, and humans[43] and human leukocytes.[44] Therefore it was of interest that we found alkylacetyl-GPC could stimulate the influx of calcium into rabbit platelets, whereas various analogs (3-hexadecylacetyl-GPC, 1-hexadecyl-2-lyso-GPC, 1-acyl-2-acetyl-GPC, or 1-hexadecanoyl-2-lyso-GPC) tested under the same conditions had much less of an effect on the calcium uptake.[45] Alkylacetyl-GPC also stimulates the uptake of calcium in polymorphonuclear leukocytes.[46] Although alkylacetyl-GPC possesses an ionophoretic-like activity, the mechanism of action accounting for the stimulation of calcium movement across the membrane by PAF is unknown. In artificial membrane systems, it has been shown that alkylacetyl-GPC does not affect calcium influx, even though other lipids (phosphatidic acid and oxidized fatty acids) selectively translocate divalent cations across liposomal membranes in the same order of magnitude as two known calcium ionophores, A23187 and ionomycin (Mn>Ca>Mg).[47]

2. Prostaglandin Relationship: Studies based on data obtained with inhibitors of prostaglandin synthesis (aspirin, eicosatetraynoic acid, and indomethacin) indicate that the effects of alkylacetyl-GPC are independent of arachidonate metabolism in stimulated basophils, macrophages, and platelets.[48,49] However, prostaglandin involvement is somewhat clouded by reports that phospholipase activity (release of arachidonate) and thromboxane synthesis are stimulated by alkylacetyl-GPC[50] and that PGI_2 and PGI_2-like activities can inhibit the aggregation of platelets and the release of [^{3}H]serotonin induced by alkylacetyl-GPC in rabbit platelets.[51] A recent investigation[52] has suggested that the biological actions of alkylacetyl-GPC on polymorphonuclear leukocytes might involve the release of arachidonic acid and the subsequent production of hydroxyeicosatetraenoic acids. Obviously, further clarification of the important relationship between arachidonic acid metabolites and alkylacetyl-GPC is needed.

3. Membrane Binding Sites: Data on the binding properties of alkylacetyl-GPC have been reported for rabbit platelets;[53,54] comparisons were made with erythrocytes, lymphocytes, and neutrophils, which also bound the active phospholipid, but to a lesser extent than platelets. The binding of alkylacetyl-GPC to platelets was saturable and was temperature- and concentration-dependent; however, binding was independent of extracellular calcium levels.[53] With saturable amounts of alkylacetyl-GPC required for maximum secretion by platelets, not all of the binding sites were occupied. The rate of binding and the secretion of alkylacetyl-GPC in platelets were identical, but the temperature-dependence studies indicated that secretion of alkylacetyl-GPC by platelets was not rate-limiting.

Physiological Responses in Target Cells and Related Studies

1. Platelets: Alkylacetyl-GPC can be formed by washed rabbit platelets after stimulation by platelet aggregating agents (calcium ionophore A23187, thrombin, or collagen) under conditions of aggregation that are not dependent on ADP release or TXA_2 production.[55] ADP, arachidonic acid, or alkylacetyl-GPC by themselves have no effect on the production of alkylacetyl-GPC. Studies with human platelets have shown that the aggregation and release of [^{3}H]serotonin by platelets is dose-dependent.[56] In this system, ADP scavengers, indomethacin, EDTA, and EGTA inhibited the aggregation response but did not affect secretion, whereas prostaglandins inhibited only the aggregation response initiated by alkylacetyl-GPC.

2. Basophils: IgE-sensitized basophils from rabbits release PAF when challenged with antigen,[57,58] but conflicting reports have appeared on the release of alkylacetyl-GPC from human basophils.[44,58-60] Betz et al.[58] and Sanchez-Crespo et al.[59] found that human basophils do not release PAF by a number of stimulants all known to be effective with rabbit basophils. In contrast, Camussi et al.[44,60] have described the alleged production of PAF by human basophils that were challenged with C5a, cationic proteins from neutrophils, anti-IgE, or Synacthen and by basophils isolated from a patient with systemic lupus erythematosus; under these conditions degranulation and release of histamine occurred.

3. Neutrophils: A calcium-dependent release of alkylacetyl-GPC that is closely linked with phagocytosis occurs in rabbit neutrophils treated with opsonized zymosan.[61] Release of PAF also accompanies the aggregation of polymorphonuclear neutrophils (humans and rabbits) induced by C5a anaphylatoxin, neutrophil cationic proteins and related peptide fragments, opsonized yeast particles, and immune complexes.[59,62,63] The chemical-physical properties of the PAF released from stimulated neutrophils and monocytes obtained from humans are identical with the properties of PAF released by stimulated neutrophils and basophils of rabbits.[6] Betz and Henson[64] have shown that the step involving the release of PAF by stimulation of neutrophils is distinctly separate from the degranulation step.

Alkylacetyl-GPC itself is also a potent stimulator of human neutrophil responses[24,25,65] that include exocytosis, migration, superoxide production, and aggregation. Approximately 10-fold higher concentrations of alkylacetyl-GPC and 1-alkyl-2-ethoxy-GPC were required for the aggregation of human neutrophils than those required for rabbit neutrophils; the relative potencies of alkylacetyl-GPC, 1-alkyl-2-ethoxy-GPC, and 1-alkyl-2-lyso-GPC with rabbit neutrophils were 10,000:1000:1, respectively.[25] In rabbit neutrophils, the degranulation response induced by PAF requires extracellular calcium.[46] Desensitization of the neutrophil response to PAF occurs when higher concentrations or preincubations of alkylacetyl-GPC or biologically active analogs are involved.[55,66] In contrast, neutrophils desensitized by preincubations with 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid were degranulated in a normal fashion by alkylacetyl-GPC,[67] but did not aggregate.[68]

4. Macrophages: A number of studies have shown that alkylacetyl-GPC could be readily released from peritoneal macrophages in the rat[69-71] and mouse.[70] Release of PAF occurs during phagocytosis of zymosan particles, antibody-coated erythrocytes, immune complexes, and Bordetella pertussis by peritoneal macrophages.[69] Stimulation of macrophages by high pH, calcium ionophore A23187, Compound 48/80, C5a anaphylatoxin, anti-IgE, and specific antigens also induced the release of PAF.[70] Granules produced by the mastocyte-degranulating compounds are phagocytized by the macrophages, which in turn induces the release of alkylacetyl-GPC. Mastocytes themselves do not appear to release PAF and are only involved indirectly in furnishing granules for the macrophage's response.[70]

Alveolar macrophages from humans, rats, and rabbits release alkylacetyl-GPC after stimulation with calcium ionophore A23187.[43] In addition the alveolar macrophages from both the rat and rabbit were found to release alkylacetyl-GPC when zymosan was phagocytized. Zymosan had no effect on PAF release in human alveolar macrophages, despite their phagocytic activity.[43]

Anaphylaxis (Respiratory and Cardiovascular Events) — The important role of alkylacetyl-GPC in anaphylaxis and acute allergic responses that are IgE-mediated has been well documented. The following sequential systemic events appear to occur during anaphylaxis induced by injection of antigen into rabbits that have been conditioned to make only IgE antibody against the antigen:[72] a) the antigen stimulates circulating IgE-sensitized basophils to release PAF, b) PAF causes intravascular aggregation of platelets and their sequestration primarily in the lung, and c) the sequestered platelets degranulate and release selective components. The platelets that return to circulation after anaphylaxis are desensitized as evidenced by their unresponsiveness to PAF-induced secreting activity.[73]

Intravenous injections of synthetic alkylacetyl-GPC to rabbits produce acute reversible intravascular changes (neutropenia, release of platelet factor 4, thrombocytopenia) that are identical to those that occur in the anaphylaxis mediated via IgE.[74,75] Similar reversible intravascular effects, including the release of thromboxane B_2, also occur in the baboon.[76] Additional physiological effects reported for rabbits injected with alkylacetyl-GPC intravenously include rapid and shallow breathing, transient apnea, a decreased dynamic compliance, an increase in pulmonary resistance, bradycardia, elevated pressure in right ventricle, and systemic hypotension;[75] attenuated responses occurred, when after recovery, the rabbits were reinjected with repeated doses of alkylacetyl-GPC.[75] As an inducer of anaphylaxis, alkylacetyl-GPC possesses the properties of a spasmogenic agent[77,78] and is an enhancer of vascular permeability.[77] In the latter studies, alkylacetyl-GPC was shown to be able to contract smooth muscle in the lung and ileum of the guinea pig[77,78] and to increase skin vascular permeability as judged by the extent Evans blue dye spread on the inner skin surface at the injection site of PAF.[77] The sustained constriction of the ileum induced by alkylacetyl-GPC was not inhibited by antagonists of histamine, acetylcholine, and slow reacting substances; also rapid desensitization occurred upon restimulation.[77,78] These results indicate that the action of alkylacetyl-GPC on smooth muscle is independent of the release of histamine or receptors for C3a and C5a polypeptides that are anaphylatoxins.

Halonen *et al.*[79] have recently documented that not all of the respiratory and circulatory events of anaphylaxis (see above) that occur in the rabbit after the intravenous administration of alkylacetyl-GPC or antigen to IgE are dependent on a platelet mechanism. Platelet depletion had no effect on the apnea, right ventricular hypertension, bradycardia, and systemic hypotension induced by either the intravenous administration of alkylacetyl-GPC or the antigen to rabbits. In contrast, platelet depletion completely abolished the responses of increased pulmonary resistance and decreased dynamic compliance induced by alkylacetyl-GPC, whereas the lung mechanical changes (and apnea) induced by IgE anaphylaxis were unaffected by platelet depletion. Thus, the conclusion was reached that the alkylacetyl-GPC released during IgE anaphylaxis is only responsible for the ventilatory and circulatory effects and does not account for the mechanical lung changes observed.[79]

In regard to the cardiovascular alterations induced by intravenous injections of alkylacetyl-GPC, it is important to note that an antihypertensive polar renomedullary lipid (referred to as APRL) isolated from rat kidney has been shown to possess biological activities and chemical properties similar to those reported for PAF.[17,80] However, rigorous proof of the chemical structure for the biologically active phospholipid from the kidney is still lacking and, therefore, it can not be said with

certainty that PAF and APRL are indeed chemically identical phospholipid species. It is of interest that PAF has recently been shown to be released in vitro from perfused isolated rat kidneys that were stimulated 10 min previously by calcium ionophore A23187;[81] the chemical-physical and biological properties of the PAF released from kidney appeared to be identical to the alkylacetyl-GPC released from stimulated leukocytes.

Conclusions - The present literature provides descriptive information on the effects and responses of alkylacetyl-GPC in target cells. In some instances, the contributions of contaminating cells have led to conflicting interpretations. Information on the biochemical mechanism of action that accounts for the biological activities associated with alkylacetyl-GPC and the chemical-physical nature of the receptors involved are still lacking. Moreover, the quantitative contributions of the synthetic and catabolic enzymes and their regulatory controls need to be correlated with the biological responses that are induced by various stimuli in target cells.

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Chapter 25. Polyamine Metabolism - Recent Developments and Implications for the Design of New Chemotherapeutic Agents

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The biosynthesis of the polyamines, putrescine, spermidine and spermine, has been extensively investigated since the pioneering work of the Tabors and Rosenthal.[1] More recently, extensive research on the fate of the polyamines in biological systems has resulted in an overall picture of the metabolism of these biosynthetic cations, as depicted in Scheme 1. This chapter will focus attention on recent developments in the biosynthesis of the three major polyamines and their subsequent acetylation, oxidation and conjugation. Consideration of the mechanisms of these reactions allows for the design of new specific inhibitors of potential use as therapeutic agents against such diverse diseases as parasitic infections, cystic fibrosis, and cancer. This chapter is not intended as a complete review of polyamine metabolism; for an excellent collection of recent reviews on this subject, the reader is referred to the monograph edited by Morris and Marton.[2]

Biosynthesis of the Polyamines - The formation of putrescine (2) can occur via decarboxylation of ornithine (1), or from arginine (4) via decarboxylation to agmatine (3) and subsequent hydrolytic (or phosphorolytic) cleavage of the guanidino moiety.[3] Most attention in recent years has focused on the putrescine-forming reaction catalyzed by ornithine decarboxylase (ODC), an enzyme with an extremely short half-life ($t_{\frac{1}{2}}$ = 10 min.), and one which is induced by a myriad of stimuli. Recent data with Physarum polycephalum indicate that an endogenous ODC inhibitor, the so-called "ODC-antizyme", may be a polyamine-dependent protein kinase.[4] Thus, regulation of ODC apparently involves a kinase-phosphorylase system, similar to regulatory systems previously associated with c-AMP dependent protein kinases.[5] These findings not only provide a mechanism for the rapid inactivation of ODC, via a kinase-mediated phosphorylation, they also suggest that some of the many activators of ODC (e.g., hormones) may act by stimulating the presumed phosphorylase. A second pathway for the synthesis of putrescine is found in plants and microorganisms. Decarboxylation of arginine (4) is the first step in this alternate route. Since arginine decarboxylase (ADC) is highly regulated,[3] and has a short half-life ($t_{\frac{1}{2}} \cong$ 30 min.) in carrot cells,[6] it may be subject to a kinase-phosphorylase regulating system similar to that described above for ODC. In other plant cells (e.g., cucumber cotyledons[7]) over much less rapidly.

The biosynthesis of the unsymmetrical triamine spermidine (7) and the symmetrical tetramine spermine (8) involves the transfer of an aminopropyl group from S-adenosyl-(3-methylthio)propylamine (5) to the nucleophiles putrescine (2) and spermidine (7), respectively. These two very similar chemical reactions are catalyzed by two distinct enzymes in mammalian cells.[8] In E. coli, only one aminopropyltransferase has been isolated, and recent steady-state kinetics studies with that enzyme have been interpreted to implicate an aminopropylated enzyme intermediate in

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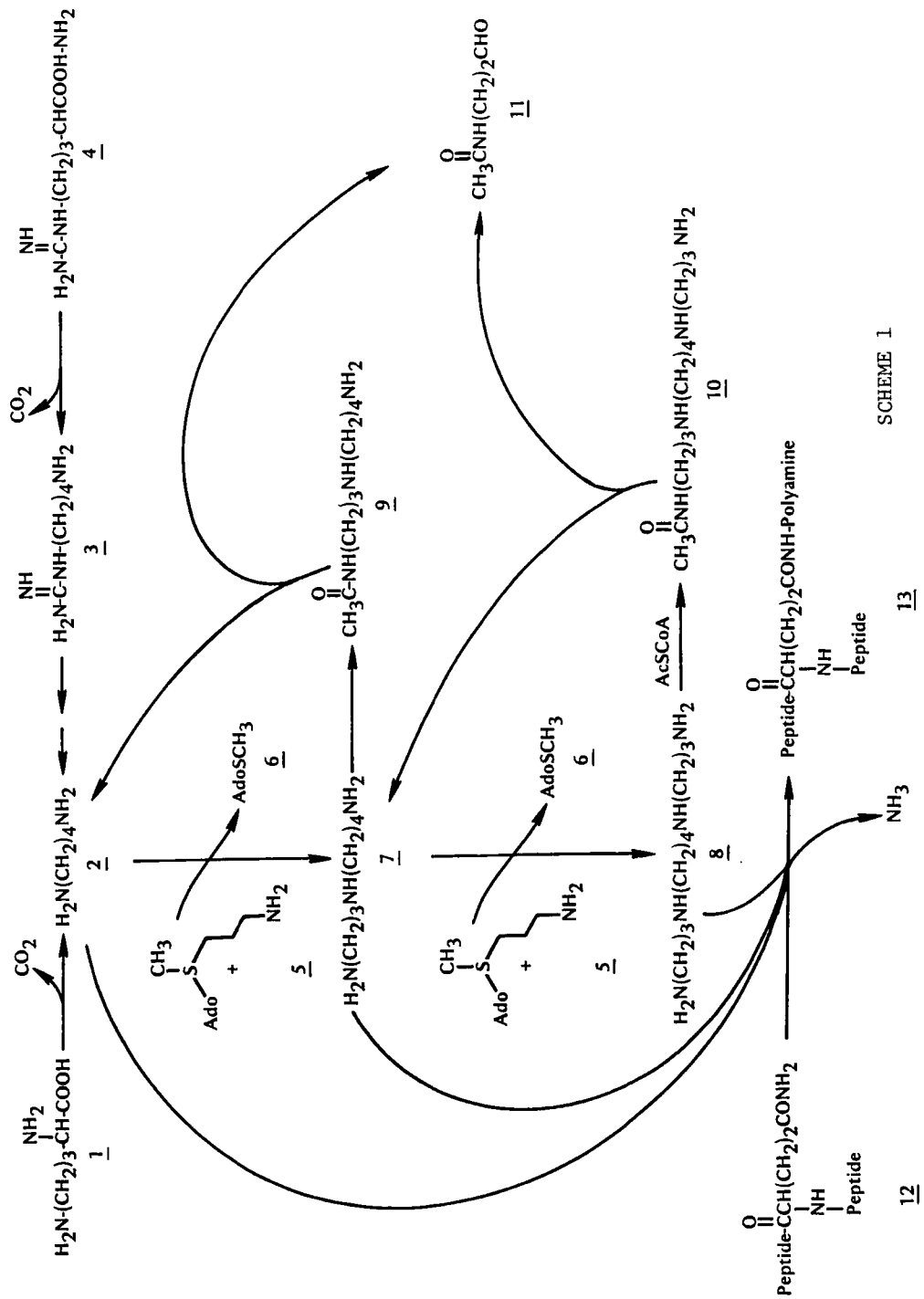
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1
H2N(CH2)4NH2
2
CH3
S
Ado
+
NH2
5
AdoSCH3
6
NH
H2N-C-NH-(CH2)4NH2
3
CO2
NH
H2N-C-NH-(CH2)3-CHCOOH-NH2
4
O
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9
H2N(CH2)3NH(CH2)4NH2
7
CH3
S
Ado
+
NH2
5
AdoSCH3
6
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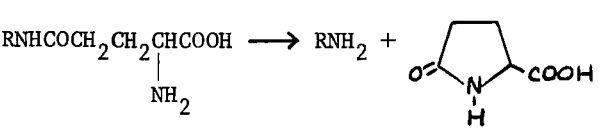
8
AcSCoA
O
CH3CNH(CH2)3NH(CH2)4NH(CH2)3 NH2
10
O
CH3CNH(CH2)2CHO
11
O
Peptide-CCH(CH2)2CONH-Polyamine
NH
Peptide
13
NH3
O
Peptide-CCH(CH2)2CONH2
NH
Peptide
12

SCHEME 1

the catalytic mechanism.[9] However, previous studies with other alkyltransferases (e.g., catechol O-methyltransferase[10]) suggest that non-kinetic methods, such as the use of chiral substrates as stereochemical probes, should be used to unambiguously distinguish between a single-displacement and a double-displacement ("ping-pong") mechanism.

Biochemical Reactions of the Polyamines - The facile oxidation of the polyamines has been known for many years.[11] Earlier studies were concerned with the oxidative deamination of polyamines to mono- or dialdehydes and subsequent β-elimination to yield acrolein and an amine (e.g., putrescine from spermidine). Alternatively, oxidative cleavage of the polyamines gives rise to an aminoaldehyde, which can cyclize to a pyrroline derivative, or can be oxidized to β- and γ-aminoacids (e.g., β-alanine, γ-aminobutyric acid). Recent studies have provided insight into the metabolic fate of polyamines in the liver[12] and brain.[13] It has been shown that N^1-acetylspermidine is readily formed by the action of an acetyltransferase on spermidine[12] and that N^1-acetylspermidine is readily converted to putrescine and 3-acetamidopropionaldehyde by a liver polyamine oxidase,[13] previously shown to be a flavoprotein.[14] That this pathway plays a significant role in the recycling of putrescine was shown by the lack of effect of the potent ODC inactivator α-difluoromethylornithine (DFMO) on putrescine content at early times (up to 6 hr), following CCl_4 treatment of rat livers.[12] Since CCl_4 stimulates spermidine N-acetyl transferase activity, with an associated increase in putrescine content, the inability of DFMO to inhibit the increase in liver putrescine is strong evidence for a non-ODC pathway of putrescine recycling. Therefore, despite the fact that only a small portion of cellular polyamines are acetylated the major route of oxidative metabolism of the polyamines may be *via* the acetylated derivatives.[15]

A second type of acylated polyamine is found in the protein-bound components (13, Scheme 1). It has been known for some time that polyamines are very effective substrates in the glutaminase-catalyzed reaction (12 → 13).[16] However, only recently have data been presented which clearly establish the presence of γ-glutamyl polyamines in mammalian cells.[17] In addition, evidence for γ-glutamylputrescine formation in rat seminal vesicles,[18,19] and covalent binding of spermidine to a specific protein (M_r = 18,000) in neuroblastoma cells,[20] suggest that conjugation of polyamines to proteins *via* the transglutaminase reaction is a common reaction. Since γ-glutamyl peptides are resistant to most proteolytic degradation,[21] it is of interest to establish how these conjugated polyamines are degraded *in vivo*. Recent work has demonstrated the existence of an enzyme, γ-glutamylamine cyclotransferase, which catalyzes the release of polyamines (RNH_2) as shown in eq. 1.[22] This enzyme acts only on



(1)

free, non-peptide glutamine derivatives. Thus, the removal of protein-bound polyamines apparently occurs after normal proteolytic degradation leads to the γ-glutamylamine derivatives, which are then cleaved by the cyclotransferase (eq. 1).[23]

Inhibition of Polyamine Metabolism - Research on the inhibition of polyamine metabolism has focused on biosynthetic pathways, and particularly on the inhibition of the decarboxylases, ODC and S-adenosylmethionine decarboxylase.[24] The latter enzyme converts S-adenosylmethionine

(AdoMet) to its decarboxylated derivative (5, Scheme 1). Inhibitors of ODC include the classical type such as α-methyl ornithine,[25] in addition to mechanism-based inhibitors such as N-(5'-phosphopyridoxyl)ornithine (PLP-Orn)[26] and α-difluoromethylornithine (DFMO).[27] Inhibitors of AdoMet decarboxylase include diguanidine derivatives such as methylglyoxal bis-(guanylhydrazone) (MGBG)[28] and α-methyl AdoMet.[29] The synthesis of α-difluoromethyl AdoMet has been reported,[30] but no biochemical data are presented. In the same paper, the synthesis of α-difluoromethyl arginine (DFMA) was described,[30] and this compound has recently been shown to be a potent irreversible inhibitor of arginine decarboxylase (4 → 3, Scheme 1).[31] The data presented suggest that DFMA inhibits arginine decarboxylase via a mechanism similar to that proposed for DFMO[28] and other enzyme-activated irreversible decarboxylase inhibitors.[32] Since mammalian cells contain little or no arginine decarboxylase, and since DFMA has been shown to inhibit that enzyme in intact microorganisms,[31] this new drug may be useful as an antiparasitic agent, with little or no host toxicity.

Of the decarboxylase inhibitors described above, DFMO[33] and MGBG[29] have received the most intense scrutiny as potential therapeutic agents. Recent studies indicate that DFMO is an effective agent against parasitic protozoa,[34,35] with no short-term toxicity to the host. Preliminary cell culture studies with DFMO in combination with cytotoxic agents, such as nitrosoureas[36] and MGBG[37], indicate that this ODC inhibitor may be of use in combination chemotherapy of cancer. Some of the decarboxylase inhibitors mentioned above, such as PLP-Orn[26] and α-CH_3AdoMet,[29] might not be expected to be taken up by cells. However, reports on the effects of α-CH_3AdoMet and its decarboxylated derivative on polyamine biosynthesis in cultured mouse lymphocytes[38] suggest that this sulfonium compound enters the cell. This conclusion needs to be unequivocably demonstrated with radioactive drug. In this connection, it should be noted that perfused rat liver appears to be impermeable to exogenous AdoMet.[39] Similarly, H-35 cells are apparently impermeable to ^{3}H-labeled PLP-Orn, and this drug had no effect on polyamine biosynthesis in this cell culture system.[40] Regardless, it would be premature to dismiss the use of charged drugs, especially in experimental cell culture systems. Methods are now available for making cells permeable to exogenous drugs, after which treatment the cells are "resealed", and allowed to grow under standard tissue culture conditions.[41]

The aminopropyltransferases, spermidine synthase and spermine synthase, are inhibited by the common product, 5'-deoxy-5'-methylthioadenosine (MTA, 6).[42,43] This product does not usually accumulate in the cell, but is rapidly cleaved by a liver or prostatic phosphorylase to yield adenine and 5-methylthioribose-1-phosphate.[19] The latter compound is converted to methionine by an unusual series of enzyme-catalyzed redox reactions, which are not yet fully characterized.[44] A similar salvage pathway has been reported to convert 5-methylthioribose to methionine in enterobacter aerogenes.[45] Based on chemical considerations, 5'-deoxy-5'-methylthiotubercidin (MTT) was synthesized as an inhibitor MTA phosphorylase.[46] As expected, MTT is not cleaved by the phosphorylase, and is a good inhibitor with Ki ≅ Km. MTT has been shown to inhibit spermidine and spermine biosynthesis in mammalian cells in culture, but the cytotoxic effects of the drug could not be blocked by co-administration of spermidine.[47,48] In contrast, the cytotoxic effects of high (5 mM) concentrations of DFMO could be blocked by co-administration of spermidine.[48] These results suggest that MTT affects biochemical processes other than inhibition of spermidine biosynthesis. MTT has also been shown to inhibit the growth of murine lymphoid cells.[49] Another

nucleoside 5'-thioether, 5'-deoxy-5'-(isobutylthio)adenosine ("S-isobutyladenosine", SIBA) has been shown to produce a large number of biological effects in cell culture systems.[50] These effects have been attributed to the inhibition of AdoMet-dependent methylases by SIBA, despite the fact that SIBA is very poor inhibitor of these methylases. However, based on recent data showing that SIBA is a substrate for MTA phosphorylase,[51] it would be prudent to consider modulation of polyamine metabolism as a possible basis for the observed biological effects of SIBA. It is appropriate to note that human malignant tumor cell lines have been shown to be deficient in MTA phosphorylase, whereas continuous cell lines from non-tumor tissue did not exhibit this deficiency.[52]

The lack of specificity observed with metabolically stable inhibitors such as MTT, and a related methylase inhibitor, S-tubercidinylhomocysteine,[53] suggested the synthesis of multisubstrate adduct inhibitors[26] for the individual alkyltransferases. S-adenosyl-1,8-diamo-3-thiooctane (AdoDATO) has been synthesized,[54] and shown to be a potent, specific inhibitor (I_{50} = 0.4μM) of spermidine synthase from rat prostate.[55] Cell culture (SV-3T3) studies have revealed that the drug (1-25μM) effects a dramatic decrease in intracellular spermidine concentration, while putrescine, spermine and decarboxylated AdoMet concentrations are increased, with little effect on cell growth.[56] At drug concentrations of 50-250μM, a dose-dependent decrease in cell growth is observed which can be blocked by spermidine but not putrescine. This is in contrast to the MTT results described above, and suggests that the effects of AdoDATO on cell growth are mediated *via* the effect of the drug on spermidine biosynthesis. The potent inhibition of spermidine synthase by AdoDATO suggested its use in combination with an ODC inhibitor in order to block two successive steps in polyamine biosynthetic pathway [Scheme 1: **1** → **2** (DFMO) and **2** → **7** (AdoDATO)]. When AdoDATO (10μM) and DFMO (5mM) were co-administered to cells in culture, both putrescine and spermidine concentrations were decreased to near the limits of detection, while spermine concentration was decreased by *ca* 70%. This effect on spermine biosynthesis could be blocked by co-administration of putrescine (25μM).[56]

In contrast to the variety of inhibitors now available for studying polyamine biosynthesis, there are few inhibitors described which are useful for studying the reactions which utilize the polyamines, such as acetylation, oxidative cleavage, and glutaminase-catalyzed protein binding. The design and synthesis of specific inhibitors of these reactions would be of considerable importance in the study of polyamine metabolism and its effect on cell biology. As a possible point of departure, the use of acetyl CoA derivatives (e.g., CoAS-SCH_3) as inhibitors of choline acetyltransferase has been reported.[57] Aminoguanidine is known to inhibit amine oxidases[15] and can be added to cell cultures to prevent spermidine toxicity caused by oxidases in the serum.[46] However, the polyamine oxidase described by Holtta,[14] thought to be the enzyme responsible for the oxidative cleavage of acetylated polyamines (Scheme 1; **9** → **2**, **10** → **7**),[12,13] is a flavoprotein. Thus, one could reasonably expect that a suitably designed acetylene might be a specific inactivator of the polyamine oxidase.[58] The transglutaminase reaction involves condensation of a polyamine and a protein (Scheme 1, **12** → **13**). The involvement of the macromolecular protein substrate complicates the design and synthesis of specific transglutaminase inhibitors. However, recent work suggests that smaller synthetic peptides can be used as substrates with monodansylcadaverine in the transglutaminase reaction.[59] Thus, it would appear that specific inhibitors of the polyamine transglutaminase reactions could be designed and synthesized for use in research on the role of these

reactions in the life of the cell.

Conclusions - The exact role of the polyamines in cell growth and differentiation is not well understood. However, results from investigations in many laboratories support the hypothesis that the polyamines, which are cationic at physiological pH, are closely associated with the synthesis and function of anionic nucleic acids. The use of inhibitors of polyamine metabolism as therapeutic agents is a relatively young field of research. Mention has already been made of some preliminary results involving the decarboxylase inhibitors, DFMO and MGBG, alone and in combination with other drugs. Another possibility is the use of inhibitors of polyamine metabolism together with drugs which effect hormone levels in organs rich in polyamines. Thus, the reduction of testosterone to 5α-dihydrotestosterone is catalyzed by the enzyme 5α-reductase (NADPH: Δ^4-3-oxosteroid-5α-oxidoreductase) in androgen-sensitive tissue, including the prostate. Recently, two new mechanism-based inhibitors of 5α-reductase have been reported. A 4-methyl-4-aza-5α-androstan-3-one derivative[60] acts as a reversible inhibitor, competitive with testosterone, with $Ki = 5 \times 10^{-9}M$. In contrast, a 4-diazo-5α-androstan-3-one derivative acts as a time-dependent, enzyme-activated (presumably irreversible) inhibitor of the reductase.[6] The 4-aza derivative has been shown to inhibit formation of DHT in rats and to have potent anti-androgenic activity in those animals.[62] It would be of interest to study the effects of inhibitors of polyamine metabolism in combination with these new 5-α reductase inhibitors. As the major roles of the polyamines in the cell become more clearly defined, possible sites of intervention for therapeutic use will become more apparent. At the present time, specific inhibitors of the reactions described in this chapter have been used, and will continue to be used, as biochemical and pharmacological tools. Such tools provide experimental approaches to elucidating the biological function of the polyamines.

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Chapter 26. Recent Developments in the Therapeutics of Disorders of Bone Metabolism

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In the past decade a variety of potent agents have become available for treatment of such diverse skeletal disorders as Paget's disease of bone, osteomalacia and rickets, and osteoporosis. In some instances the drugs produce a definitive cure of the disorder. One example is the use of calcitriol (1α, 25-dihydroxycholecalciferol) in patients with vitamin D-dependent rickets in which an apparent renal enzyme deficiency results in reduced blood levels of this most potent vitamin D metabolite. Suppression of disease activity, but not eradication, occurs in patients with Paget's disease who are treated with calcitonin or disodium etidronate (disodium ethane-1-hydroxy-1,1-diphosphonate). In the present review, the mode of action and pharmacological effects of selected agents will be detailed.

Calcitonin - Calcitonin is a 32 amino acid polypeptide hormone whose structure shows considerable variability among the species in which it has been elucidated (Figure 1).[1] However, identity of amino acid sequences is found

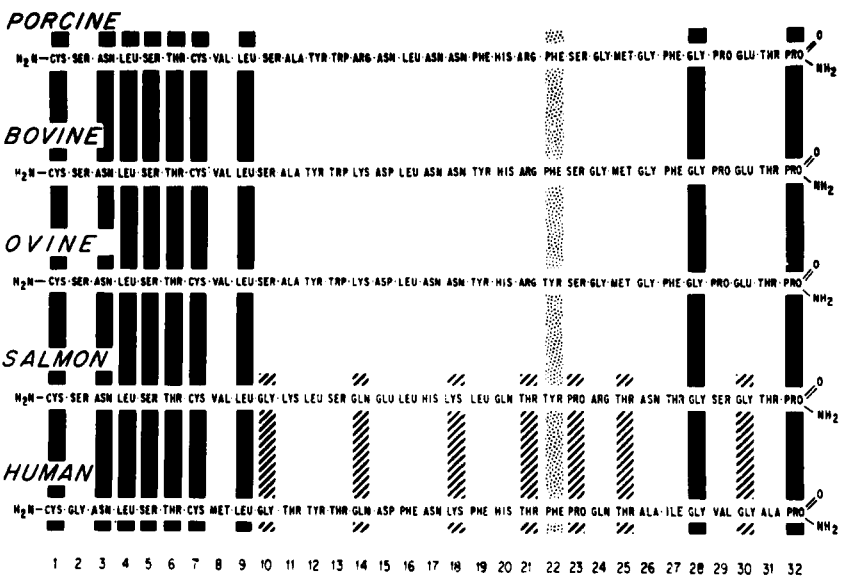

Figure 1: Comparison of amino acid sequences of porcine, bovine, ovine, salmon I and human calcitonin. Solid bars indicate sequence positions homologous among all five molecules; cross-hatched bars indicate salmon and human calcitonin. From reference 1 by permission of Excerpta Medica

at positions 1-7, 9, 28 and 32. Calcitonins of mammalian origin are less potent than those of fish origin such as salmon and eel calcitonin as judged

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by standard bioassays of acute hypocalcemic response in the rat.[2,3] The exact structural determinants which confer the greater potency on the fish calcitonins have not been identified. Studies have been done in which the effects of single and multiple amino acid substitution in the human calcitonin molecule have been examined.[4-8] The biological activity of human calcitonin could be increased by four to fivefold by replacing a single amino acid in position 8, 12 or 22 by the corresponding amino acid found in the dominant form of salmon calcitonin extracted from ultimobrachial glands (two minor forms of salmon calcitonin also exist).[2,7] A tenfold increase in potency was produced by replacing the three aromatic amino acids found at positions 12, 16 and 19 with the three leucines found in salmon calcitonin.[7] The most potent analogue of human calcitonin so far reported[7] resulted from the substitution of the three leucines in combination with the replacement of PHE^{22} with $TYR.^{22}$ This analogue was 15-20 times more potent than human calcitonin. Although little is known about the secondary structure of calcitonin, it has been postulated, based on several predictive methods, that the central region of ultimobrachial calcitonins has a helical structure potential.[9] Since several of the substitutions reported above are likely to induce an α-helix conformation in the altered molecule, this may prove to be a major link between the biological potency and possible secondary structure of calcitonin.

Although early studies had indicated that the entire calcitonin molecule was required for biological activity,[1] a fully active analogue of salmon calcitonin has been synthesized lacking the serine residue at position 2.[10]

The mode of action of calcitonin appears to depend, at least in part, upon activation of membrane-bound adenylate cyclase.[11] This occurs in concert with binding of the hormone to specific high affinity receptors.[11] In addition to bone cell receptors,[11,15,17,18] specific calcitonin receptors have been demonstrated on kidney cells,[11,16,18] brain cells,[19-22] testicular cells,[23] and on lymphocytes.[24,25] An unusual feature of the binding of labeled calcitonin to receptors of kidney and brain cells is a slow and incomplete reversibility of binding.

The concept that calcitonin action is also dependent on membrane calcium transport has received some experimental support. In both isolated bone cells[26,27] and kidney cells,[28] calcitonin produces an increase in intracellular calcium concentration, primarily in the mitochondria. A third proposed mode of action is that calcitonin causes a rapid accumulation of free phosphate in specific bone cells and this initiates calcitonin action in an unknown manner.[29,30]

Calcitonin has become an established agent in the management of Paget's disease of bone, a disorder in which osteoclast proliferation appears to be the prime abnormality.[31] Chronic parenteral administration of porcine, salmon or human calcitonin produces a variety of clinical effects, including relief of bone pain, reduction of elevated skin temperature over affected extremities, reduction of elevated cardiac output, reversal of neurological deficits, stabilization of hearing loss and improvement of mobility.[31] An impressive benefit of calcitonin therapy is improvement of the bone architecture, particularly in respect to resolution of osteolytic lesions demonstrated radiographically.[32,33] Histologic studies also indicate a reduction in the number of osteoclasts and a more normal microscopic structure of bone.[31] Biochemical improvement is also found with an average decrease of serum alkaline phosphatase activity and urinary hydroxyproline excretion of 50% from pretreatment levels.[31] Side effects from calcitonin injections include nausea, facial flushing, a metallic taste

sensation, polyuria, diarrhea and very rarely tetany.[34] The severity of these symptoms occasionally requires discontinuation of the drug. Another rare complication of salmon calcitonin therapy is the development of allergic reactions, although antibodies to salmon calcitonin can be detected in the sera of more than 50% of treated patients.[35] In patients with high titers of anti-salmon calcitonin antibodies, resistance to the continuing therapy usually develops because of neutralization of hormone action.[35] Human calcitonin is effective in patients who become resistant because of calcitonin antibodies, but inexplicably a few patients have become resistant to calcitonin therapy in the absence of antibodies.[35,36]

Calcitonin has been used with some degree of success in the treatment of hypercalcemia, although in many patients with primary hyperparathyroidism or cancer the hypocalcemic effect is slight to moderate or short-lived.[37] More impressive responses have been observed in patients with less common causes of hypercalcemia, such as immobilization.[38] The treatment of postmenopausal osteoporosis with calcitonin has also produced equivocal results.[39,40]

A number of other pharmacologic effects of calcitonin have yet to be evaluated in clinical trials. In man, calcitonin has been demonstrated to increase renal excretion of sodium, inhibit gastric acid secretion, and inhibit pancreatic enzyme secretion.[41] Stimulation of fracture healing[42] and endochondral bone formation,[43] stimulation of wound healing,[44] reduction of vascular permeability,[45] inhibition of inflammation,[46] inhibition of eating,[47] and induction of a central analgesic effect[48] have been demonstrated in experimental animals.

Diphosphonates - The diphosphonates are synthetic compounds which are structurally similar to inorganic pyrophosphate (Figure 2), but because of chemical modification are resistant to enzymatic and chemical hydrolysis. Interest in producing such compounds was stimulated by the evidence that pyrophosphate could inhibit the precipitation of calcium phosphate from solution and might regulate calcification and rates of bone resorption and formation *in vivo*.[49]

The initial concept of the mode of action of diphosphonates in bone was that binding to surface crystals of hydroxyapatite resulted in inhibition of bone resorption and formation.[50] While this phenomenon may be of prime importance in diphosphonate action other direct or indirect effects of these compounds may contribute to their influence on mineral metabolism. A cellular effect has been suggested by observations that morphology of osteoclasts has been altered, both in culture[51] and *in vivo*.[52] Diphosphonates have been shown to inhibit adenylate cyclase *in vitro*.[53] However, diphosphonate inhibition of bone resorption stimulated by parathyroid hormone did not reduce bone cyclic AMP levels.[54] Diphosphonates, in particular Cl_2MDP, inhibit the lysosomal enzymes acid phosphatase and pyrophosphatase.[55,56] Surprisingly, Cl_2MDP stimulates an increase in alkaline phosphatase activity in cultured bone cells.[57] Other metabolic events in bone, which are influenced by diphosphonates, include decreased glucose consumption and lactic acid production,[58,59] an unexpected increase of bone and cartilage collagen biosynthesis,[60] increased proteoglycan biosynthesis by isolated chondrocytes,[61] and inhibition of the synthesis of prostaglandins in cultured calvaria cells.[62] Since cellular uptake of EHDP and Cl_2MDP has been demonstrated *in vitro*,[59] it is possible that some or all of the above phenomena can be explained by cellular effects of the compound. In addition, extraskeletal effects of diphosphonates suggest a cellular basis for the mode of action. EHDP and Cl_2MDP slow the release of calcium from kidney mitochondria,[63] EHDP depresses Na^+-dependent phosphate uptake

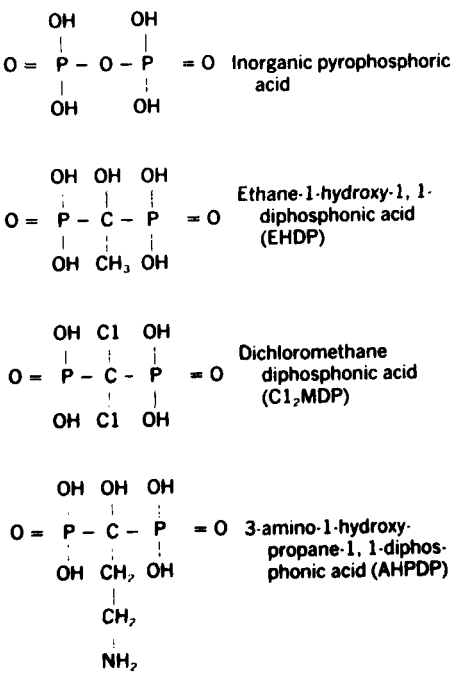

Figure 2: Chemical structure of diphosphonates. Modified from Fleisch, H., Arthritis Rheum., 23, 1162 (1980) and published with permission from the Arthritis Foundation.

by renal brush border vesicles,[64] and antiinflammatory effects have been reported.[65] It should also be appreciated that there are significant differences in potency, as well as metabolic effects, among the three diphosphonates that have been in clinical use. In general the order of potency is AHPDP>Cl_2MDP>EHDP.[66] Cl_2MDP and EHDP inhibit glucose consumption and lactate production as previously indicated,[58,59] but AHPDP does not.[66] Cl_2MDP stimulates bone and cartilage collagen and proteoglycan biosynthesis, but EHDP does not.[60,61]

The pharmacologic effects of diphosphonates have been extensively documented in experimental animals and in man. These compounds have been demonstrated to inhibit various types of experimental soft tissue calcification including renal stones[67] and nephrocalcinosis,[68] aortic calcification[68] and calcium deposits associated with adjuvant induced arthritis.[69] In growing animals, EHDP inhibits the mineralization of cartilage and bone[70] and produces a state grossly resembling vitamin D deficiency rickets. However, the histology of the epiphyseal plate does not resemble classical rickets and treatment of animals with 1,25-dihydroxycholecalciferol does not reverse the mineralization defect.[71] Large doses of EHDP do produce a decrease in intestinal calcium absorption due to impaired formation of 1,25-hydroxycholecalciferol, an abnormality which is corrected by treatment with the vitamin D metabolite.[72] Despite the great potency of Cl_2MDP in preventing soft tissue calcification and stabilizing crystal growth, Cl_2MDP has minimal effects on bone mineralization.[70]

Diphosphonates have been demonstrated to markedly reduce bone resorption and bone remodeling, particularly in young animals. This has been documented by histologic, radiologic and ^{45}Ca kinetic analyses. AHPDP is the most potent compound *in vivo*[66,73] despite *in vitro* studies indicating Cl_2MDP to be most effective.[51] With high doses the degree of bone resorption can be reduced to such an extent as to mimic the bone lesions of congenital osteopetrosis of the grey-lethal mouse.[74]

In man, the pharmacologic effects of diphosphonates have been most extensively studied in patients with Paget's disease of bone. The greatest experience is with EHDP.[75,76] Using oral doses of 5 mg/kg/day, symptomatic relief is produced in 60% of patients and serum alkaline phosphatase activity and urinary hydroxyproline excretion are reduced to 50% of pretreatment values.[75] At higher doses (10-20 mg/kg/day), a mineralization defect occurs which may lead to pathologic fractures.[77] Mineralization defects have not been observed in patients treated with Cl_2MDP[78] or AHPDP.[79] In general, diphosphonates produce the same clinical benefits as calcitonin and may result in prolonged remission after cessation of therapy. However, calcitonin appears to be far more effective in producing healing of osteolytic lesions.[80]

Both Cl_2MDP and AHPDP have been shown to be effective in partially or completely reversing hypercalcemia in patients with increased bone resorption.[81,82] Some success has been achieved with EHDP in preventing heterotopic ossification in spinal cord injury patients[83] and after total hip replacement,[84] but ectopic calcification complicating dermatomyositis or scleroderma is not controlled.[85] EHDP has been demonstrated to reduce the frequency of calcium nephrolithiasis in one study, but the dose used was in the range associated with defective bone mineralization.[86] In a preliminary trial, AHPDP produced clinical and biochemical improvement in five patients with rheumatoid arthritis.[87]

EHDP, but not the other diphosphonates, increases the renal tubular maximum for phosphate reabsorption and thereby produces hyperphosphatemia.[88]

Diphosphonates do not appear to undergo metabolism after administration. They either are found in bone or are excreted intact by the kidneys.[49]

Vitamin D Metabolites - The discovery of 25-hydroxycholecalciferol in the laboratory of De Luca[89] and the ensuing knowledge of other metabolites of the inert parent cholecalciferol (Figure 3) has led to the development of potent new agents for the treatment of a variety of disorders of mineral metabolism.

After biosynthesis of cholecalciferol (vitamin D_3) or ingestion of ergocalciferol (vitamin D_2) a series of hydroxylations take place in the liver and kidneys which converts these sterols to biologically active forms of vitamin D[90] (Figure 3). Although the exact physiological role of vitamin D is still a controversial subject, it is agreed that a major function is in the regulation of intestinal calcium and phosphorus absorption. The mode of action of the most active metabolite, $1,25(OH)_2D_3$, has been studied extensively and appears identical to that of steroid hormones.[91] Target organs such as the intestine contain specific cytosol receptors which bind $1,25(OH)_2D_3$. The $1,25(OH)_2D_3$-receptor complex can then penetrate into the nucleus and stimulate the synthesis of specific messenger RNA and proteins which are responsible for initiation of the biological response. In addition to the intestine, specific receptors are found in

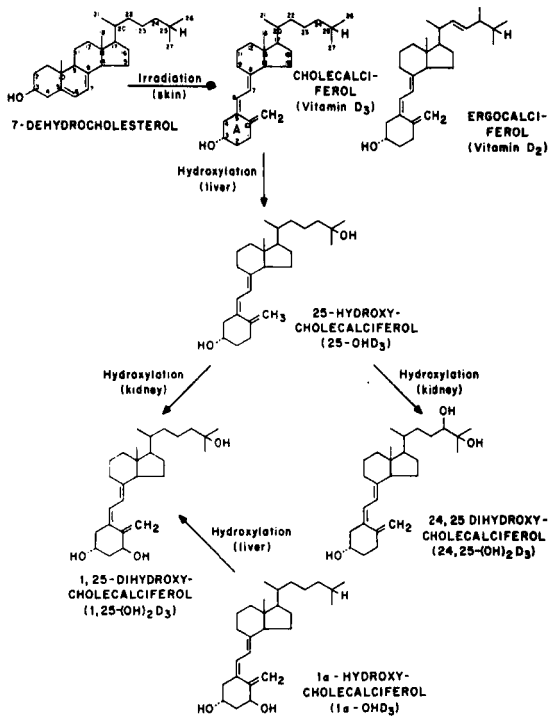

Figure 3: Chemical structures of the major vitamin D metabolites and 1α-OHD_3. Modified from Broadus, A.E., in: "Endocrinology and Metabolism", P. Felig, J.D. Baxter, A.E. Broadus, and L.A. Frohman, Eds., McGraw-Hill, New York, 1981, p. 963 and published by permission of the publisher.

bone[92] and kidney cells,[93] and unexpectedly in a wide variety of organs, including the pancreas, pituitary gland, placenta, parathyroid gland[94] and skin.[95] The function of vitamin D in respect to these organs has yet to to be clarified fully.

Both $25OHD_3$ and $1,25(OH)_2D_3$ have been approved by the Food and Drug Administration for the treatment of hypocalcemia in patients with chronic renal failure. $1,25(OH)_2D_3$ is particularly effective in the treatment of hypocalcemia and osteitis fibrosa associated with secondary hyperparathyroidism.[96] Inexplicably, results of $1,25(OH)_2D_3$ treatment of renal osteodystrophy patients with predominant osteomalacia have been less satisfactory.[97] It has been suggested that $25OHD_3$ in large doses was more effective than $1,25(OH)_2D_3$ in correcting the mineralization defect in vitamin D deficient subjects.[98] However, this observation has not been confirmed.[99] Preliminary trials with $24,25(OH)_2D_3$ in combination with $1,25(OH)_2D_3$ in osteomalacic renal failure patients have yielded promising results,[97] but further studies will be needed to definitively assess clinical efficacy. Previously, 24,25(OH2D3 has been considered a metabolically inactive metablite, rather than an important physiological regulator of bone mineralization.[100] However, this remains a point of considerable controversy.[101] 1α OHD_3 has also been used in the treatment of renal osteodystrophy,[102] but it has to undergo 25-hydroxylation by the liver to be active and has no advantage over $1,25(OH)_2D_3$ (Figure 3).

Vitamin D metabolites have been used less extensively in a variety of other disorders.[103] Vitamin D-dependent rickets type 1 is a rare disorder which probably arises from a deficiency of the renal 1α-hydroxylase. Treatment of these patients with $1,25(OH)_2D_3$ in near physiologic doses has been demonstrated to correct the biochemical abnormalities present before therapy.[104] $1,25(OH)_2D_3$ treatment has also been suggested to be of benefit in combination with phosphate salts in patients with hypophosphatemic rickets.[105] The use of 1α-hydroxylated vitamin D metabolites in patients with hypoparathyroidism has allowed more rapid resolution of hypercalcemia in patients who are overtreated.[106,107] Patients with liver disease,[108] gastrointestinal disorders,[109] and osteoporosis[110] have received vitamin D metabolite therapy with varying degrees of benefit.

Future studies may lead to therapeutic roles for two novel vitamin D related compounds. A difluorinated form of $1,25(OH)_2D_3$, 24,25-difluoro-1α,25 dihydroxyvitamin D_3 has been reported to be four times more potent than $1,25(OH)_2D_3$ *in vitro*.[111] Ultraviolet exposure of 1α,25-dihydroxy-7-dehydrocholesterol applied to skin of humans or rats resulted in photoproduction of $1,25(OH)_2D_3$ in the skin and release into the circulation.[112]

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Chapter 27. Substance P and Neurotensin: Actions in the Gastrointestinal Tract

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Introduction - Substance P (SP) was first discovered in equine intestinal and brain extracts by von Euler and Gaddum over 50 years ago.[1] The partially purified substance markedly reduced blood pressure and contracted gastrointestinal (GI) smooth muscle. In 1970, SP was rediscovered by Chang and Leeman as a sialogogic (i.e. salivation-inducing) principle from bovine hypothalamic extracts.[2] These investigators later completely purified this activity and found it to be a peptide of 11 amino acids with the sequence indicated in Table 1.[3] SP bears a C-terminal sequence and spectrum of biological activities similar to those of other peptides collectively named "tachykinins" (Table 1). These peptides have largely been isolated from amphibian skin with the exception of eledoisin, which is present in octopus salivary glands.[4]

TABLE 1

STRUCTURES OF SP, NT AND NATURAL HOMOLOGS

SP	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Uperolein	pGlu-Pro-Asp-Pro-Asn-Ala-Phe-Tyr-Gly-Leu-Met-NH_2
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Phyllomedusin	pGlu-Asn-Pro-Asn-Arg-Phe-Ile-Gly-Leu-Met-NH_2
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2
NT	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH
Xenopsin	pGlu-Gly-Lys-Arg-Pro-Trp-Ile-Leu-OH

In the course of purifying SP, another substance was discovered which produced cutaneous vasodilation and cyanosis and was chemically distinguishable from SP. This preparation was named neurotensin (NT) and was subsequently determined to be a tridecapeptide.[5,6] A peptide structurally similar to NT, xenopsin, has been found in amphibian skin.[7]

Both SP and NT have a dual localization in the central nervous system and the periphery; in the latter they are present in particularly high concentrations within the GI tract. Both peptides are widely, albeit unevenly distributed throughout the central nervous system where they may subserve roles in neurotransmission. The activities of SP and NT in the central nervous system are reviewed in chapter 4 and will not be further considered here. Rather, we shall focus upon the anatomical and physiological characteristics of these peptides in the GI tract and structural requirements for their biological activity in this and other peripheral systems.

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Substance P

Distribution in the Gut - SP-like immunoreactivity (SPLI) has been detected in high concentrations in both endocrine cells and neurons of the GI tract. SPLI is present in large, pleomorphic granules within enterochromaffin cells (Type 1) where it may play a paracrine or hormonal role.[8,9] Indeed, a large portion of SPLI circulating in the plasma is contributed by the gut.[10]

In addition, SPLI is contained in neurons which are most numerous in the myenteric and submucosal plexuses, the longitudinal and circular muscle, and cells of the gut mucosa.[11-14] Most of these neurons appear to be derived from cell bodies within the gut wall.[15,16] However a few SPLI-containing nerve fibers which may originate from outside the GI tract appear to innervate submucous blood vessels and the submucous plexus.[17,18] SPLI-neurons have been classified as "p-type" (i.e. peptidergic) because they bear a morphological resemblance to peptide containing, neurosecretory neurons of the posterior pituitary;[19] within this classification, SPLI-neurons form a distinct subpopulation.[20] SP appears to be released from intestinal nerve terminals by electrical field stimulation[21] and can depolarize myenteric neurons.[22] These SP neurons may function in sensory processing, motor control, or as interneurons in the GI nervous system.[18]

Biological Activity - As indicated above, SP and other tachykinins have numerous effects in the GI tract and related systems. For example, SP dilates small blood vessels causing hypotension and increased blood flow, and may be involved in the modulation of vascular resistance in the intestine.[23] SP also stimulates salivary[24] and pancreatic[25] enzyme secretion, probably through an interaction with specific binding sites which serve to promote calcium mobilization in acinar cells.[26-28] On the other hand, the peptide diminishes pancreatic and gastric secretion elicited by other stimuli.[29] Recently, SP has been found to enhance fluid absorption in the upper small intestine,[30] and to stimulate intestinal electrolyte transport in the guinea-pig ileum by a direct, calcium-dependent action upon mucosal cells.[31]

One of the most frequently studied--and exploited--activities of SP has been its ability to powerfully contract smooth muscle, particularly of the ileum, but also of the colon, stomach, vas deferens and larger blood vessels. This contractile response is largely due to a direct effect of SP on smooth muscle, as local anesthetics or tetrodotoxin, which interrupt nervous transmission, do not abolish the SP response.[32-34] However, SP may secondarily release acetylcholine from enteric nerves which could contribute to the overall contractile effect.[14,35] Nevertheless, SP appears to act at distinct binding sites in most types of smooth muscle studied (ileal, colonic, tracheobronchial, vascular and vas deferens smooth muscle) as its effects are not inhibited by a wide variety of specific neurotrasmitter inhibitors.[32-38] SP binding sites have been identified in the brain,[39,40] pancreas[26] and parotid gland,[28] but not so far in the gut.

Desensitization (tachyphylaxis) to repeated or prolonged administration rapidly occurs to many of the effects of SP on smooth muscle and other systems. Because of the lack of a specific SP antagonist, the phenomena of autodesensitization has been utilized in elucidating a role for SP as a neurotransmitter.[21,41-43] However, the specificity of SP tachyphylaxis has been questioned.[44] There have recently been several claims of a specific SP antagonist, (D-Pro(2)-D-Phe(7)-D-Trp(9))-SP, but its inability to induce tachyphylaxis and complete specificity for SP

receptors have yet to be fully assessed.[45-48]

Structure-Activity Relationships

The SP Molecule - From a number of investigations, it has become clear that the C-terminal portion of the SP molecule is essential for its activity. Removal of the N-terminal amino acids to Gln(6) does not reduce the bioactivity of SP (Table 2). Indeed, the octa-, hepta-, and hexapeptide C-terminal fragments of SP actually exhibit increased potency in most test systems, but have somewhat reduced potency in inducing salivation.[24,51,64] A dramatic reduction in potency occurs in the C-terminal pentapeptide.[24,51-54] Furthermore, removing or substituting other amino acids for amino acids near the C-terminus results in partial or complete loss of activity.[54-57] This suggests that the C-terminal hexa- or pentapeptide possesses the minimum structural requirements for interaction with SP binding sites. Furthermore, removal of the C-terminal amide also reduces the biological activity of the SP molecule, but its replacement with a methyl ester restores activity, which suggests that hydrophobicity of the C-terminus may be an important determinant of SP bioactivity.[58]

Although the C-terminus of the SP molecule has been emphasized in structure-activity studies, the polar N-terminus may be of importance in stabilizing SP from rapid degradation.[59] Furthermore, the N-terminal tetrapeptide sequence may play a role in inflammatory processes as it exhibits chemotactic activity[60] and the ability to release histamine from mast cells at least at high concentrations.[61-63] The notion that both the N- and C-terminal portions of SP may possess biological activity is interesting in view of a proposed degradative scheme for SP involving cleavage of the N-terminal tetrapeptide by an endopeptidase succeeded by further cleavage of the C-terminal sequence by an aminopeptidase.[59]

TABLE 2

RELATIVE BIOLOGICAL ACTIVITIES OF SP AND ITS FRAGMENTS (SP=100)

Fragments[a]	Receptor Affinity[b] Rat	Rabbit	Ileum Contractility[c]	Blood Flow[d]	Sialogogic Activity[e]
SP	100	100	100	100	100
SP(2-11)	30	---	60	70	25
(3-11)	8	0.1	160	130	15
(4-11)	5	1	200	160	40
(5-11)	50	5700	125	100	30
(6-11)	170	250	110	65	40
(7-11)	1	1	2	4	3
SP-COOH	3	---	---	---	0.03

[a] From N-terminus.

[b] Transformed from IC_{50} values in displacing ^{3}H-SP from rat[40] and rabbit[39] brain membranes. IC_{50} for SP=3 nM in both preparations.

[c] Transformed from ED_{50} values in contracting isolated guinea-pig ileum[49]; ED_{50} for SP=2.5 nM.

[d] Dose-ratio of each fragment relative to SP in increasing femoral artery blood flow in dogs.[49] ED_{50} for SP=140 fM.

[e] Transformed from ED_{50} values in inducing salivation in the rat.[50] ED_{50} for SP=18 nM.

Natural Analogs of SP - SP shares a common C-terminus, Phe-X-Gly-Leu-Met-NH_2, with other naturally-occurring tachykinins (Table 1). A number of studies have compared the biological activities of SP with those of other tachykinins in a variety of test systems. The rank orders of potency of the peptides appear to vary with the biological system under examination. SP exhibits potent hypotensive effects in the dog and rabbit with an order of potency: SP > physalaemin > uperolein = phyllomedusin > eledoisin > kassinin.[65,71] However, physalaemin has greater affinity for ^{125}I-physalaemin- and ^{3}H-SP-labelled binding sites in the guinea-pig pancreas[25] and rat brain,[40] respectively; the order of affinities is physalaemin > SP > eledoisin. Similarly, physalaemin > SP ≃ eledoisin >> kassinin in stimulating salivation.[24,66] In the stomach, eledoisin has a potent spasmogenic action, with eledoisin > phyllomedusin > physalaemin > uperolein > SP.[67,68] A similar rank order of potency exists in contracting smooth musculature of the intestine and the urinary bladder with eledoisin and kassinin in some cases being exceedingly potent relative to physalaemin and SP.[65,66,69] Interestingly, Kachur et al.[31] have found that kassinin > physalaemin > eledoisin ≥ SP in stimulating ion transport across the guinea-pig ileal mucosa.

These marked differences in the potency of tachykinins among the various test systems strongly suggests a heterogeneity of receptors for this family of peptides. It has recently been proposed that at least two forms of the "SP receptor" exist: one subtype with a high affinity for physalaemin and SP and a second with greater selectivity for eledoisin and kassinin.[70] Indeed, among the various tachykinins, eledoisin and kassinin are most similar in their spectrum of activities and in their slower, but more sustained pharmacological effects.[66] Since the C-terminus of the tachykinins is highly conserved, it is probable that differences in the N-terminal amino acid sequence determine the potency and intrinsic activity of these peptides in various biological systems. This possibility should be considered in the design of more selective SP analogs.

Neurotensin

Distribution in the Gut - Most of the body's content of NT is present in the GI tract.[71] A few enteric nerve fibers may contain NT-like immunoreactivity (NTLI) and these are limited to the myenteric plexuses of the esophagus, stomach and duodenum, the circular muscle of the stomach and caecum, and the longitudinal muscle and lamina propria of the stomach.[12] The major portion of gut NT exists in endocrine cells of the GI mucosa.[72,73] These NT containing cells represent a unique cell type, the N cell.[74,75] The N cells are generally elongated and triangular in appearance with a tufted apex extending into the intestinal brush border and a basal portion containing electron-opaque secretory granules in close proximity to the serosal aspect of the intestine.[74-76] In mammals, they are distributed in increasing frequency from the distal jejunum to the distal ileum and are virtually absent in the stomach, colon and pancreas.[73,77-80] The N cells are predominantly localized to the villi although they can occur in small numbers in the crypts.[77,80]

NTLI can be detected circulating in the plasma at levels comparable to those of other hormones. Most of the NTLI represents authentic NT although other, yet unidentified, substances bearing C-terminal homologies to NT also appear to be present.[81] Increases in plasma NTLI have been observed following the ingestion of food, particularly fat.[82-84] This suggests that NT may function in the digestive processes of the intestine.

Plasma NTLI is also elevated in certain clinical syndromes, such as coeliac disease,[85] which is manifested by intestinal malabsorption of nutrients, and the dumping syndrome, a condition which often occurs after gastrectomy and is characterized by the rapid emptying of liquids from the stomach.[86,87] Some NTLI-containing pancreatic tumors may also increase the amount of NTLI in the plasma.[88,89] Although most NTLI appears to be released from the small intestine into the blood, at least one study has demonstrated that NTLI may be released into the lumen of the intestine as well; the teleological reasons for this luminal release of the peptide are undetermined at present.[90]

Biological Activity - Like SP, NT has potent effects on GI and other smooth muscles. The peptide contracts the rat stomach fundus[91] and guinea-pig ileum,[93-95] but relaxes the human lower esophageal sphincter,[95] and rat and human duodenum.[91,92,96] The contractile effect of NT on guinea-pig ileum appears to be neuronally-mediated as it is attenuated by atropine and abolished by tetrodotoxin.[94,97] Opioid peptides, somatostatin, and prior SP autodesensitization also attenuate NT-induced contractions of the ileum, suggesting that this effect may involve both cholinergic and non-cholinergic neural components.[41,98,99] On the other hand, the relaxation of GI smooth muscle by NT appears to be a direct effect of the peptide on muscle cells.[94,100] NT inhibits peristalsis probably through the relaxation of smooth muscle and disruption of organized myoelectrical activity in the small intestine.[96,101]

NT also relaxes venous smooth muscle, producing hypotension and increased blood flow. Indeed, the peptide dilates intestinal blood vessels and increases blood flow in the muscular layers of the ileum at physiologically relevant concentrations.[102,103] Furthermore, NT increases vascular permeability.[104] Both the hypotensive and vascular permeability effects of NT may involve its release of histamine, possibly from mast cells, as the effects are attenuated by histamine antagonists or prior mast cell degranulation.[104-106,132] Indeed, NT appears to bind to specific sites on mast cells.[107]

NT also produces a number of other peripheral effects, other than those on smooth muscle. For example, the peptide inhibits pentagastrin-stimulated gastric acid secretion in dogs with Pavlov pouches.[108] In the pancreas, low doses of NT elevate bicarbonate output and release pancreatic polypeptide and insulin, whereas higher doses reduce water and protein secretion.[109-112] NT also elevates plasma cholesterol levels, possibly by increasing either cholesterol transport through the intestinal wall or hepatic secretion of lipoproteins.[113,114] Furthermore, NT stimulates active electrolyte secretion in the guinea-pig small intestine. This effect occurs through a neuronal mechanism, apparently involving the release of SP from enteric neurons.[31] Finally, NT has numerous endocrine effects, the most striking being its hyperglycemic action.[115]

Many of the effects of NT, like SP, undergo rapid tachyphylaxis. This is particularly true with regard to NT action on smooth muscle and intestinal ion transport. The peptide appears to produce its effects, in many cases, through an interaction with specific binding sites on nervous or muscular tissue. Such NT "receptors" have been identified on rat brain membranes,[116-119] intestinal longitudinal muscle,[120] a human colon carcinoma cell line,[119] and mast cells.[107] NT binds to these sites with high affinity (K_D = 1 to 8 nM), although the peptide binds to mast cells with somewhat lower affinity ($K_D \simeq 150$ nM).

Structure-Activity Relationships

NT Fragments - As with SP, the biological activity of NT resides largely in the C-terminal portion of the molecule. NT bioactivity in many test systems is at least partially preserved after removal of the first seven N-terminal amino acids, leaving the hexapeptide NT (8-13) (Table 3). The C-terminal pentapeptide NT (9-13), although much reduced in potency, is the smallest NT fragment retaining full intrinsic activity.[124,125] Elimination of amino acids from the C-terminus almost completely abolishes NT bioactivity. In this regard xenopsin, which possesses a C-terminal pentapeptide sequence identical to that of NT (Table 1), is only slightly less potent than NT in the few systems in which it has been tested.[7,31,126]

Thus, the C-terminal pentapeptide sequence of NT contributes to most of its biological activity. Moreover, this activity is dependent on the presence of the free carboxyl group of Leu(13); substitution of this group with an amide moiety considerably attenuates NT bioactivity.[126-128]

TABLE 3

RELATIVE BIOLOGICAL ACTIVITIES OF NT AND ITS FRAGMENTS (NT=100)

Fragment[a]	Receptor Affinity[b] Brain	H-29 cell	Atrium[c]	Heart[d]	Portal Vein[e]	Stomach Fundus[f]	Ileum[g]
NT	100	100	100	100	100	100	100
NT(2-13)	---	---	94	87	---	90	73
(4-13)	---	---	99	92	---	85	59
(6-13)	---	---	73	84	---	78	21
(8-13)	15	120	52	102	94	69	21
(9-13)	1	1	1	2	2	11	---
(10-13)	0	0	0	0	0	0	---
(1-12)	0	0	0	0	0	0	---

[a] From N-terminus.

[b] Transformed from IC_{50} values in displacing ^{3}H-NT from rat brain membranes and a human colon carcinoma cell line (H-29).[119] IC_{50} for NT = 1.5 - 2.0 nM in both preparations.

[c] Transformed from ED_{50} values for positive inotropic activity in isolated, spontaneously-beating guinea-pig atria.[121] ED_{50} for NT = 9 nM.

[d] Transformed from ED_{45} values for increasing coronary perfusion pressure in the isolated, perfused rat heart.[122] ED_{45} for NT = 21 nM.

[e] Transformed from ED_{50} values in contracting isolated rat portal vein.[123] ED_{50} for NT = 16 nM.

[f] Transformed from ED50 values for contracting rat fundic strips.[121] ED_{50} for NT = 11 nM.

[g] Transformed from a 4 point assay line for NT in contracting the isolated guinea-pig ileum.[93]

NT Analogs - Numerous studies have been conducted to ascertain which amino acid determinants in the NT molecule are responsible for its affinity for specific NT binding sites and its intrinsic biological activity. In general, the N-terminal eight amino acids and Ile(12) and Leu(13) appear to modulate the affinity of NT for binding sites. The intrinsic activity

of the peptide, on the other hand, is dependent upon the tripeptide sequence Arg(9)-Pro(10)-Tyr(11).[129] For example, substitutions of D-amino acids for N-terminal residues have little effect on or may even increase the biological potency of the molecule.[31,128] Also, Gln(4)-NT, which may actually represent the naturally-occurring peptide, has biological activity similar to NT.[91,92,102,127] In contrast, substitutions in the C-terminal residues markedly decrease biological potency and intrinsic activity. Thus, in a number of test systems, the activity of D-Arg(8)-NT is similar to NT, whereas that of D-Arg(9)-NT is much diminished.[119,120,125,128] Interestingly D-Trp(11)-NT, which has been proposed as a competitive NT antagonist in the perfused rat heart and isolated portal vein has little activity in these systems.[130,131] However, it does possess full intrinsic activity, but low potency, in other preparations; this discrepancy does not support its characterization as a true antagonist.[129] It is conceivable that other substitutions in this region of the NT molecule will yield analogs possessing high affinity for NT binding sites but no detectable intrinsic activity, i.e., pure antagonists.

Summary

SP and NT, peptides with a dual localization in the mammalian brain and gut, have many potent actions upon GI and other peripheral organ systems. Recent investigations have disclosed determinants within their structures responsible for biological activity; the C-terminal amino acids of both peptides are important in this respect. The design and synthesis of novel peptide analogs may yield biologically-stable compounds of high potency for the treatment of disorders of intestinal motility or ion transport, cardiovascular disease or endocrine abnormalities. Conversely, selective peptide antagonists would be useful in the clinical management of disorders associated with the pathological expression of peptide activity and as pharmacological tools in the elucidation of SP and NT receptors and their roles in physiological processes.

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Section VI - Topics in Chemistry and Drug Design

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Chapter 28. Quantitative Structure Activity Relationships Applied to Drug Design

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Introduction - This review covers research published during 1978 to 1981 on the application of quantitative structure-activity relationship (QSAR) studies to drug design, extending the review published three years ago.[1] The topic of computerized pharmacophore mapping, also previously reviewed in this series, will not be discussed here.[2] QSAR research has continued to increase as evidenced by the publication of monographs[3,4] and other review chapters.[5] The consequences of the Hansch approach have been reviewed,[6] as have multivariate statistical[7] and topological approaches.[8,9] The proceedings of meetings devoted to QSAR[10-14] and a review of interpretation of QSAR relationships[15] have been published. QSAR studies of drug metabolism and distribution have been reviewed.[16]

Methods - The early work which used the bilinear model for nonlinear structure activity relationships has been extended[17] and reviewed.[18] A new double parabolic model has also been presented.[19] Methods have been described in which the three dimensional conformation of the molecule is used to generate descriptors. These techniques include conformational energy minimization[20] and exhaustive computerized search of conformational space with computation of ligand and receptor geometry.[21,22] Computation of the steric difference between the ligand and a hypothetical receptor generated from the most active compound in a series has also been discussed.[23] The significance of the shape descriptors resulting from these techniques are usually analyzed by regression analysis techniques.

Further work has been done on mathematical methods applicable to series design, including criteria for measurement of the suitability of a proposed series[24] and two-dimensional mapping of descriptors.[25] Suggestions on mathematical methods for normalization of variables to give more interpretable results have been made.[26,27] The usefulness of the "jackknife" confidence interval estimator as applicable to QSAR has been discussed.[28]

The pattern recognition technique of adaptive least squares has been applied to the discrimination of categorical biological data.[29] The SIMCA (Simple Modeling of Chemical Analogy) pattern recognition technique has been reviewed.[30] A simple method for solving the Free-Wilson model has also been presented.[31] Some of the statistical hazards associated with multiple regression analysis were discussed. A

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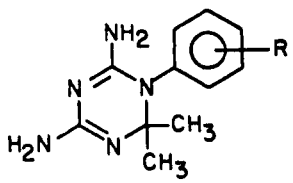
particular distinction must be made between the variables investigated for possible correlation and those included in a particular equation.[32]

Parameters - Partition coefficient, expressed either as log P or as π is still the most important parameter considered in developing QSAR relationships. Hansch has published a book describing the method for calculation of log P from substructures of the molecule.[33] Rekker has expanded on his system of fragment constants.[34,35] The Pomona College Medicinal Chemistry Project has continued to add significant numbers of new experimentally determined log P values to their database.[36]

Since it is rather tedious to measure log P by the solvent partition method, recent workers have concentrated upon the correlation between partition coefficients and other more accessible physical parameters.[37] Chromatography as a tool for determining parameters useful in QSAR has been reviewed.[38] Chromatographic constants such as R_m derived from TLC reverse phase systems for ionic molecules such as amines[39] and aryl-alkyl acids[40,41] have been correlated with partition coefficient by a number of investigators. Reverse phase HPLC has been correlated with log P using phenols,[42] and with π using nonionic pesticides.[43] Gas chromatography behavior has been correlated with partition coefficients for volatile compounds using oleyl alcohol as the stationary phase.[44] A series of correlation equations has related log P in octanol and various buffers with log P determined in octanol and water.[45] An excellent correlation was obtained between log P in an octanol-N,N-dimethyloctylamine containing buffer system and HPLC R_m for a series of lipophilic phenothiazines.[46] Log P has also been correlated with aqueous solubility,[47] connectivity,[48] passive intestinal absorption rates,[49] and kinetic transport rates.[50] The connectivity parameter χ has been correlated with GLC retention times.[51,52]



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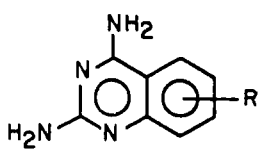

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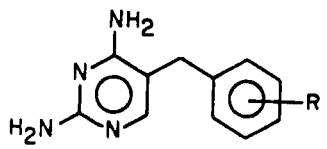

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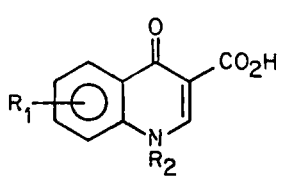
Applications of QSAR Techniques - QSAR techniques have been widely used to study inhibitors of dihydrofolate reductase (DHFR). Molecular properties, obtained from CNDO/2 molecular orbital calculations have been used to correlate the inhibition of DHFR by substituted quinazolines.[53] Regression analysis was applied to a series of quinazolines as inhibitors of DHFR from human and mouse leukemia cells.[54] Hopfinger has correlated the DHFR inhibition activity of a series of 2,4-diaminotriazines (1) with three descriptors of molecular shape and π. The shape descriptors were determined by use of the CAMSEQ-II software system.[55] Molecular shape analysis studies have also correlated the DHFR inhibition activity of quinazolines (2) and 5-benzyl-2,4-diaminopyrimidines (3).[56,57] Recent efforts by Hansch and coworkers have begun to approach the difficult problem of rational design of mammalian vs. bacterial species specific enzyme inhibitors. Series of compounds were prepared which have less correlation between molecular descriptors and span a wider range of substituents. Physical chemical parameters responsible for the differences in inhibition between the DHFR enzymes for bovine liver and E. coli. were described[58,59] Using substituted 5-benzyl-2,4-diamino-

pyrimidines (3), the equations clearly show the differences in space of the active site of the enzyme, since good correlations were obtained with different physical chemical parameters.[58,59] Phenyl 4,6-diamino-s-triazine (1) inhibitors of mammalian DHFR enzymes give bilinear correlations with π of the substitution in the 4-position of the phenyl group.[60]

Studies on chymotrypsin have continued with investigation focused on the binding of L-alanine analogs and their effect on the ρ3 area of the chymotrypsin active site.[61] Work has been done on the binding of alkyl phosphonates to chymotrypsin.[62] Other proteases have been studied with correlations investigating bovine tryptic proteases[63] and human serine proteases.[64] These studies point up differences in the binding site regions and illuminate the difficulty of designing specific inhibitors for related enzymes.

The antifibrinolytic activity of a series of arylacetic acids was correlated with lipophilicity. Estimation of lipophilicity by reversed phase thin layer chromatographic correlated poorly with antifibrinolytic activity, but tabulated π values correlated well.[65] Lipophilicity of a series of quinoline-3-carboxylic acids (4), as expressed by the HPLC retention index, was the superior parameter correlating with inhibition mitochondrial but not cytoplasmic dehydrogenase enzymes.[66] For example inhibition studies of cholinesterase by a large series of carbamates suggest that the most important parameter for binding is molar refraction.[67] Studies of rifamycins as inhibitors of various mammalian and viral DNA polymerases indicate that the partition coefficient correlates best with inhibitory potency.[68]

Studies using pattern recognition techniques in the antibacterial area have been designed to make comparisons of biological test systems on a quantitative basis. Coates has studied pyrimidines which are reversible or irreversible folic acid antagonists.[69] The antibacterial properties of a series of antibiotics have been classified by cluster analysis.[70] Darvas used a principal component methodology to investigate



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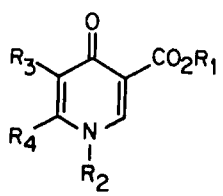

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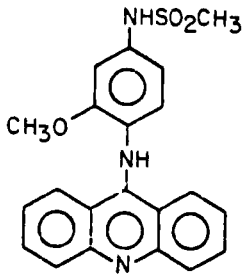
the mechanism of action of a series of antibacterial γ-pyridone-β-carboxylic acids (5). Two principal components account for more than 80% of the bacteriostatic activity.[71] Parabolic relationships have been developed for partition coefficient and antibacterial activity of substituted rifamycin analogs[72] and biguanides.[73]

Applications of QSAR techniques in the area of cancer chemotherapy have been reviewed.[74] DNA-dependent inhibition of DNA polymerase, DNA binding, mammalian toxicity and tumor selectivity of a series of bisguanylhydrazones was described.[75] The mammalian toxicity of these compounds was effectively correlated with chromatographically determined R_m values. In a series of studies concerned with DNA binding, mutagenic-

ity, toxicity, and antitumor efficacy of a series of 9-anilinoacridines related to the clinical antitumor agent 4'-(9-acridinylamino)methanesulfonanilide (m-AMSA) (6), chromatographically determined R_m values were used to model partition coefficients. In a large series of m-AMSA analogs, the D_{40}, (molar dose required to give 40% extension of life in treated animals over untreated controls) correlated with pK_a and R_m.[76,77] Mutagenicity correlated with partition coefficient as the dominant factor. Correlation equations suggested that mutagenicity and antitumor activity can be separated by appropriate choice of substituents and adjustment of the partition coefficient.[78] Studies of a series of anthracyclines suggest that most structural modifications which lead to an increased partition coefficient, increase both antitumor activity and cardiac toxicity.[79] Studies of of a series of nitroaromatic compounds show that one electron reduction potential and lipophilicity correlate with the radiosensitizing activity.[80] Partition coefficient proved to be the most important parameter for correlating the antitumor activity of a series of substituted phenyl-3,3-dimethyltriazines.[81] In another study, equations correlating toxicity were similar to those correlating antitumor activity,[82] suggesting that the therapeutic ratio cannot be improved in this series. In a similar study of a series of nitrosoureas, comparison of the correlation equations of the partition coefficient and an indicator variable suggests that toxicity and antitumor activity can be separated by proper adjustment of lipophilicity.[83] The antileukemic activity of a series of colchicine analogs against P388 cells was correlated with partition coefficient.[84] The activity of a series of nitrosamine carcinogens has been correlated with water-hexane partition coefficient and electronic factors represented by σ.[85] The mutagenicity of a similar set of compounds tested in the Ames bacterial mutagenicity assay has been correlated with molecular connectivity.[86] The mutagenicity of a large series of polycyclic heterocyles evaluated in the Ames test correlated with partition coefficient and the minimal topological difference parameter defined by Simon.[87,88] Finally, the mutagenicity of a series of substituted o-phenylenediamineplatinum dichlorides has been correlated with σ^-.[89]



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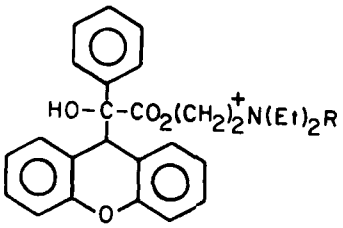
Pattern recognition techniques using descriptors based upon substructure, connectivity, and geometry have been used to correlate a heterogeneous set of carcinogens,[90] and a large series of carcinogenic aromatic amines.[91] Parameters investigated in these two studies include substructure, molecular connectivity, and geometric descriptors, such as the principal moments of inertia and the molecular volume. The carcinogenic activity of a set of polycyclic aromatic hydrocarbons was classified using the SIMCA pattern recognition technique. Twenty three parameters, including theoretical parameters derived from quantum mechanical computations and measured parameters, such as ionization potential, were used.[92]

The partition coefficient between buffer and erythrocytes for a series of antimalarial bis-arylsulfonamides was correlated with lipophilicity, as described by R_m, and the pK_a.[93] A series of 646 arylcarbinols with a 1000-fold range of activity against P. berghei in mice was corre-

lated. The equation covering all compounds contained a series of indicator variables to account for the different relative contributions of the aromatic ring systems, and the partition coefficient and σ of the ring substituents as significant parameters.[94]

QSAR prediction of high muscarinic receptor antagonist activity for a series of quaternary diethylaminoethyl-xanthenylmandelates (7) was not confirmed.[95] The muscarinic receptor binding activity of a series of aryl substituted alkyltrimethylammonium quaternary agents (8) has been



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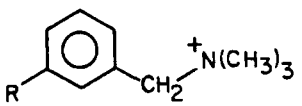

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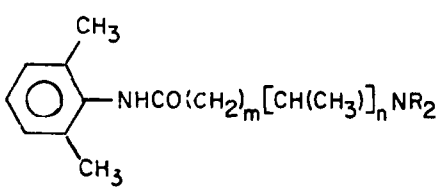
correlated with π for the aromatic side chain and a bulk parameter for side chain substituents. An indicator variable accounts for differences in the fit of the aromatic ring on the receptor site.[96] A study of a series of 100 muscarinic antagonists and agonists using molecular connectivity parameters showed that the quaternary ammonium group contributes equally to agonist and antagonist activity, while the structure of the side chain strongly influences antagonist activity.[97]

Correlations between molar volume and inhibition potency against mouse-brain synaptosomal lysophosphatidylcholine acyltransferase has been observed in a series of psychoactive cannabinoids. Use of a molar volume parameter allowed separation of nonspecific lipophilicity effects from intrinsic binding affinity to the membrane bound enzyme.[98] Chromatographic R_m values from a reverse phase system correlated well with measured or experimental log P values for a series of benzodiazepines.[99] The CNS activity of these compounds as measured by exploratory and conflict behavioral tests, correlated with R_m values and structural indicator variables. Different slopes for the R_m term in the correlation equation suggest a different dependence upon partition coefficient, and a different mechanism of action for exploratory and conflict behavior.[99]



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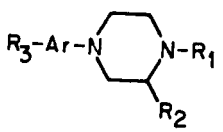
In a study of a series of aminoxylidines (9), anti-arrhythmic activity and acute CNS toxicity, characterized by ataxia, were correlated with measured partition coefficient and pK_a. Correlation equations suggest that increases in the pK_a of this class of compounds would increase the therapeutic index. Anti-arrhythmic activity correlated with partition coefficient alone.[100] Pattern recognition techniques identified connec-

tivity fragments and substructure descriptors as the significant structural parameters which correlate with duration of action of a large series of barbiturates.[101] The anticonvulsant and CNS-depressant activity, and toxicity of a series of antiepileptic drugs, as measured, respectively, by the maximal electroshock and pentylenetetrazol seizure tests, and median toxic dose, was correlated with log P and dipole moment values.[102] The duration of action of a series of phenylsuccinimide anticonvulsants, active against maximal electroshock seizures, was correlated with the hydrophilicity of the substituent on the nitrogen.[103] Similarly, the anticonvulsant activity of a series of 4-arylpiperazines



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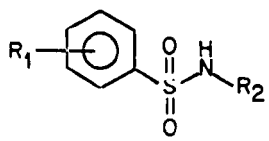

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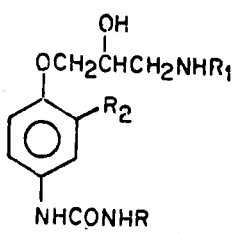
(10) correlated with computed log P. Increased lipophilicity directly correlated with increased potency.[104] Protection against maximal electroshock seizures by a series of substituted aromatic sulfonamides (11) was correlated with Swain and Lupton's F constant and π. Steric parameters for substituents on the ring, and substituents on the nitrogen improved the correlations.[105] The cardiotoxicity of a series of steroidal aglycones was mapped by use of the minimal steric difference correlation method.[106] The optimized superposition of the molecule provides a map of the site of the cardiotoxic receptor, which has a high degree of predictability.[106] Pattern recognition techniques were used to classify a series of steroids into five therapeutic categories. A template for each class and first-order molecular connectivity parameters



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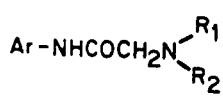

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were useful for classification.[107] Free-Wilson analysis of a large series of ureido phenoxy-3-amino-2-propanol (12) β-adrenergic blocking agents suggested that regression analysis would require inclusion of indicator variables for specific substitution patterns.[108] A significant relationship was developed between sigma and π, and the indicator variables.[108]

The local anesthetic activity of a series of N,N-disubstituted aminoacetylarylamines (13) correlated best with molar refraction giving a parabolic relationship significantly superior to equations with partition coefficient. Intravenous toxicity based on LD_{50} data correlated with partition coefficient in a parabolic relationship.[109] A multiple regression model using substructural parameters based upon the Edgewood Arsenal fragment code should be useful in ranking of potentially toxic

compounds.[110]

The pharmacokinetic parameters, elimination rate constant, clearance, and protein binding of a series of 2-sulfapyridines have been correlated with physicochemical parameters such as chromatographically derived partition coefficients, pK_a, and steric parameters. Protein binding increases with lipophilicity and pK_a. The steric effect of substituents on the binding of the compounds to bacterial enzymes is opposite that of binding to serum proteins.[111] Pharmacokinetic data from studies of the metabolism of N-substituted amphetamines in humans has been correlated with lipophilicity and structural parameters describing the nitrogen substitution patterns. Partition coefficients measured in n-heptane-pH 7.4 buffer gave a better correlation with urinary excretion than calculated octanol-water partition coefficients.[112] The binding of a series of barbiturates to cytochrome P-450 and their hepatic clearance have been correlated in a parabolic relationship with calculated log P and the volume of the 5-substituent. The regression equations for P-450 binding were similar to those for hepatic clearance.[113] The activity of a series of pyridinecarbonyldithiocarbazates, which uncouple oxidative phosphorylation, was correlated with lipophilicity and indicator variables.[114] The sweetness of a series of aspartyl dipeptide methyl esters was correlated with π.[115]

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Chapter 29. Structure Elucidation and the Total Synthesis of the Leukotrienes

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Introduction — The subject of this review is a group of "non-cyclized C_{20} carboxylic acids with one or two oxygen substituents and three conjugated double bonds" which were initially discovered in leukocytes and are now generally referred to as the leukotrienes.[1] The family of compounds consists of the arachidonic acid metabolites Leukotriene A_4 (LTA_4), **4**; and Leukotriene B_4 (LTB_4), **5**; the naturally occurring slow reacting substances of anaphylaxis Leukotriene C_4 (LTC_4), **6**; Leukotriene D_4 (LTD_4), **7**; and Leukotriene E_4 (LTE_4), **8**, as well as other eicosanoid metabolites.[2] The term "slow reacting substance" (SRS) was introduced by Feldberg and Kellaway[3] in 1938 for a substance isolated from a perfusion of guinea pig lung with cobra venom as well as more conventional allergens.[4] This acronym was chosen since the isolated substance caused a powerful slow contracting effect on the guinea pig ileum. Brocklehurst[5,6] later used the term "slow reacting substance of anaphylaxis" (SRS-A) to describe material produced by lungs upon immunological challenge by antigens since it was unknown whether this substance and the previously described SRS were identical. Today, the acronyms SRS and SRS-A can be used interchangeably since the chemical substance in question has been shown to be the same whether produced by non-immunological challenge, immunological challenge or chemical synthesis. Reviews on the discovery and structure elucidation as well as the biological properties of the leukotrienes have appeared.[7-20] The arachidonic acid lipoxygenase cascade was reviewed in chapter 20 of volume 16 of Annual Reports[21a] and this subject has been further updated this year.[21b] This review will focus on the *chemistry* leading to the complete stereochemical structural elucidation of the leukotrienes, as well as more recent synthetic advances.

In a series of papers (1977-1979), Samuelsson *et al.*[22-26] reported the isolation and characterization of a group of dihydroxy eicosatetraenoic acids from polymorphonuclear leukocytes, one of which was enzymatically produced and termed LTB_4. These diols were generated from arachidonic acid presumably via an intermediate (LTA_4) whose structure was unknown at that time. From alcohol trapping and oxygen incorporation experiments and in collaboration with Professor Corey, a structure was suggested for LTA_4 as a 5,6-oxido-7,9,11,14-eicosatetraenoic acid.[27] In an effort to determine the double-bond geometries and the configuration at positions C(5) and C(6), Corey *et al.* synthesized (±)-5,6-oxido-7,9-trans,11,14-*cis*-eicosatetraenoic acid as a mixture of *cis/trans* epoxides.[28] The synthetic and natural material were shown to be converted to the same mixture of products upon solvolysis with water and methanol. The complete structure determination of the elusive LTA_4 came shortly thereafter when Corey *et al.*[29] completed a stereospecific synthesis of 5(S)-*trans*-5,6-oxido-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid. Since the synthetic material was converted in neutrophils enzymatically to LTB_4, the double-bond geometry and configurations at C(5) and C(6) were presumed to be identical to the natural product.[30]

The final structural elucidation of the naturally occurring SRS-As (LTC_4, LTD_4 and LTE_4) was intimately linked to the discovery and characterization of the above leukotrienes. Early structural work demonstrated that SRS was a polar lipid[31,32] with a strong ultraviolet

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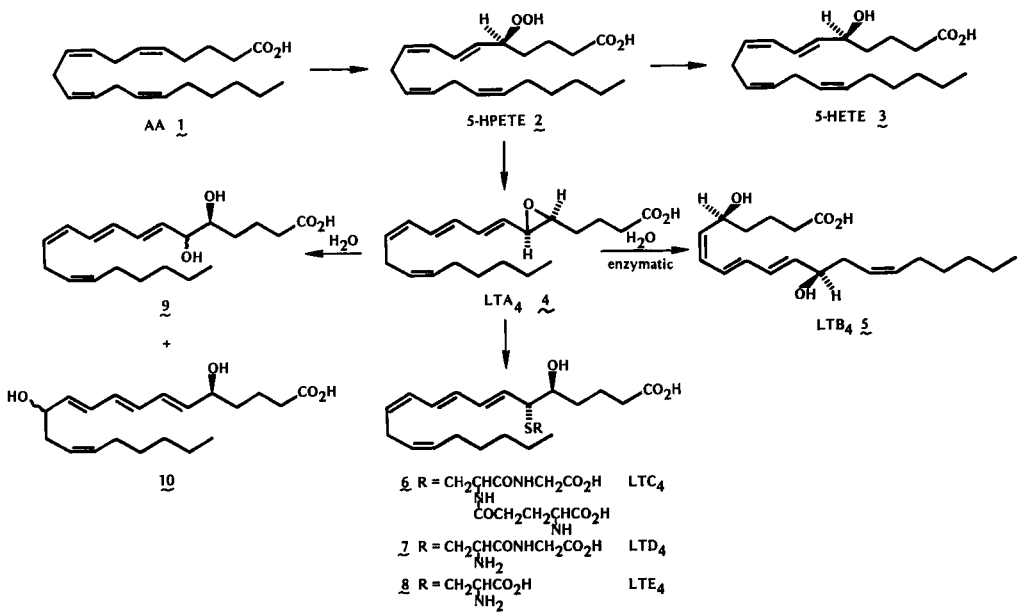
absorbance and possibly containing sulfur.[33] Cysteine[34] and various thiols[35,36] were subsequently shown to stimulate SRS production, and Parker[37] and others[38] demonstrated that radiolabeled arachidonic acid could be incorporated into SRS. Morris *et al.*[39,40] pioneered the use of HPLC purification of SRS and showed that pure material had an ultraviolet absorbance characteristic of the leukotrienes.[41] Sirois[42-44]proposed that SRS contained a

SCHEME 1

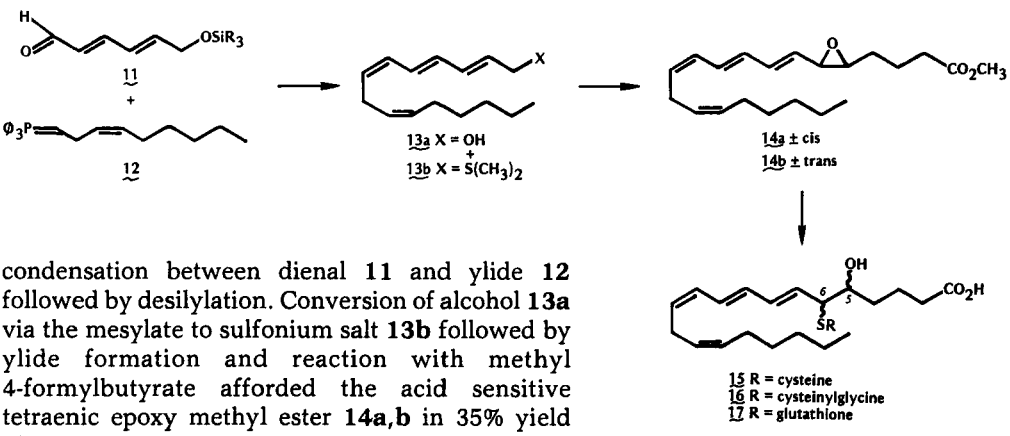


cis,cis-1,4 pentadiene unit since rat SRS-A was inactivated by a lipoxygenase. A valuable insight into the structure of SRS came in 1979 when Samuelsson demonstrated that both tritiated arachidonic acid and ^{14}C-labeled cysteine were incorporated into an SRS isolated from mouse mastocytoma cells (LTC_4). Further elegant degradative work led him to propose that LTC_4 was a 5-hydroxy-7,9,11,14-eicosatetraenoic acid which was substituted at C(6) by cysteine or some cysteine containing peptide.[27] The unknown peptide in LTC_4 was later identified as glutathione (γ-glutamylcysteinylglycine) by the synthesis of the glutathione conjugate from the racemic mixture of *cis/trans* epoxides.[46]The complete stereochemical structure of LTC_4 was extablished by conversion of (-)-methyl *trans*-5(S),6(S)-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoate (LTA_4 methyl ester) to the 5(S)-hydroxy, 6(R)-glutathionyl adduct which was shown to be identical to native LTC_4 by comparison of HPLC retention time, ultraviolet λmax= 280 nm, biological activity on guinea pig ileum, and inactivation by soybean lipoxygenase.[29,47] The structure of LTD_4 followed shortly thereafter when three groups reported cysteinylglycine to be the peptide on an SRS isolated from rat basophil leukemia cells[48,49] and rat peritoneal cells.[50] LTD_4 was also shown to be derived from LTC_4 by the action of γ-glutamyl transpeptidase.[49] The SRS-A released upon immunological challenge of sensitized guinea pig lung was shown to be identical to the SRS from non-immunological challenge with the aid of electron-impact mass spectrometry.[45] Synthetic LTD_4 of complete structural certainty was demonstrated to be identical to SRS-A isolated from rat peritoneal cavity and human lung.[51,52] Finally, LTE_4 was identified as a naturally occurring SRS-A by comparison of the synthetic cysteinyl adduct to a third SRS-A component from rat peritoneum.[53]

Early Synthetic Routes to LTA_4, LTC_4, LTD_4, LTE_4 — The assignment of stereochemistry and structure to a trace, biologically active mammalian metabolite by combination of synthesis, enzymic studies and spectroscopy is rare in modern synthetic chemistry. The early

synthetic efforts in the SRS structure determination were by necessity very flexible since initially three questions were unresolved: (1) What is the nature of the peptide moiety? (2) What are the absolute configurations at C(5) and C(6)? (3) What is the triene stereochemistry? The simplest consideration of investigating three different R groups, the four possible configurations at C(5) and C(6), and two triene geometries gave twenty-four target structures. Accordingly, the initial synthesis of the structurally undefined LTA_4[28] generated a racemic mixture of *cis/trans* epoxides with the most likely triene geometry of 7,9-E,11-Z (Scheme 2). The key intermediate tetraenic alcohol **13a** was prepared by Wittig

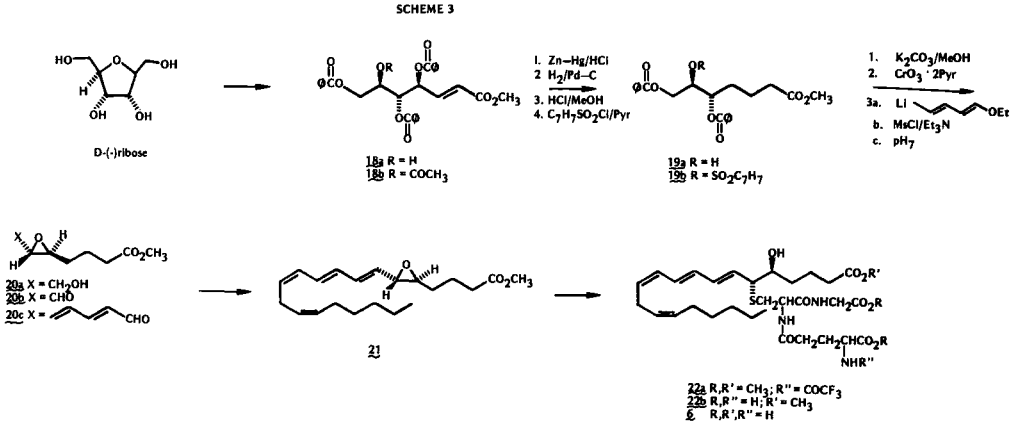
SCHEME 2



condensation between dienal **11** and ylide **12** followed by desilylation. Conversion of alcohol **13a** via the mesylate to sulfonium salt **13b** followed by ylide formation and reaction with methyl 4-formylbutyrate afforded the acid sensitive tetraenic epoxy methyl ester **14a,b** in 35% yield after purification on triethylamine deactivated silica gel as a 1/1 *cis/trans* mixture. A similar reaction sequence was later reported by a group from Merck.[54] Reaction of the epoxide mixture **14a,b** with various protected thiols in triethylamine/methanol followed by base hydrolysis gave all four diastereomers of the amino acid or peptide conjugates at C(6). Comparison of these adducts by HPLC to the SRS isolated from mouse mastocytoma cells led to the identification of glutathione as the peptide moiety in LTC_4.[46]

The configurations at C(5) and C(6) in LTC_4 as 5(S),6(R) were established after LTA_4 was shown to be the *trans*-5(S),6(S)epoxide. The starting material for this historic synthesis,[29] D-(-)ribose, was converted to the key intermediate (5S,6R)-epoxy aldehyde **20b** in 75% yield after 12 steps (Scheme 3). Four carbon chain extensions followed by Wittig condensation produced LTA_4 methyl ester, **21**, (35%). Reaction of **21** with dimethyl N-trifluoroacetylglutathione or glutathione followed by base hydrolysis afforded a single diastereomer (5S,6R) which was identical to LTC_4. Correspondingly, LTD_4[51,52] and LTE_4[53] were later synthesized in a similar manner from methyl ester **21**.

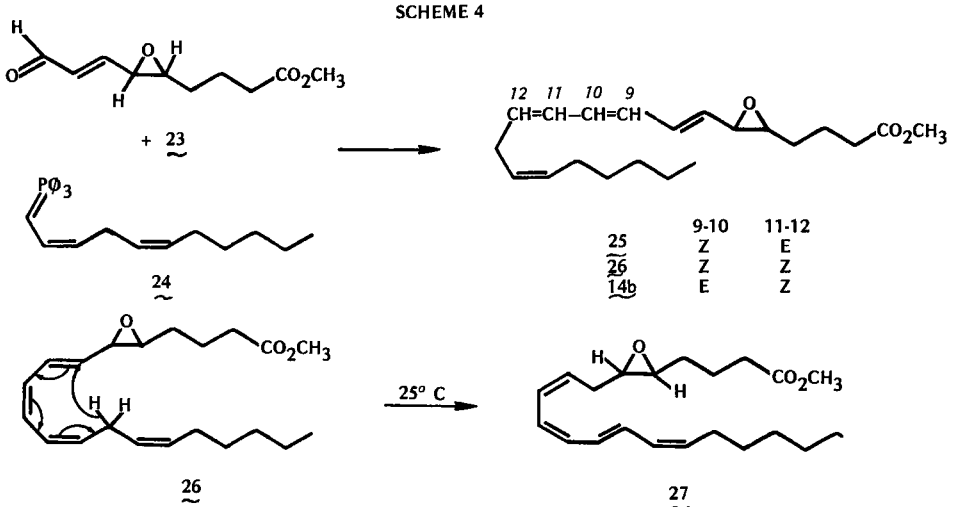
SCHEME 3



Several isomers of LTC_4 were prepared in an effort to rigorously exclude them as possible structures. A second SRS isolated from mouse mastocytoma cells (originally termed LTC-2) was shown to be 11-*trans*-LTC_4[26] by a series of experiments culminating with synthesis and direct comparison with an authentic sample of 11-*trans*-LTC_4 and other hypothetical possibilities such as 6-epi-LTC_4.[52,55] This natural SRS was derived from the corresponding 11-*trans*-LTA_4 which was prepared by modification of the conditions for the final Wittig condensation in Scheme 3.

The synthesis of LTA_4 methyl ester (racemic and optically active) by Wittig condensation of nine carbon enal-epoxide **23** and eleven carbon Z,Z-diene phosphonium salt **24** has been reported by three groups (Scheme 4).[29,56,57] Baker[57] reported the isolation of three triene isomers whose ratios were solvent dependent. HPLC separation and detailed 270 MHz ^{1}H-NMR analysis showed these trienes to be isomeric at the C(9) and C(11) double bonds. His group also found that the 7-E,9,11,14-Z isomer **26** readily underwent a rearrangement at room temperature to the conjugated tetraene **27**. A similar rearrangement presumed to proceed by a 1,7 hydrogen shift was reported earlier by Rokach.[54] The glutathione adduct of the 7-E,9-Z,11-E,14-Z isomer (originally misassigned as 7-E,9,11,14-Z isomer) has been distinguished from LTC_4 by HPLC and ultraviolet spectroscopy.[29] The observed instability of the 7-E,9,11,14-Z system precluded this geometry as a viable possibility for the SRS structure. An isomer of LTC_4 with the glutathione moiety at the C(12) position was synthesized[58] since an early report in the literature suggested this structure,[59] but this compound was easily distinguished from LTC_4.

SCHEME 4



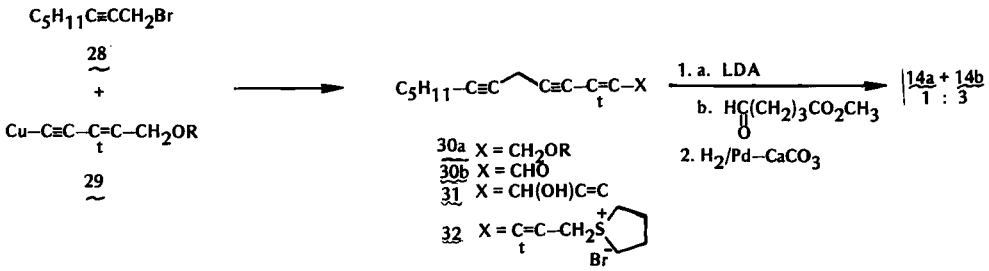
26 27

A second synthesis of racemic LTA_4 methyl ester, **14b**, and LTE_4 was reported by Rosenberg at Hoffmann LaRoche.[60] The key intermediate sulfonium salt **32** was prepared as shown in Scheme 5. Reaction of the sulfonium ylide and methyl 4-formylbutyrate gave a mixture of epoxides (*cis/trans* 1/3) which were separated by HPLC. The *trans* epoxide underwent catalytic hydrogenation with Lindlar catalyst to afford racemic LTA_4 methyl ester. Reaction of L-Cysteine methyl ester with $\pm LTA_4$ methyl ester gave a separable mixture of diastereomers which upon saponification gave the cysteine adduct LTE_4. This compound was reported to have marked spasmogenic activity on guinea pig ileum characteristic of the natural SRS-A'S, but no comparison with authentic SRS was reported.

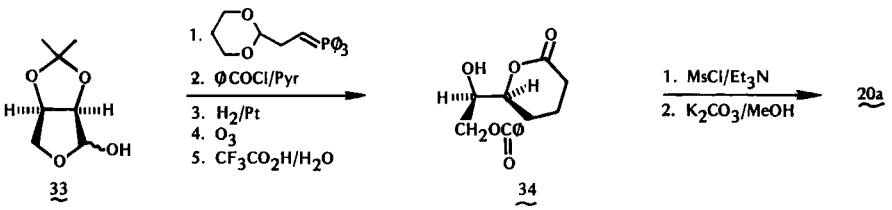
Recent Synthetic Routes to LTA_4 — Several recent syntheses of **LTA_4** methylester, **21**, or the key intermediate epoxy-aldehyde methyl ester **20b** have appeared using various sugars as starting materials. A Hoffmann LaRoche group[61] has reported the synthesis of **20b** starting from D-araboascorbic acid and L-diethyl tartrate (Scheme 6). 2,3-0-Isopropylidene-D-erythrose, **33**, was prepared by modification of known methods and was converted to lac-

tone **34** by straightforward procedures. Mesylation followed by lactone opening and epoxide formation generated alcohol **20a** which was oxidized to aldehyde **20b**. The *cis*-(5*S*,6*R*)-epoxide was prepared by a similar reaction sequence.

SCHEME 5

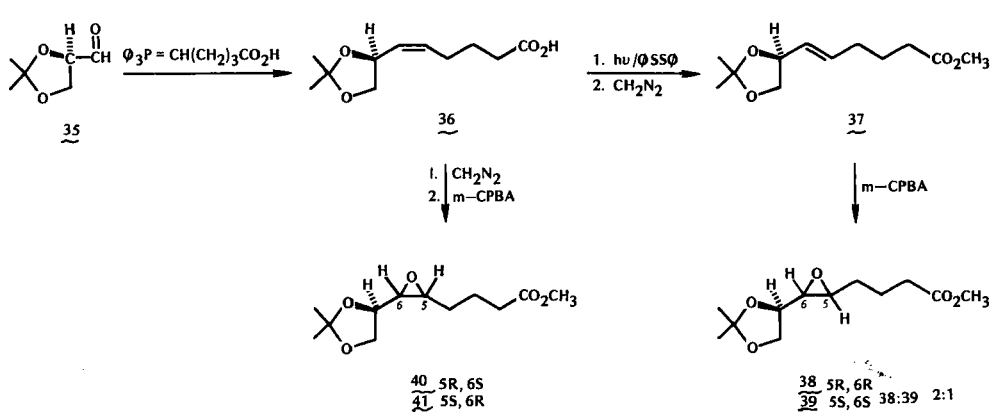


SCHEME 6



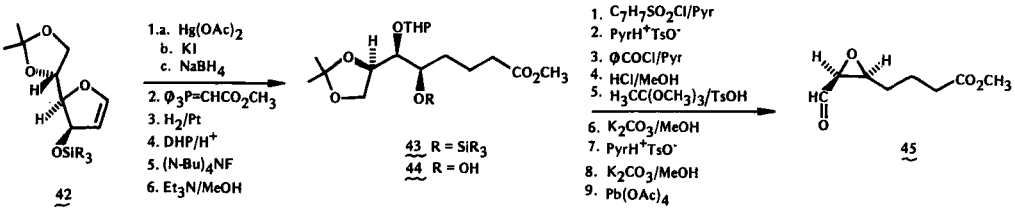
Rokach[62] (Merck) has reported the synthesis of the four enantiomerically pure stereoisomers of **20b** from D and L-glyceraldehyde (Scheme 7). The acetonide of D-glyceraldehyde, **35**, was converted to the *trans* olefin **37** in three steps. Reaction with m-CPBA afforded a mixture of separable diastereomeric epoxides which unfortunately favored the isomer **38** with the unnatural absolute stereochemistry at C(5) and C(6). The same reaction sequence starting from the acetonide of L-glyceraldehyde prepared from L-arabinose afforded the isomer with natural LTA_4 configuration as the major product. The *cis* olefin **36** was also epoxidized to form a separable mixture of corresponding *cis* epoxides **40** and **41**.. Oxidation of each epoxide diastereomer with periodate afforded the corresponding enantiomerically pure isomer of **20b**. The natural 5(S),6(R) isomer was further converted to LTA_4 methyl ester by a succession of two carbon Wittig reactions and subsequently to leukotrienes C_4, D_4 and E_4.

SCHEME 7



Corey[63] has also reported a synthesis of 6-epi-leukotrienes A_4, C_4 and D_4 using D-(+)-mannose as starting material (Scheme 8). The glycal monoacetonide **42** was prepared by known procedures and further converted to hydroxy ester **44**. Protecting group exchange afforded a benzoate tosylate which was readily transformed to *cis*-epoxy aldehyde **45**. Formation of 6-epi-leukotrienes A_4, C_4 and D_4 proceeded in the standard manner.

SCHEME 8



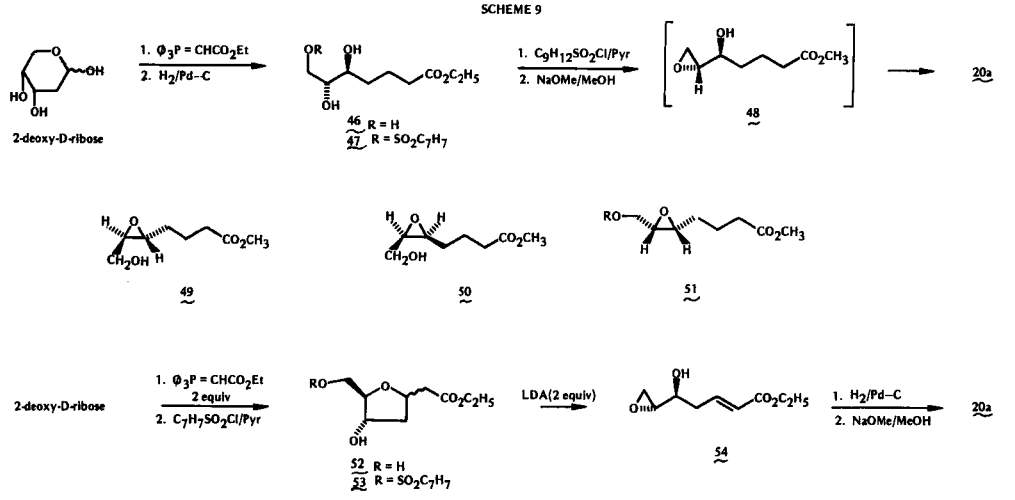
42

43 R = SiR_3
44 R = OH

45

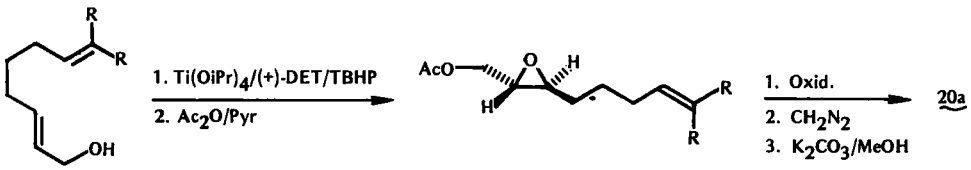
Several syntheses of LTA_4 methyl ester, **21**, as well as 5-epi-LTA_4, 6-epi-LTA_4, and 5-epi, 6-epi-LTA_4 using 2-deoxy-D-ribose as starting material have been reported. Rokach's[64] synthesis of LTA_4 methyl ester, **21**, in seven steps is outlined in Scheme 9. Wittig reaction of 2-deoxy-D-ribose followed by hydrogenation afforded triol **46** in 64% yield. Selective activation of the primary alcohol and treatment with base generated epoxide **20a** through the intermediacy of epoxide **48**. This substrate was converted to LTA_4 methyl ester in the routine manner. Rokach ingeniously adapted this route to prepare the remaining enantiomerically pure diastereomers **49**, **50**, and **51**. Modification of the initial Wittig reaction conditions of 2-deoxy-D-ribose affords C-glycoside **52**[65] Tosylation followed by anion formation and β- elimination afforded epoxide **54** in 50% yield. Hydrogenation and treatment with sodium methoxide produced epoxide **20a**. The diastereomeric epoxides **49** and **51** were also prepared from C-glycoside **52**. A Fison's group[66] has utilized 2-deoxy-D-ribose in a reaction sequence which parallels the original synthesis of LTA_4.

SCHEME 9



An alternative approach to the synthesis of chiral epoxide **20a** utilizes the recently discovered asymmetric epoxidation procedure of Sharpless.[67] Both Sharpless[68] and Corey[69] have reported syntheses of **20a** utilizing different substrate allylic alcohols (Scheme 10). The enantiomeric excess was excellent in both cases (95% ee and 93% ee, respectively).

SCHEME 10

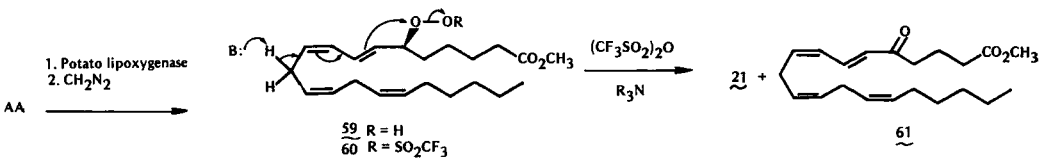


55 R = H
56 R = CH_3

57 R = H
58 R = CH_3

An efficient chemical synthesis of (±)-5-HPETE and an enzymatic synthesis of the natural (S)-5-HPETE from arachidonic acid[70] has led to a simple biomimetic synthesis of LTA_4 and LTC_4 (Scheme 11).[71] Conversion of hydroperoxy ester **59** to the activated peroxytrifluoromethane sulfonate **60** in the presence of a sterically hindered base 1,2,2,6,6 pentamethyl piperidine led to 1,7 elimination to form LTA_4 methyl ester, **21**, and 1,2 elimination to dienic ketone **61**. The LTC_4 prepared as previously described[29] was again shown to be identical to natural LTC_4. Sih[72] later reported a similar procedure which gave in addition to **21** and **61** the isomeric epoxide **26**.

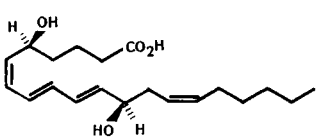
SCHEME 11



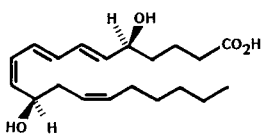
59 R = H
60 R = SO_2CF_3

61

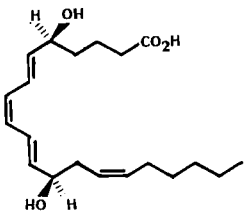
Synthesis of LTB_4 — Although LTB_4 was the first leukotriene whose gross structure was known, the final structural details of the triene geometry were not established until Corey *et al.*[73] compared several synthetic isomers with native material.[74] These studies demonstrated that the triene geometry of LTB_4 was 6-Z,8,10-E and that the isomeric trienes 6-E,8-Z,10-E,**63**, and 6,8-E,10-Z,**62**, could be readily distinguished from LTB_4.[75]



LTB_4 5



62

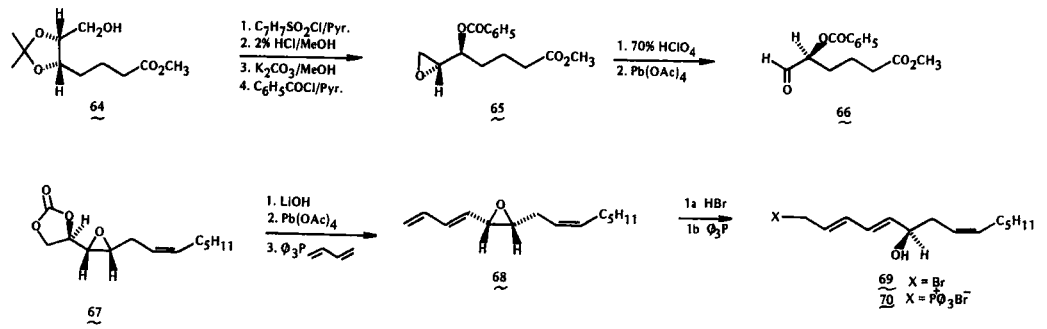


63

The original synthesis of this potent chemotactic agent (maximal activity 0.1-1.1 ng/ml)[20a,b] utilized 2-deoxy-D-ribose for construction of the C(1)-C(6) segment **66** and D-(+)-mannose for the C(7)-C(20) unit **70** (Scheme 12). Wittig reaction of the acetonide of 2-deoxy-D-ribose followed by hydrogenation afforded hydroxy ester **64** which was readily transformed to epoxide **65** and subsequently to aldehyde **66**. A previously synthesized[63] cyclic hemiacetal of D-(+)-mannose was converted in five steps to *cis* epoxide **67**. Carbonate hydrolysis, oxidation and Wittig reaction afforded epoxide **68** which was transformed to phosphonium

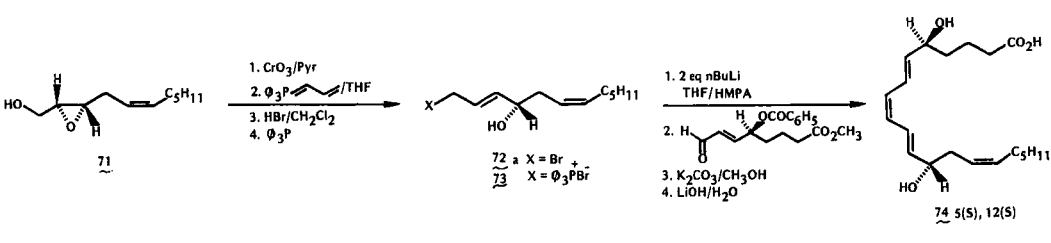
salt **70** by reaction with HBr followed by triphenylphosphine. Wittig condensation between **70** and aldehyde **66** generated the 5-benzoyl methyl ester derivative of **5**. Hydrolysis of the benzoate and methyl ester with lithium hydroxide afforded LTB_4 which was identical to native material as judged by ultraviolet spectroscopy, reverse phase HPLC retention time and bioassay.

SCHEME 12



In a companion paper, Corey *et al.*[75] reported the synthesis of the trienic isomers **62** and **63**. Both diastereomers **62** and **63** were distinguishable from LTB_4 by bioassay. Since the diol **63** was synthesized as a racemic mixture of epimers, the relative configurations at C(5) and C(12) could not be absolutely assigned and thus, a stereospecific synthesis of the 5(S), 12(S) isomer **74** was completed (Scheme 13).[76] This enantiomer has been identified as a new human arachidonic acid metabolite isolated from mixed peripheral blood leukocytes.[77]

SCHEME 13

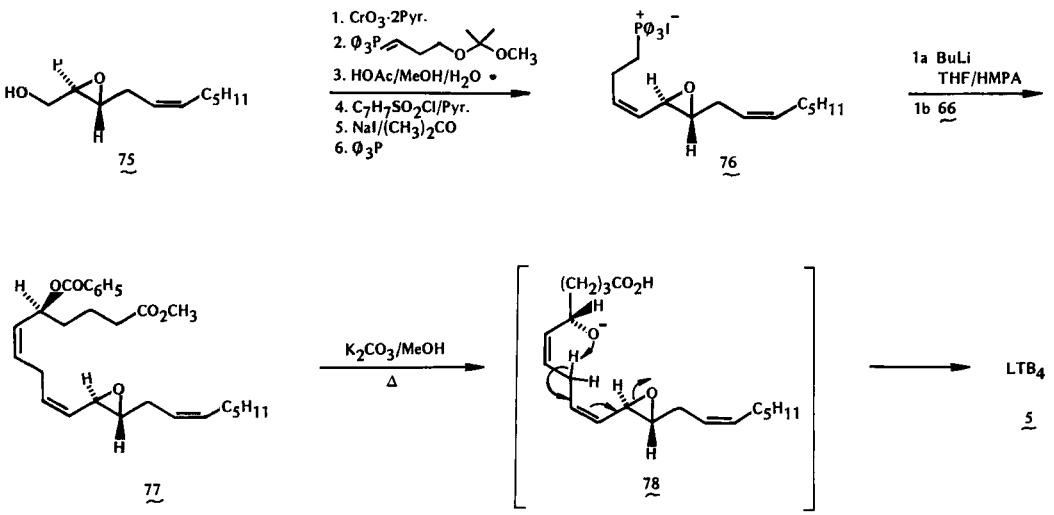


A second, more efficient synthesis of LTB_4 by the Corey group[78] utilized a novel internally promoted elimination reaction of epoxy ester **77** (Scheme 14). This intermediate was synthesized by Wittig condensation of the aldehyde **66** and phosphonium salt **76**, prepared from the previously synthesized[75] epoxy alcohol **75.**

Synthesis of the 12(S)- and 12(R)-forms of 6-*trans*-leukotriene B_4, have been reported utilizing β-hydroxyphosphonium salts.[79]

Additional Leukotrienes — Several additional leukotrienes have been identified from oxygenation of arachidonic acid at various positions of the hydrocarbon backbone as well as other eicosanoic acids. Samuelsson[80] has reported isolation of isomeric 14,15-dihydroxy-5,9,11,13-eicosatetraenoic acids and 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acids formed by initial oxygenation of arachidonic acid at C(15). Diols formed by hydrolysis of epoxides of arachidonic acid at C(14),C(15) and C(11),C(12) have been reported.[81] An epoxyhydroxy metabolite, 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid and triol hydrolysis products have been identified in human blood platelets.[82] Finally, leukotrienes

SCHEME14



derived from 5,8,11-eicosatrienoic acid[83] (LTC_3) and 5,8,11,14,17-eicosapentaenoic acid[84,85,87] (LTC_5) have been isolated and an isomer of leukotriene C_3 formed from 8,11,14-eicosatrienoic acid is known.[86]

Conclusion — The recent discovery of the leukotrienes has significantly increased our knowledge of arachidonic acid metabolism. Synthetic chemists have played a key role in the structure elucidation of various leukotrienes and have for the first time made available ample quantities of pure material. Pharmacologists and medicinal chemists now have the tools accessible to them to explore the biological import of this fascinating family of molecules.

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Chapter 30. Strategies in the Discovery of Drugs from Natural Sources

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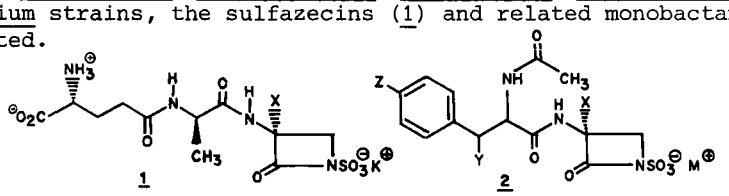
INTRODUCTION

Perspective views of strategies utilized up to the end of the nineteen sixties for the discovery of such drugs as reserpine and vincaleukoblastine from plant sources,[1] the steroid hormones and prostaglandins from animal sources,[2] and the antibacterial antibiotics from microbiological sources,[3] have been published. Decreasing numbers of clinically useful drugs following major systematic searches of plants and microbial sources over the last three decades have led to despondency in the belief that such ventures can be engaged in profitably.[4,5] Natural products, nevertheless, remain one of the few de novo sources of drug discovery, yielding unorthodox and often unexpected chemical structures. These have offered novel points of departure for molecular modification leading to clinically available drugs.[4] The success rate of discovering new drugs from natural sources is, indeed, dependent on the conception and implementation of ingenious, comprehensive strategies that exploit the untapped potential of the natural sources. This review, therefore, purports not so much to describe new naturally-occurring bioactive structures, which have been adequately reviewed recently,[6-12] but to highlight the major strategies that have been employed in the past three to five years in the discovery of drugs with potential clinical utility. The emphasis will be placed on "winning" strategies with microbial and plant sources. Discoveries from animal sources will be alluded to only briefly, as the strategies leading to new drugs exemplified by the peptide hormones, interferons, angiotensin-converting enzyme inhibitors, enkephalins and leukotrienes have been included in recent previous volumes of these reports.

MICROBIAL SOURCES

Antibiotics - Several reviews on the prospects of and strategies in obtaining biologically active molecules have been published recently.[13-20] The basic strategies can be summarized under the following sub-headings:

A. Isolation of novel genotypes from terrestrial and marine ecosystems - Though there are more than 5000 antibiotics and biologically active molecules known today, the majority have been isolated from a single family, the Streptomycetaceae. Other so-called "neglected" families of Actinomycetales and of the orders Eubacteriales and Pseudomonadales, neglected classes of fungi other than fungi imperfecti (Fig.1), and marine microorganisms are now being recognized as genetically versatile sources of a variety of novel secondary metabolites.[21-30] Major success has come from the screening of Eubacteriales, combined with sensitive detection techniques. From Pseudomonas, Gluconobacter, Acetobacter, Chronobacter and Agrobacterium strains, the sulfazecins (1) and related monobactams (2) were isolated.



1

X = OCH_3, H.

2

Y = Z = H, OH, $OSO_3^{\ominus}$ $M^{\oplus}$ M = Na, K.

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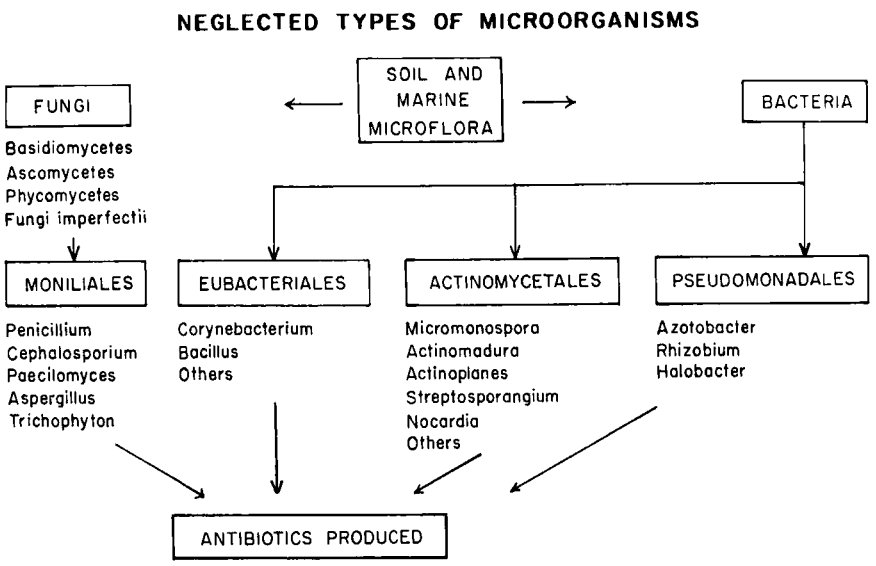


Figure 1

B. Creation of novel or altered genotypes by genetic engineering - Genetic engineering has become a major tool in the design of microorganisms tailored to produce specific metabolites.[18,33-35] The methods used are: mutation, exchange of genetic material by recombination or protoplast fusion, and gene cloning by the use of plasmid vectors.

Mutation to yield idiotrophs, followed by the feeding of precursor analogs, has led to the hybrid biosynthesis or mutasynthesis of a variety of antibiotics such as aminoglycosides, macrolides and penicillins.[36] Mutants can also be used for the microbial conversion of inactive metabolites, such as anthracyclinones, to active moieties such as anthracycline antibiotics by glycosidation.[37]

Intraspecific and interspecific recombinations have been carried out in rifamycin-producing strains and in aminoglycoside-producing strains using double auxotrophs and conventional techniques.[38-41] However, the major advance in recombinant DNA techniques has come with the use of protoplast fusion. The two important manipulations are protoplast fusion which allows *in vivo* genetic recombinations with high frequencies, and transformation or transfection of protoplasts with plasmid DNA or actinophage DNA.[42-46] Interspecific recombination by protoplast fusion shows the greatest potential in the synthesis of hybrid molecules. Significant numbers of recombinants have been obtained in fusions between *S. coelicolor* and *S. lividans*, *S. fradiae* and *S. bikiniensis*, but not in others, for example, *S. coelicolor* and *S. parvulus*.[43-47]

Gene cloning using two plasmids such as SCP2 and SLP1.2 as vectors is further facilitated by the introduction of antibiotic resistance markers in the plasmids. These genetic methods hold considerable promise in obtaining novel structures, though the results are only in the preliminary stage at present.

C. Biochemical manipulation of selected pathways - Pathways of secondary metabolism can be altered or directed by precursor feeding, inhibition of selected enzymes, alteration of media constituents, and controlled manipulation of physical parameters such as pH, temperature, aeration.[48-50] Thus,

oganomycins, new 7-methoxycephalosporin antibiotics, can be produced by S. oganonensis Y-G19Z by feeding heterocyclic thiols. By using cerulenin, a specific inhibitor of fatty acid and polyketide synthesis, it is possible to convert the macrolide, tylosin, to a new compound by the spiramycin producing strain, S. ambofaciens KA 1028.[51]

D. Supersensitive and specific detection methods and screening for varied bioactivities - With the use of unconventional test strains and the testing for unusual activities, a number of novel compounds have been isolated (Fig.2). The antibiotic haloquinone, anthelmintic avermectins, the insecticidal and ascaricidal milbemycins, aspiculamycin and orthosomycins, have been detected.[52-56]

NOVEL DETECTION METHODS

METHOD	TYPE OF ANTIBIOTIC DETECTED
1. CROSS RESISTANCE / SENSITIVITY PATTERNS USING E. coli STRAINS [76]	BROAD SPECTRUM ANTIBIOTICS
2. SUPER SENSITIVE STRAINS β-LACTAMASE INHIBITION / SENSITIVITY. SPHEROPLAST FORMATION. [19, 57-61, 78]	CELL WALL ACTIVE COMPOUNDS, MAINLY BETA LACTAMS
3. INHIBITION AND/ OR RESISTANCE TO INACTIVATING ENZYMES [62-64]	ENZYME INHIBITORS AND NOVEL ANTIBIOTICS
4. ANTIBIOTIC POTENTIATION [77]	CELL WALL ACTIVE COMPOUNDS
5. DIFFERENCE ASSAYS USING B. subtilis AND S. viridochromogenes [19]	NARROW SPECTRUM COMPOUNDS
6. COMBINATION ASSAYS WITH CHELATING AGENTS SUCH AS EDTA [19]	IONOPHORES
7. TESTS FOR BIOLOGICAL ACTIVITIES OTHER THAN GROWTH INHIBITION [19]	NOVEL COMPOUNDS

Figure 2

There are also several "target" specific assay systems (Fig.3) that facilitate the detection of certain types of compounds.

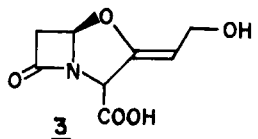
TARGET SPECIFIC ASSAY SYSTEMS

METHOD	TYPE OF ANTIBIOTICS
1. INHIBITION OF PROTEIN SYNTHESIS USING INDUCTION OF β-GALACTOSIDASE IN E. coli	BROAD SPECTRUM ANTIBIOTICS
2. DIFFERENCE ASSAY USING B. subtilis AND Acholeplasma laidlawii [79]	CELL WALL ACTIVE
3. INHIBITION OF DIAMINO PIMELIC ACID INCORPORATION USING E. COLI DAP^- LYS^- [79]	CELL WALL ACTIVE
4. INHIBITION OF SPECIFIC CELL WALL SYNTHESIS ENZYMES SUCH AS TRANSPEPTIDASE, DD-CARBOXYPEPTIDASE [80-82]	CELL WALL ACTIVE

Figure 3

The most successful strategy has been the use of strains super sensitive to β-lactams coupled with the use of different β-lactamases as discriminative probes.[57-62] This has resulted in the discovery of clavulanic acid (3),[63] the trans-olivanic acids,[62] PS-5,6,7 types of antibiotics and thienamycin (4),[64] the cis-olivanic acids and carpetimycins (5),[62,65] and the asparenomycins (6).[68]

Through testing for anticancer agents, an antitumor compound, anguidin (7),[87] has been isolated from a fungi and is in phase II clinical trials.



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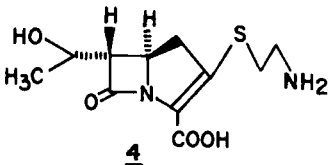

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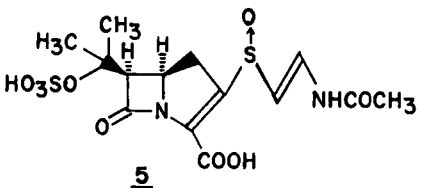

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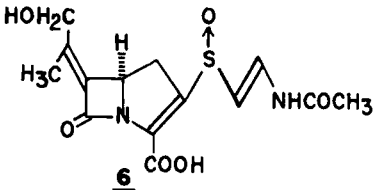

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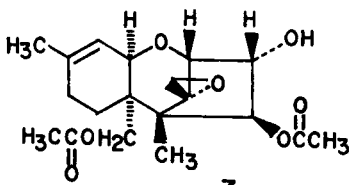
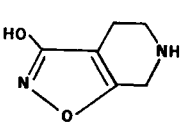


7

Pharmacologically active compounds - A search for inhibitors of specific enzymes has provided microbial metabolites with cardiotonic, hypocholesterolemic, antiinflammatory, antihypertensive and neuromuscular blocking activities.[15,69-74] Dihydromevinolin[74] is a potent inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase from A.terreus with hypocholesterolemic activity. A new amylase inhibitor, HOE 467 (8), has been isolated from Streptomyces tendae.[75] The compound reduces bodyweight and cholesterol levels and can be used as supporting treatment in diabetes. Lead development of muscimol,[83] a GABA agonist detected in Amanita muscaria, has led to the analgetic compound THIP (9).



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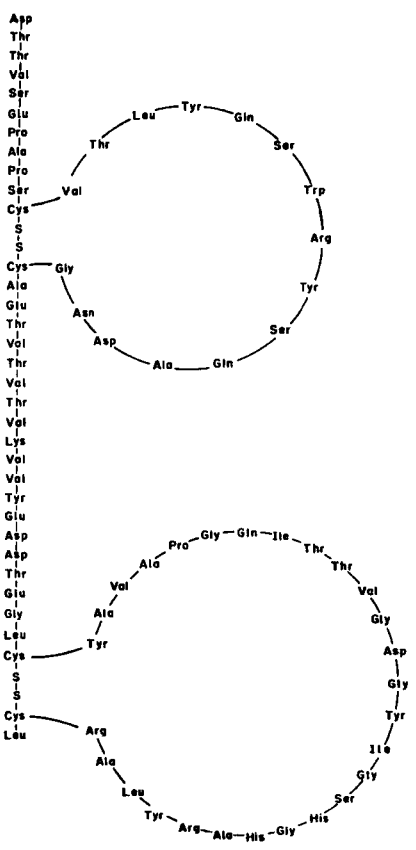


8

PLANT SOURCES

The increasing number of symposia, congresses and literature reports on the theme of medicinal agents from plants emphasises the interest and worldwide activity in this field.[7-12,84-86] Only a very limited number of reports, however, describes strategic long-term, mission-oriented programs to discover novel drugs.[86-95] The variable parameters in the different strategies may be summarized under the following sub-headings.

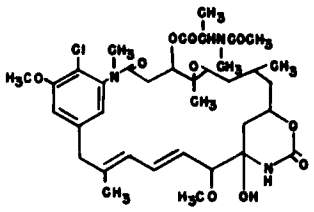
Plant Selection Criteria - The various approaches include selections based on ethnotherapeutic and folkloric considerations, botanical or phytochemical considerations, and random choice. Their advantages and drawbacks in the evaluation of plants for anticancer,[87,96] antiinflammatory[96] and antimicrobial[90] activity have been discussed. The random selection approach has been preferred for screening of anticancer activity[87] and for collection of Australasian marine species.[94,95] A combination of folkloric, botanical and phytochemical criteria has been employed with success in other programs.[91,96,97] One of the most modern approaches being used in the selection of plants for fertility regulation is based on a computer-generated, rank-ordered list of priority plants. The data base, priority designator codes, pharmacological data for rank-orders and weighting criteria used for analysis by the NAPRALERT system, have been described.[92,93]

Preparation of Plant Extracts for Bioassay - The nature and number of extracts are strategic features of some programs, in which considerations of economy of time, finances and tests to provide the highest number of active extracts play an important role. Single and multiple solvent/aqueous extracts, room temperature percolation/soxhlet extraction, and solubilization of insoluble extracts are factors which influence the bioassay results.[87,96] A procedure for solubilizing most plant extracts, involving the formation of a co-precipitate with polyvinylpyrolidone 1000, has been found very useful.[93]

Biological Screening Approaches - One or more of a wide range of *in vitro* and *in vivo* pharmacological and microbiological screens[7-9,11,85-96] are used, largely based on those also employed for the evaluation of synthetic compounds. Past and current pharmacological approaches to primary screening of plant extracts have been reviewed.[98] Single technique-single goal screening, screening using a battery of specific procedures, single technique-multiple goal screening, and combinations of specific and multiple-purpose procedures have been supplanted in drug companies by multidimensional primary screening. Two techniques widely employed presently are the rat "hippocratic" screen, including computerised evaluation, and the mouse multidimensional screen. For the secondary screening of extracts and pure compounds, the value of the dog pharmacodynamic screen and of using hippocratic variants in intact, unanaesthetized larger animals is emphasised.[98] Some screening techniques recently introduced for plant extracts or those that have resulted in compounds under clinical trials will be briefly described below.

The *in vitro* bioassay technique for isolating enzyme inhibitors from microbial cultures has been applied to the random screening of Chinese medicinal plant drugs. Onjisaponins, lignans pinoresinol and trachelogenin, and the norlignan *cis*-hinokiresinol were identified as cAMP phosphodiesterase inhibitors and were suggested to be the active principles causing depression of the central nervous system. A lichen depside, 4-O-methyl cryptochlorophaeic acid, was identified as a prostaglandin synthetase inhibitor, ten times more active than indomethacin.[99,100]

Different tumor systems are used as primary screens for anticancer activity. These have changed over the years from the sarcoma 180, adenocarcinoma 755, Walker 256 carcinosarcoma, and L1210 leukemia systems to the current ones of the KB cell line and the P388 leukemia system. The more important current compounds in clinical trials are the ANSA macrolide maytansine (10), the quassinoid bruceantin (11), and the alkaloid indicine N-oxide (12). Ellipticine, taxol, homoharringtonine, tripdiolide and bouvardin are newer potential clinical trial candidates.[87] From a Chinese herb, the rubescensines (13) were found clinically effective for treatment of aesophageal, liver and pancreas carcinomas.[101]



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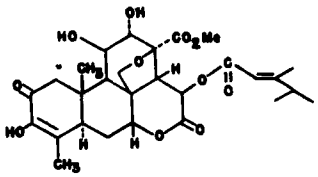

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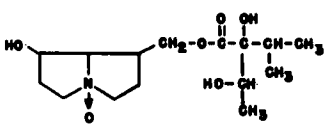

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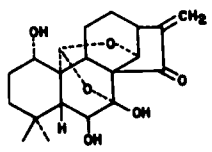
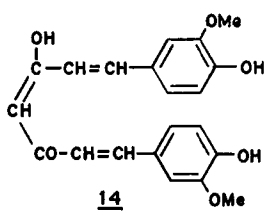


13

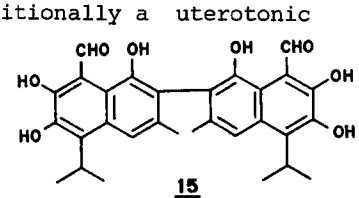
Through screening for hypolipidemic activity,[102,103,105,106] guggulipid, the hypolipidemic principle of Commiphora mukul, comparable to clofibrate but with additional platelet aggregation inhibition and anti-inflammatory properties, has proved to be safe and well-tolerated in human volunteers.



14

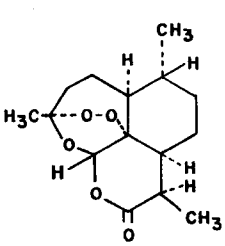
Curcumin (14), the antiinflammatory principle of Curcuma longa, comparable in its efficacy to phenylbutazone, is in Phase II clinical trials.[89,104]

For fertility-regulating programs, the uterotonic in vitro assay (rats) is used[93,107,108] very frequently. Bioassay protocols are characterized by a priority sequence and include additionally a uterotonic assay in situ (rabbit), a 29-day pregnant rabbit bioassay, and postcoital bioassays (rat, hamster).[93] The dimer gossypol (15) found in the cotton plant (Gossypium sp.) and used as a male contraceptive, has provided a stimulus for a new search from plants for orally active antispermatogenic agents.[109,110]



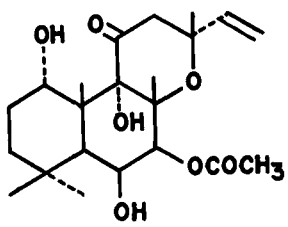
15

Screens for the detection of antiparasitic agents have yielded the antimalarial sesquiterpene peroxide Qinghaosu (16) as the active principle of the Chinese herb Artemisia annua L. It has a high level of blood schizontocidal activity against chloroquine-resistant malaria parasites, both in laboratory models and in the clinic.[111,112]



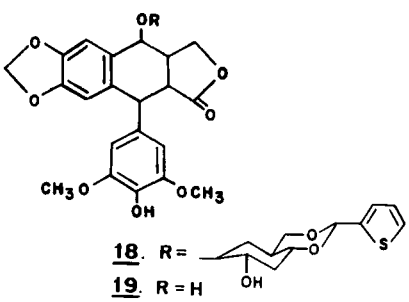
16

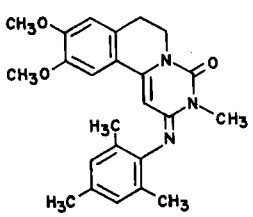
Phylogenetic considerations in plant selection, coupled with multidimensional screening oriented towards cardiovascular active compounds, has provided the diterpenoid, forskolin (17) from the Indian plant Coleus forskohlii. It has cardiotonic, antihypertensive and vasodilatory properties, acts by unique adenylate cyclase activation, and is now scheduled for clinical trials.[91,113-116]



Investigations of indigenous drugs used in the treatment of Diabetes mellitus have resulted in the isolation of a hypoglycemic peptide, polypeptide-p, from the fruit, seeds and tissue of Momordica charantia Linn. It has displayed an hypoglycemic effect in juvenile and maturity-onset diabetic patients when administered subcutaneously.[117]

Active Principle Isolation, Characterization and Development - Bioassay directed purification of extracts, newer separation methods such as droplet counter-current chromatography[118] and centrifugal thin layer chromatography,[119] and refinements in microspectrometric methods of structure analysis constitute important elements of current-day strategies.[87] Lead development and refinement through semi-synthetic and synthetic programs using drug design principles are essential features of different strategically oriented programs.[87,91] The anticancer podophyllotoxin analogs, VM-26 and VP-19 (18,19), and the antihypertensive vasodilator HL-725 (20) are recent examples of drugs under clinical testing which have resulted through such comprehensive strategies.[87,120]





20

ANIMAL SOURCES

Similar strategies to exploit the biomedical potential of marine species have provided hosts of unusual new lead compounds with potent antiviral, antimicrobial and pharmacologically active properties.[94-6,121-128] Other approaches utilize the frog histrionicotoxins and pumiliotoxins, and snake venom toxins as tools in physiological and receptor site studies.[129-130] The skin of an Australian frog has provided ceruletide, a cholecystokinin-like decapeptide, as a clinically useful analgesic drug.[131,132] The high number of interesting new drugs from animal/mammalian sources, as mentioned here and in the introductory paragraph, is a pointer to the strategic value of engagement in the investigation of such sources.

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Chapter 31. Herbicides and Insect Control Agents

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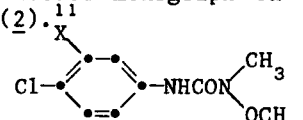
Introduction - With the exception of a review on the design of DDT analogs,[1] reviews of pesticides have not appeared in this series, in spite of the obvious similarity of interests, training, and thought processes of medicinal and agricultural chemists. The most important agents generally understood to be included within the meaning of the nearly interchangeable terms "pesticides" and "agrochemicals" are herbicides, insect control agents, fungicides, and plant growth regulators. Space limitations preclude the inclusion of all these and thus the two most important in terms of global use, herbicides (an estimated $4.9 billion in sales at the user's level in 1980)[2] and insect control agents ($3.9 billion)[2] were chosen for review. Work largely published in the past few years will be discussed, although some background information is included where appropriate.

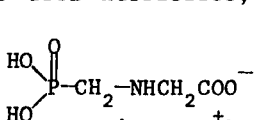
An excellent general reference on pesticides is "The Pesticide Manual",[3] which consists of a compendium of pesticidal structures, as well as synopses of the history of the development of pesticides and their properties, processes of manufacture, uses, toxicology, formulations, and methods of analysis.

Herbicides

General - Global use of herbicides continues to increase. A number of new chemical classes, as well as variants of established herbicides, have been introduced in the past few years. Monographs on herbicides edited by Kearney and Kaufman[4] and by Audus[5] appeared in the mid-1970's, the former being of substantial interest to medicinal and agricultural chemists. A short review on herbicides appeared in mid-1981.[6] A new edition of a handbook containing the structures of herbicides in current use, as well as describing their utility, was published.[7] A book on synthesis and mode of action appeared.[8] The mechanisms of herbicidal action were reviewed, involving chloroplast-associated reactions, mitochondrial electron transport and phosphorylation, membrane interactions, and cell division and synthesis of nucleic acids and proteins.[9] Proceedings of a workshop on the action of pesticides on photosynthesis were published,[10] as was a multifaceted monograph on the urea herbicides, linuron (1) and monolinuron (2).[11]



1: X = Cl
2: X = H



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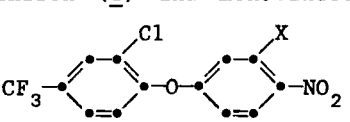

4: X = COONa
5: X = OC_2H_5

Among concepts and practices newly introduced or come of age are the induction of crop selectivity chemically through the use of "safeners" or "antidotes"[12] or mechanically *via* ropewick applicators[13] or recirculating sprayers.[14] The use of the nonselective systemic herbicide glyphosate (isopropylamine salt:3) has grown rapidly for both annual and perennial

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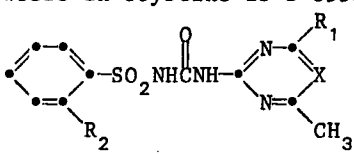
weeds. There is a trend toward more potent compounds, allowing the use of less pesticide per unit area; examples are the diphenyl ethers acifluorfen (4)[15] and oxyfluorfen (5),[16] and a new compound, chlorsulfuron (6),[17] which is active at rates as low as 10 g/ha. The advent of newer diphenyl ethers, such as 4, as well as a number of other new herbicides, might signal a trend toward application to foliage of growing plants (postemergence treatment), as opposed to application to the soil (preemergence treatment).

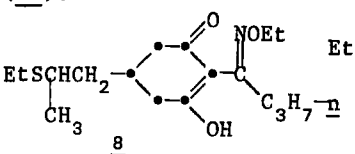
Rapid screening systems developed to discover herbicidal activity *in vitro* include a tissue culture screen[18] and utilization of algae.[19]

New Herbicides - Among new compounds that have been broadly field tested in the past few years is DPX 4189 (6), the first member of a new class of sulfonylureas. It is systemically active in the range of 10-25 g/ha on broadleaved weeds in wheat, oats, barley and rye.[17,20] Mode of action studies indicate it is a potent inhibitor of cell division and does not interfere with cell elongation, photosynthesis, respiration, or protein or RNA synthesis.[21] A second highly active member of the series, DPX 5648 (7), was recently disclosed[22] and appears promising for total control of vegetation. Sethoxydim (8) is a selective herbicide for postemergence use on annual and perennial grassy weeds in broadleaved crops.[23] NC 20 484 (9), a preemergence or soil-incorporated herbicide, has been evaluated for selective control of annual and perennial weeds in cotton, sugar cane, tobacco, and rice.[24] It is particularly effective on sedges. Diclofop-methyl (10) has proved to be an important model for new synthesis work. Two related compounds recently announced for controlling grasses in broadleaved crops by postemergence treatment are fluazifop-butyl (11)[25] and CGA 82725 (12).[26] Both of these are translocated in plants and are active against annual and perennial weeds. UBI-S734 (13) is a soil-applied herbicide which controls grasses and sedges in dicotyledonous crops.[27] A compound which has been reported for postemergence control of broadleaf weeds in soybeans is S-3552 (14).



6: R_1= OCH_3, R_2= Cl, X = N

7: R_1= CH_3, R_2= CO_2CH_3, X = CH



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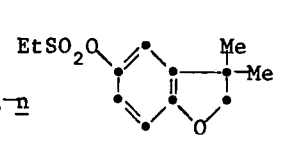

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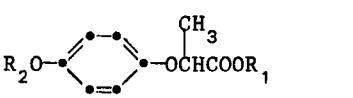
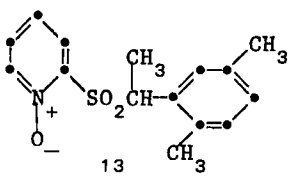


10: R_1= CH_3, R_2= 2,4-$Cl_2C_6H_3$

11: R_1= Bu, R_2= 5-CF_3-2-pyridyl

12: R_1= $CH_2C{\equiv}CH$, R_2= 3,5-diCl-2-pyridyl



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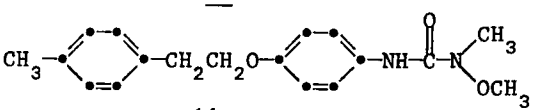
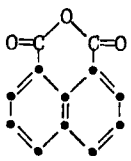


14

The large number of chemists involved in herbicide synthesis notwithstanding, synthesis and structure-activity papers other than those related directly to new products or probable new products are rare. Among chemical series recently discussed are aryloxyethydialkylamines as photophosphorylation uncouplers,[28] quaternary salts of pyridylheterocycles,[29] 2-alkyl-2-cyanoacetanilides,[30] rigid acetanilides,[31] arenesulfinamides,[32] and quaternaries derived from bispyridyl thiadiazoles and oxadiazoles.[33]

Quantitative structure-activity work was done on N-aryl-tetrahydrophthalimides[34] and phenylalkoxyphenyl ureas.[35]

Safeners - The concept of decreasing herbicidal damage to crops through the use of a second chemical compound, called a "safener", a "herbicide antidote", "herbicide antagonist", or "crop protectant" is attributed to Hoffman, who found that 1,8-napthalic anhydride (15), used as a seed dressing, decreased injury to corn caused by such thiocarbamate herbicides as EPTC (16) and chloroacetanilides such as alachlor (18).[36] A group at Stauffer Chemical[37] discovered that N,N-diallyl-2,2-dichloroacetamide (19, R-25788), when applied to the soil in admixture with 16 prior to planting corn, protected the corn from the phytotoxic effects of the herbicide but did not reduce damage to weeds. Mixtures of 19 with 16 and with 17 (butylate) are commercially available for use on corn. The mode of action of 19 in safening EPTC has been related[38] to its ability to raise both glutathione and glutathione-S-transferase levels in corn, causing more rapid detoxification of EPTC via its sulfoxide metabolite.



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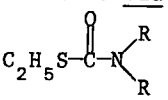
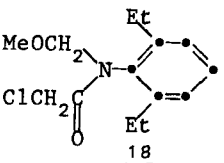


16: R=n-C_3H_7

17: R=i-C_4H_9



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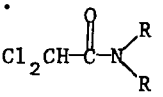
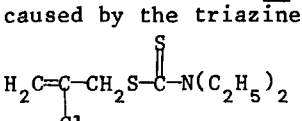


19: R=allyl

20: R=n-C_3H_7

In a structure-activity study[39] of analogs of 19 in a soil-free medium, the saturated analog 20 was found to afford the greatest protection to corn seedlings from EPTC injury. The activity of R-25788 is not confined to thiocarbamates; it also safens corn against the dithiocarbamate sulfallate (21), the chloroacetanilide alachlor (18), and the carbamate barban (22).[40] Evidence was obtained that the mechanism of antidotal action for chloroacetanilides does not involve glutathione-S-transferase but may be related to increased levels of glutathione, which undergoes a non-enzymatic reaction with chloroacetanilides.[41] Recent work with corn cell cultures suggests that the protective effect of R-25788 on EPTC is unrelated to glutathione levels, but rather is due to a series of interactions, the most rapid causing decreased EPTC uptake.[42] Grain sorghum was protected from metolachlor (23) injury by cyometrinil (24), preferably as a seed treatment, at rates of the herbicide as high as 4 kg/ha.[43] Mon 4606 (25) also safens chloroacetanilides in sorghum.[44] With alachlor (18), it was used successfully as a seed treatment, granular soil treatment, or as a mixture with the herbicide in the spray tank. The dinitroaniline herbicides trifluralin (26) and pendimethalin (27) reduced injury to soybeans caused by the triazine herbicides 29 (atrazine) and 30 (metribuzin).[45]



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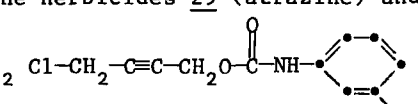

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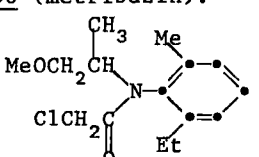

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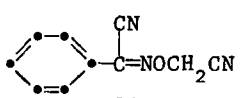

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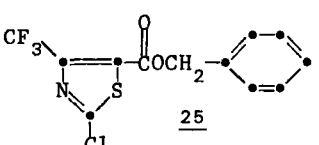

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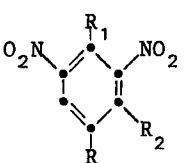

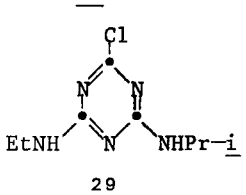

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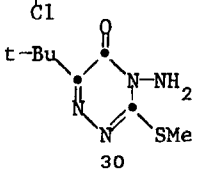


30

	R_1	R_2	R_3
26	$N(Pr\text{-}n)_2$	H	CF_3
27	$NHCHEt_2$	CH_3	CH_3
28	$N(Pr\text{-}n)_2$	H	SO_2NH_2

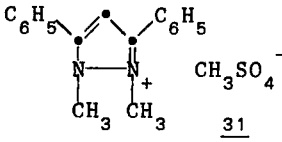
Mode of Action - A substantial amount of work was done in an attempt to elucidate the mode of action of glyphosate (3) and the dinitroaniline herbicides. Inhibition of aromatic amino acid biosynthesis has been suggested as the mechanism of glyphosate action via inhibition or suppression of chorismate mutase and/or prephenate dehydratase.[46] More recently strong evidence for interference at an earlier stage, namely, the shikimate-to-chorismate conversion, has been found, specifically, inhibition of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthetase.[47] Studies of the effect of glyphosate on corn[48,49] and soybeans[50,51] revealed that increased levels of phenylalanine ammonia-lyase accompany the lowering of phenylalanine concentrations. The finding of a significant glyphosate-induced decrease in the content of 8-amino levulinic acid in barley and corn, opens a new area, since this amino-ketoacid is a precursor of porphyrins in chlorophyll, cytochromes, peroxidases and other vital plant constituents.[52] Glyphosate was also found to inhibit transpiration in bean leaves.[53]

Work with the alga Chlamydomonas led to the suggestion that the widely used dinitroaniline trifluralin (26) disrupts normal cell division in the affected tissue via an interaction with tubulin that prevents tubulin assembly into spindle microtubules.[54] A study of the site of binding of ^{14}C-labeled oryzalin (28) in corn root homogenate suggested that this dinitroaniline is bound to lipid-like rather than proteinaceous material.[55] The hypothesis was put forward that oryzalin interacts with cellular membranes, affecting their permeability.[56] Work with 12 dinitro-anilines in mung bean mitochondria showed interference with electron transport and phosphorylation and also led to the conclusion of binding at a lipoidal site;[57] the suggestion was made that dinitroanilines partition into the inner mitochondrial membrane, decreasing its fluidity and altering its permeability.

Diphenyl ether herbicides are known to inhibit respiration and photosynthesis.[58] This inhibition apparently occurs between photosystem I and photosystem II.[59] It was suggested that destruction of carotenoids and then chlorophyll was the primary mode of action of certain of those diphenyl ethers such as oxyfluorfen (5) that cause rapid bleaching effects in plants.[60] Activation by light is necessary for diphenyl ethers; photo-synthetic electron transport was found to be necessary for the activation process.[60] Earlier work with chloroacetanilides that correlated activity with inhibition of protein synthesis was confirmed.[61,62] In the case of metolachlor (23), the primary cause was found to be inhibition of amino acid uptake rather than a direct effect on the process.[61] Multiple sites of action were proposed for metolachlor as a result of work indicating an effect on terpenoid biosynthesis.[63] The wild oat herbicide difenzoquat (31) inhibited DNA synthesis substantially more in a wheat cultivar susceptible to its herbicidal effect than in a tolerant cultivar.[64] The ranks of herbicides inducing photosynthetic electron transport inhibition was joined by buthidazole (32), which was found to have a major site of inhibition in photosystem II and a minor one in photosystem I.[65] Studies on four herbicides known to be inhibitors of photosynthesis suggested they exerted their toxic action through formation of singlet oxygen.[66] Three herbicides known to prevent the formation of carotenoids in plants were found also to cause destruction of α- and β-carotene under aerobic conditions in algae.[67]

Resistance to Herbicides - Genetic resistance of previously susceptible weeds to herbicides is a relatively recent phenomenon and confined almost entirely to fields with a long history of s-triazine applications, particularly of atrazine (29).[68] The subject was reviewed.[69] At least 10

weed species have been reported resistant to triazines.[70] The mechanism of atrazine resistance in common groundsel was found not to be differential metabolism, uptake, or translocation, but rather an alteration in the receptor at the site of herbicidal action on the thylakoid membrane of the chloroplast.[71] Two new bioassays were reported for ready detection of triazine resistance.[70,72] A mathematical model was derived for the development of herbicide resistance which explains the low incidence of resistance in terms of low selection pressure, decreased fitness of resistant strains, and other factors.[73] Although genetic resistance to herbicides other than s-triazines has not been confirmed, resistance to 2,4-D (33) and MCPA (34) was reported in Russia.[74]



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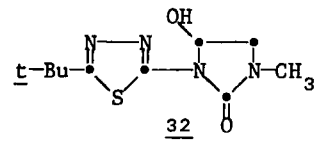

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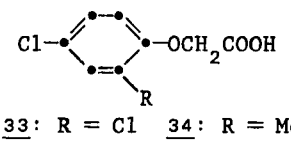


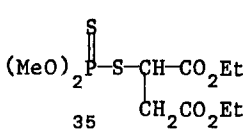
33: R = Cl 34: R = Me

Insect Control Agents

General - Although insecticides remain the number one means of preventing destruction of many crops, and of controlling vectors for a number of diseases, environmental aspects and the serious consequences of insect resistance are of major concern to all in the field of discovering and developing these materials. Much of the controversy relative to improper use and the plea for better management has been summarized.[75] The problem of resistance and prospects for its management was also reviewed.[76] An excellent overview of the status of insecticide development and thoughts on the future have been provided,[77] as well as a more general look at pesticide research.[78] Towards the goal of better understanding the target, an outstanding collection of papers dealing with the organization of the insect nervous system and its mediation by neurotransmitters and interaction with chemicals is available.[79] A review of advances in understanding the mode of action of insecticides has just issued.[80] A developing means of insect control is the use of pathogens, particularly Bacillus thuringiensis. Their use has been reviewed[81,82] and interest in new bacterial strains remains high.[83,84] The wide range of chemical types used as acaricides has been reviewed.[85] The anthelmintic avermectins show interesting insecticidal properties.[86]

Organophosphates - Although overshadowed by the recent rapid development of pyrethroids and interest in other areas of insect control, the organic phosphates as a class remain the most broadly used agronomic insecticides.[87] They vary widely in structure and in acute oral toxicity as represented by malathion (35, LD_{50} 2800 mg/kg),[88] phorate (36, LD_{50} 1.6-3.7 mg/kg),[89] and chlorpyrifos (37, LD_{50} 135-163 mg/kg),[90] among many in use.[91] A more recent useful addition to the OP's is acephate (38) which, by virtue of adding the acetyl group to the amide nitrogen of methamidophos (39), markedly reduces toxicity, without destroying insecticidal effectiveness.[92] Another recent discovery is the improved activity, especially against certain hard-to-kill Lepidoptera, of a series of O-aryl, O-alkyl, S-alkyl phosphates and phosphorothioates, as shown by sulprofos (40)[93] and profenofos (41).[94] Although the mode of toxic action of the OP's in insects is generally attributed to inhibition of acetylcholinesterase,[95] recent studies have suggested that other effects caused by uncontrolled release of neurosecretory hormones may contribute to death.[96] Delayed irreversible neurotoxicity, unconnected with acetylcholinesterase inactivation, is a serious side effect of some OP's in mammals and birds. Active research on improved detection and understanding of the neuropathy involved continues.[97,98]

Carbamates - The major insecticidal phenolic-type N-methylcarbamates are represented by carbaryl (42) and carbofuran (43).[87] As with OP's, these compounds function as acetylcholinesterase inhibitors and a recent QSAR



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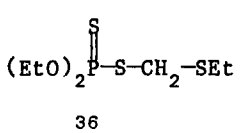

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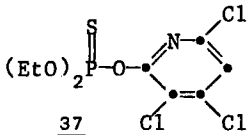

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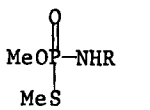
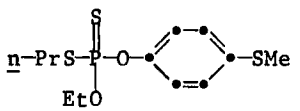


38: R = COMe 39: R = H



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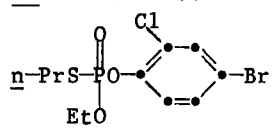
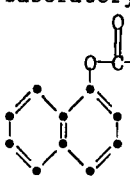


41

study of 269 substituted N-methylcarbamates using housefly head cholinesterase inhibition was reported.[99] The other important group comprises those derived from certain oximes, most importantly methomyl (44)[100] and aldicarb (45).[101] Improvements such as reduced mammalian toxicity, reduced phytotoxicity, better formulation properties, and longer residual action of a number of these products have resulted from appropriate derivatization at the carbamate nitrogen atom, e.g. carbosulfan (46)[102,103] and thiodicarb (47).[104] Much of this work has emanated from T. R. Fukuto's laboratory, and work there[105] and by others[106,107] continues.



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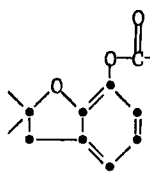
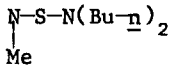
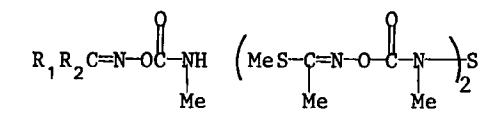
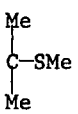


43: R = NHMe
46: R = 



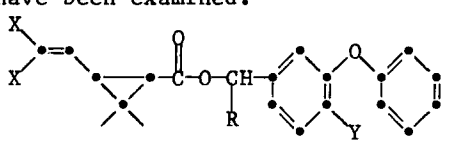
44: R_1 = Me, R_2 = SMe

47

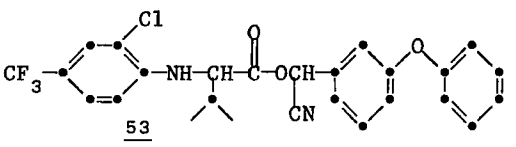
45: R_1 = H, R_2 = 

Pyrethroids - The odyssey begun by Staudinger and Ruzicka in the early part of this century in unravelling the key structural features of the natural pyrethrins reached a climactic episode in 1973 with the announcement by Elliott and coworkers of a synthetic analog, permethrin (48), sufficiently photostable and active to offer promise as an agronomic insecticide.[108] From this group rapidly followed cypermethrin (49) and decamethrin (50), the latter compound being a single crystalline isomer having the cis-(1R,3R) configuration for the cyclopropane ring and (S)-chirality at the cyanoalcohol locus.[109] Against many insects, decamethrin is the most potent insecticide ever synthesized.[109] All three compounds are in commercial use, attesting to the importance and rapid development of these new insecticides. An analysis of the structural features important for insecticidal activity was reported,[110] and the potential of the pyrethroids for insect control discussed.[111] New synthesis schemes required to provide the cyclopropyl acid intermediates on a large scale, including efforts at stereoselective syntheses and the search for some novel acids, have been reviewed.[112] Highly effective "non-cyclopropane" structural types, represented by fenvalerate (51),[113] flucythrinate (52),[114] and fluvalinate (53),[115] are in commercial use or undergoing development. As with the cyclopropanes, appropriate chirality is important for activity[116] and, in the case of flucythrinate, only the active (S)-acid is used in the final esterification.[114] Despite much synthetic effort, attempts to find light-stable active esters derived from alcohols other than the m-phenoxybenzyl structure common to compounds 48-53 have met with little success. Even substitution on the m-phenoxybenzyl

aromatic rings has been mostly unrewarding, although appropriate fluorine placement has afforded the active cypermethrin analog, FCR 1272 (54), which is being widely tested.[117] Also, replacing aromatic with heterocyclic rings has generally not worked to give esters with agronomic potential, an exception being Dowco 417 (55) which incorporates a pyridine nucleus.[118] A Hansch-type evaluation of structural parameters for meta-substituted benzyl esters of type 56 did reveal that phenyl (in place of phenoxy) substitution gave an ester about half as active as permethrin.[119] Among other efforts to exploit the structural features of the esters described, most notable has been the synthesis of a number of oxime-ethers of the type represented by 57.[120,121] The (E)-configuration shown is important to insecticidal activity and to pyrethroid-like physiological response on isolated nerves.[120] The potential build-up of resistance to pyrethroids is of real concern and mechanisms of resistance in house flies have been examined.[122]



48: X = Cl, Y = H, R = H
49: X = Cl, Y = H, R = CN
50: X = Br, Y = H, R = CN
54: X = Cl, Y = F, R = CN



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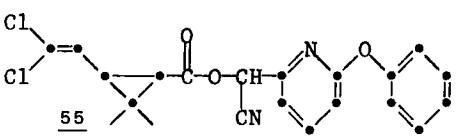

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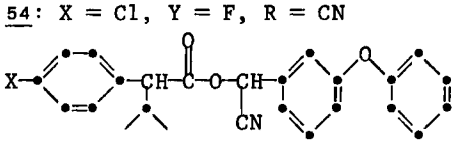
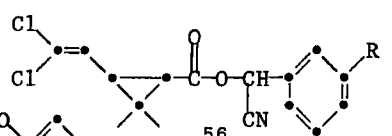


51: X = Cl
52: X = CHF_2O



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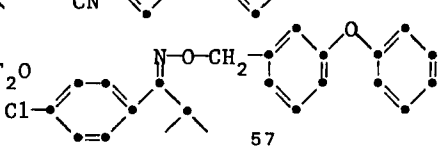
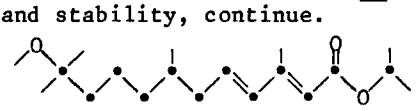


57

Juvenile Hormone Analogs (Juvenoids) - Intensive work has proceeded in many laboratories over the past 15 years aimed at finding compounds which mimic the action of natural juvenile hormones utilized by insects to prevent unregulated molting from immature to adult forms. However, for a variety of reasons, they have not reached the level of application anticipated and only one compound, methoprene (58), is fully registered in the U.S. Summaries of the activity spectrum of methoprene and other juvenoids and field experience with some of these are reported.[123,124] Despite the problems of inapplicability to the more destructive agronomic pests, reports of new, highly active compounds such as the ether (59)[125] and the amide RO 13-5223 (60),[126] which may offer advantages in activity and stability, continue.



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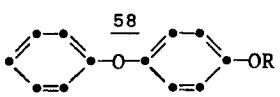
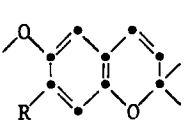
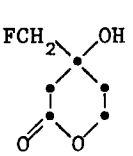


59: R = $CH_2C_6H_4$-4-Et

60: R = $CH_2CH_2NHCOOEt$



61: R=H, Precocene 1
62: R=MeO, Precocene 2



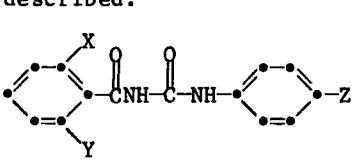
63

Anti-Juvenile Hormone Agents - In view of the importance of juvenile hormones to insects, disruption of their generation or utilization has become an attractive goal, particularly since Bowers sought out and discovered in plant material the potent anti-JH precocenes (61,62).[127] The mode of action, which involves in situ epoxidation of the precocenes in the

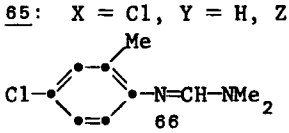
insect corpora allata, the source of JH, and subsequent rapid chemical degradation of the gland in certain insects, has been established.[128,129] Interference with the JH biosynthetic pathway, as currently understood[130] and described as a possible means of insect control earlier,[131] was demonstrated with fluoromevalonate (63) on Lepidoptera.[132]

Pheromones - Great strides have been made in recent years in recognizing, isolating, identifying, and synthesizing a wide range of insect pheromones with the objective of using them to monitor the build-up of insect populations. Much effort is being placed on their use for mating disruption with some reports of limited success, e.g., control of western pine shoot borer, Eucosma sonomana, in pine and of tussock moth, Orgyia pseudotsugata, in fir.[133] In the former case, the male sex attractant is a 4:1 mixture of (Z-) and (E-)-9-dodecenyl acetate; the latter is (Z)-6-heneicosen-11-one. The general state of the art relative to monitoring, mass trapping, mating disruption, and formulation has been discussed.[133] The potential of pheromone use in insect control has been appraised.[134] Structural features important for pheromone activity including geometric, positional, and optical isomerism, and the importance of blends, have been reviewed.[134,135]

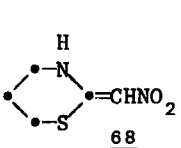
Other Types - The benzoylurea, diflubenzuron (64), is a recently developed commercial insecticide which exhibits larvicidal and ovicidal activity by affecting normal chitin development.[136] Since its discovery, a variety of analogs have been reported and broadly tested, e.g. SIR 8514 (65).[137] A recent report, which summarized earlier work by others, concludes that inhibition of chitin synthase is not the mode of action of the benzoylureas.[138] Another study supports this, but points to other known insecticides and fungicides which act in this manner.[139] The formamidine chlordimeform (66), noted for its unusual effects in controlling certain insect species and acarina, is a potent octopamine agonist (as the N-demethyl form).[80] A novel hydrazone, 67,[140] has replaced mirex as the major toxicant for control of fire ant. Although none of a series of insecticidally active but photochemically labile nitromethylene heterocycles represented by the especially potent thiazine (68) has been developed,[141] a cyanomethylene analog, SN 72129 (69), is undergoing field evaluation.[142] The benzimidazole EL-919 (70) and its precursor, EL-968 (71), are effective ectoparasitic agents.[143] The latter (as EL-468) is also reported active against fire ants and termites.[144] Efforts to find new insecticidal materials from natural products are proceeding in a number of laboratories. Modeling based on phenolic materials isolated from wood extracts has led to such potent mosquito growth inhibitors as 72.[145] Although not currently used in insect control, antifeedants are of considerable interest and a variety of naturally occurring types have been described.[146]



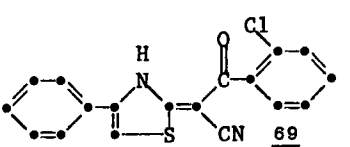
64: X = Y = F, Z = Cl
65: X = Cl, Y = H, Z = OCF_3



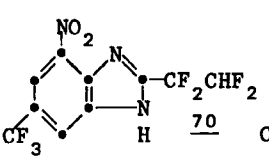
66



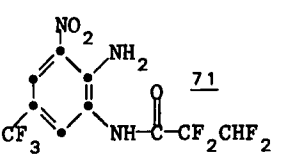
68



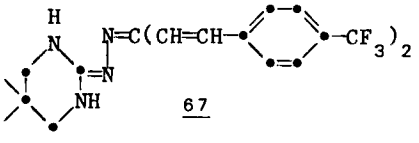
69



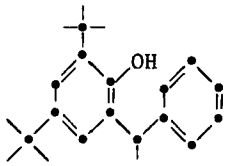
70



71



67



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Chapter 32. Nonnutritive Sweeteners. The Search for Sucrose Mimics.

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Introduction - The deleterious health effects of high level carbohydrate sweetener consumption have been cause for many to turn to nonnutritive sweeteners. In order to be of general utility, a nonnutritive sweetener should show high sweetness potency, taste quality which mimics sucrose, undisputed safety for human consumption, thermostability, hydrolytic stability, high and rapid water solubility, favorable economics, "body" or viscosity effects similar to sucrose, and noncariogenicity. Although safety is an ultimate concern, it cannot be overstated that a useful nonnutritive sweetener must be a very effective mimic of sucrose, the consumers standard. For this reason, this report will confine itself to classes of compounds of current interest which have a reasonably sucrose-like sweet taste and which are judged to have potential for sucrose replacement in food. The subject of design of nonnutritive sweeteners has been comprehensively reviewed.[1]

Sensory Evaluation of Experimental Compounds - A major difficulty in comparing compounds which are reported to be sweet is the lack of consistent methods of sensory evaluation. Sweetness potency (P), relative to a surose reference (R), is the most commonly reported descriptor for sweet compounds. It is strongly and inversely dependent on the concentration of R employed. Thus, for example, saccharin P estimates range from 190 to 675, the extremes being relative to 15% and 1.6% R, respectively.[2] In this review, P will be indicated relative to use levels (5-15%) of R whenever possible and reported as P(X)/R(Y) where X is the value of P and Y=%R.

Qualitative descriptors of a sweetener's taste are also commonly reported. Although a rigorous treatment of taste quality theory would consider taste as a continuous phenomenon,[3] it is experimentally useful to describe a taste stimulus as a combination of the four so-called primary tastes; sweet, sour, salty, and bitter. In most cases, compounds with sweet taste show only bitter taste as a secondary taste component, with the occasional presence of other taste qualities, such as menthol-like or anise-like. Thus, in order to provide an indication of taste quality, compounds will be additionally described, wherever possible, with a sweet to nonsweet (S/N) taste component ratio. By this method, sucrose, by definition, has S/N=100/0. Sensory analytical procedures[4,5] for obtaining such potency and S/N estimates, as well as their application,[6-8] have been reported.

Sucrose taste is characterized by a very rapid onset or appearance time (AT), followed by a rapid disappearance or extinction time (ET). For many compounds, this is not the case. It has been suggested that a compound's temporal sensory properties are determined by how rapidly it forms "orderly queues" at the taste receptor (AT), followed by how rapidly it passes through these queues (ET).[9] Alternatively, it has been suggested that more than one type of receptor exists for sweet substances, some of which result in sucrose-like temporal properties and some of which result in delayed AT and long ET sweet taste.[10] Sensory analytical procedures for estimation of AT and ET in experimental sweeteners have been reported.[7,11,12] Very recently, a much simplified procedure was described for measurement of

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AT and relative ET, the latter being defined as the time required for disappearance of sweet taste once a maximum intensity has been reached.[13] Sucrose was determined to have AT=4s and ET=14s by this method. In summary, therefore, in order to judge the utility of an experimental sweetener, it is necessary to have estimates of sweetness potency [P(X)/R(Y)], taste quality (S/N), and temporal taste properties (AT,ET). It is unfortunate that such information is available for only very few sweet compounds described in the literature.

Sensory evaluation of experimental compounds using human panelists has doubtless been a concern for some workers. Recently, an in vitro method of sweetness potency estimation was suggested.[14] Antibodies raised to the protein sweetener thaumatin were found to exhibit cross-reactivity to other sweet compounds in a manner quantitatively consistent with potencies determined by human sensory studies.

Sweet Taste Mechanism - Current knowledge on this subject has been reviewed.[1,15,16] In essence, it is believed that the sweet taste response is induced following efficaceous interaction of an active substance with a receptor protein located on the external periphery of a taste cell. Such binding causes taste cell depolarization and initiation of a coded sweet taste signal to the brain.

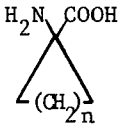
The puzzling diversity of structure which exists among sweet tasting compounds has made difficult the formulation of a general SAR. The most commonly quoted theory, which assumes binding of all sweet compounds to common receptor functionality, states that all sweet compounds contain A-H and B hydrogen bonding groups, where the H and B atoms are 2.5-4.0 A° distant.[17] This theory was expanded for the case of high potency sweeteners to include a third binding site (X) located 3.5 A° and 5.5 A° from the A and B atoms, respectively.[18] The generality of this A-H/B/X theory unfortunately renders it of little value. Innumerable nonsweet compounds are known which conform to the A-H/B/X formula. Additionally, for many sweet compounds, such as perillartine and nitroaniline sweeteners, it is not possible to find the A-H site in any sensible manner; for others, such as the dihydrochalcones, multiple A-H/B units are possible. Thus, rationalization of the sweet taste of the many classes of sweet compounds by the existence of a common essential pharmacophore is not possible.

A more reasonable approach to the rationalization of common activity of the structurally diverse sweetener classes is the suggestion of multiple receptors. Arguments in favor of this concept have been reviewed.[1,19] Electro-physiological studies with the fruitfly Drosophila melanogaster have added support to the multiple receptor concept,[20] as do gerbil behavioral studies.[21] The results of human psychophysical studies, employing multidimensional scaling methods, also support the existence of multiple receptor sites.[22,23] Biochemical studies on human circumvallate tissue preparations suggest that most, if not all, sweet taste receptors may be on the same protein.[24] A model of the sweet taste receptor consistent with all experimental information, has been described[25] in which many dissimilar binding sites are suggested to exist in the hydrophobic α-helical region of a cell membrane bound receptor protein. Binding at any of these sites may induce a protein conformational change and initiation of the sweet taste response.

The existence of multiple receptors for sweet substances complicates the task of sweetener design. It is reasonable, however, that members of the same class of sweeteners may bind to the same receptor functionality. Thus for the following discussion, sweeteners will be grouped in classes that are likely to be interacting with a common receptor site.

Structural Classes of Sweeteners of Current Interest

Amino Acids - The sweet achiral cyclic amino acids 1 (n=1-8) were recently reported;[26] maximum potency [P(5-15)/R(0.6)] was observed for the six-membered ring congener (n=3). D-trytophan (2) is the most potent of the simple amino acids; 6-chloro-D-trytophan (3) is dramatically more potent.[27] N'-Acetyl kynurenine (4), a tryptophan metabolite, is also sweet [P(35)],[28] as is the D,L-chloro derivative 5 [P(73)/R(8)].[29]



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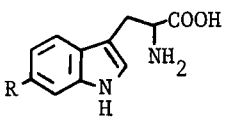
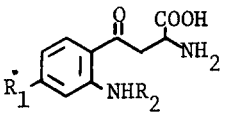
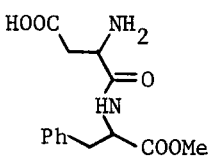


2, R=H
3, R=Cl

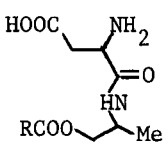


4, R_1=H, R_2=Ac
5, R_1=Cl, R_2=H

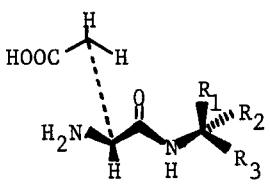
Dipeptides and Related Compounds - The dipeptide aspartame (6) was very recently approved for food use in the U.S. Aspartame exhibits a sweetness of high potency [(P(130)/R(10)] and quality (S/N=100/0),[30] with sucrose-like temporal properties (AT=5s, ET=19s).[13] It is, therefore, an excellent sucrose substitute. Solution stability, however, due to diketopiperazine formation, is a limitation. Analogs 7 [P(220)] and 8 [P(240)] have been



6



7, R=c-C_4H_7
8, R=t-Bu

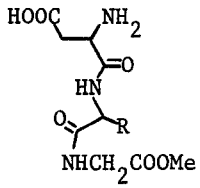


9, R_1=COOMe, R_2=H, R_3=CH_2Ph
10, R_1=H, R_2=CH_2Ph, R_3=COOMe

suggested as alternatives to overcoming this problem.[31] An improved method of preparation for aspartame, involving coupling L-CBZ-Asp to L-Phe-OMe by means of immobilized thermolysin was recently reported.[32] The active conformation of 6 has been suggested to be 9, based on potential energy calculations, nmr measurements, and SAR information from other types of sweet compounds.[33] The same authors have attempted to show how a receptor site having topography consistent with 9 may also efficiently interact with saccharin, oxime, nitroaniline, and oxathiazinone dioxide sweeteners.[34] In later work,[35] 10 was suggested to be a much more likely active conformer based on SAR studies in a range of sweet L-isoasparaginine derivatives. Conformer 10 was also suggested by QSAR studies employing steric substituent parameters.[36] Very recently, using a very much larger selection of L-isoasparaginine sweeteners, QSAR studies suggested 9 to be the active conformer.[37]

SAR studies on a series of o-, m-, and p-hydroxy and methoxy phenyl substituted analogs of 6 underline the importance of steric constraints in receptor binding of dipeptide sweeteners.[38] All were less sweet except for the ortho analogs, a fact which was rationalized by steric inhibition of receptor binding. The suggestion[37] that the N-H of 6 is involved in specific receptor interaction is supported by the fact that the ester, N-methyl, and C=O/N-H inverted analogs all lack sweet taste.[39] Although steric factors and certain binding functionality appear to be essential for efficaceous receptor binding, net hydrophobicity also appears to be a factor. A series of tripeptides 11 (R=Me,Et,i-Pr) has been reported, the members of which appear to contain all the requisite functionality for sweet taste.[40] Very low sweetness potencies [P(0-3)] were observed for these compounds. This observation was explained by excessive hydrophilicity. In aspartame, the conformation of the L-Asp moiety is restricted by formation

of a hydrogen bonded six-membered ring. For this reason, rigid aspartame analogs 12 (X=CONHNH, CH_2NHNH, COCONH, CH_2CONH, CH_2CH_2NH, $(CH_2)_3$) were evaluated.[41] All lacked sweet taste. Interestingly, it has been found that L-Ala-L-Phe-OMe, which is not sweet, exhibits strong sweetness in the presence of acetic acid.[42] Presumably, the latter provides, externally, the necessary carboxyl binding functionality endogenous in 6.



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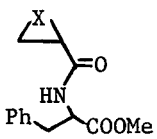

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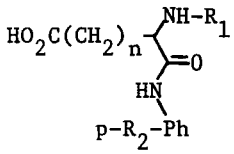
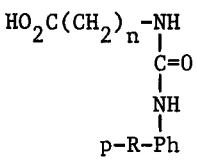
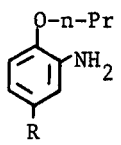


13, n=1, R_1=$COCF_3$, R_2=CN
14, n=1, R_1=$COCCl_3$, R_2=CN
15, n=1, R_1=$COCF_3$, R_2=NO_2
16, n=1, R_1=$COCHCl_2$, R_2=NO_2
17, n=1, R_1=$COCCl_3$, R_2=NO_2
18, n=1, R_1=$COCH_3$, R_2=NO_2
19, n=2, R_1=$COCF_3$, R_2=CN
20, n=2, R_1=$COCF_3$, R_2=NO_2

A group of compounds which appear related to the dipeptide sweetners are L-acylamidosuccinanilic acid derivatives. A substantial amount of SAR work has been carried out around 13 [P(3000)/R(2)] and 14 [P(3000)/R(2)].[43] Potent sweet taste was observed in the nitro analogs 15 [P(100)/R(2)], 16 [P(50)/R(2)], and 17 [P(3000-4500/R(2)].[44] The importance of halogen in these compounds is indicated by the absence of sweet taste in 18 and the different potency of 16 and 17. L-Glutamyl can be substituted for the L-aspartyl moiety without loss of potency (19, [P(3000)/R(2)]; 20, [P(100)/R(2)]).[45] As is true in the case of dipeptide sweeteners, the central amide moiety appears to be essential for sweet taste. Substitution of NHNH for NH in 13-18 eliminates sweet taste.[46]



21, R=NO_2
22, R=CN



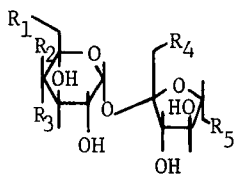
23, R=CN, NO_2

Suosan (21) [P(700)/R(2)] and 22 [P(450)/R(2)], its cyano analog, are potent aromatic urea sweeteners which appear structually related to 13-20.[45] Some general conclusions have been drawn with respect to the SAR of these and other sweet compounds (e.g. 23).[47]

Proteins - Thaumatin is the sweet proteinaceous principle of the West African plant Thaumatococcus daniellii. This material has been found to be a mixture of at least five sweet proteins.[48,49] The major protein, thaumatin I [P(3000)/R(10)][14] is a 207 amino acid polypeptide (M=22,209) with eight disulfide bridges and an isoelectric point of ca. 12.[50] Reduction of the disulfide bridges with dithiothreitol caused autodigestion and elimination of sweet taste.[51] Sequential acetylation resulted in proportional decrease of the isoelectric point, with concommitant decrease in sweetness potency. The thaumatin proteins were recently suggested to originate biochemically from potently sweet glycoproteins isolated from the same plant source.[52]

Monellin, the sweet principle [P(2000)/R(10)][14] of the West African plant Dioscoreophyllum cumminsii, is a protein (M=10,700) containing 94 amino acids. It consists of two nonidentical polypeptide chains linked noncovalently. It has been reported that antibodies raised to thaumatin I show cross reactivity with monellin.[14]

Carbohydrates - Potently sweet analogs (24-28) were obtained by substitution of the hydroxyl groups in sucrose by chloro groups.[53,54] A study of the effects of chloro substitution in other carbohydrates has been reported.[55] Significant sweet taste was found only in 1,6-dichloro-1,6-deoxy-β-D-fructose. It was suggested to be the essential pharmacophore in 24-28. Analogs 29-41 in which the sucrose hydroxyl groups were replaced by other lipophilic groups, surprisingly lacked significant sweet taste. Thus the Cl groups of 24-28 are apparently involved in a very specific receptor interaction.

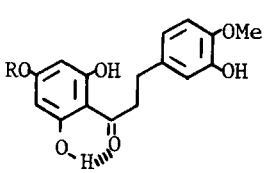


Cpd	R_1	R_2	R_3	R_4	R_5	Taste Char.	P(X) R(5)
24[a]	Cl	Cl	H	OH	Cl	d	10
25[b]	OH	Cl	H	Cl	Cl	d	500
26[a]	OH	H	OH	Cl	Cl	d	100
27[b]	Cl	Cl	H	Cl	Cl	d	200
28[c]	OH	H	Cl	Cl	Cl	d	100
29[c]	OH	H	OMe	OMe	OMe	e	1
30[c]	OH	H	OH	OMe	OMe	e	1
31[c]	OH	H	H	H	H	f	0
32[c]	OH	N_3	H	N_3	N_3	e	30
33[c]	OH	H	OH	N_3	N_3	e	15
34[c]	OH	H	OH	OCOt-Bu	OCOt-Bu	g	--
35[c]	OH	H	OCOt-Bu	OCOt-Bu	OCOt-Bu	f	0
36[c]	OH	H	OH	OCOEt	OCOEt	g	--
37[c]	OH	H	OH	OCOc-C_6H_{11}	OCOc-C_6H_{11}	f	0
38[c]	OH	H	OH	OCOi-Pr	OCOi-Pr	g	--
39[c]	OH	H	OH	OCOPh	OCOPh	e	--
40[c]	OH	H	OH	O-n-Pr	O-n-Pr	f	0
41[c]	OH	H	OH	i-Am	i-Am	g	--

(a) Ref. 53, (b) Ref. 54, (c) Ref. 56, (d) sweet, (e) bittersweet, (f) tasteless, (g) bitter

Sulfamates - The SAR in the structurally simple sulfamate ($RNHSO_3M$) class of sweeteners has been summarized.[57] In general, a free N-H and a hydrogen atom attached to the α-carbon are required for activity. This has been interpreted to indicate that the dihedral angle separating N-H and S-O moieties must be ca. 60°, since full α-carbon substitution causes a compression of this angle.[58] Sweet sulfamates must also contain an R group having dimensions <0.7 nm and >0.5 nm.[59] The upper size requirement was interpreted to suggest the presence of a spacial barrier >0.7 nm from the N-H binding site, while the lower size requirement was interpreted to indicate a minimum amount of requisite hydrophobic interaction. A simple semi-quantitative method of correlating sulfamates SAR has been proposed.[60]

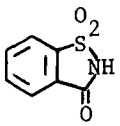
Cyclamate, the best known of the sulfamate sweeteners, exhibits high sweetness quality (S/N=94/6), but only modest potency [P(22/R(10)].[30] Sucrose-like temporal properties (AT=4s, ET=14s) explain its success in food applications.[13] Metabolism of cyclamate to cyclohexylamine is believed responsible for its observed toxicity. Since cyclamates of ring size >6 are also sweet, investigation of their metabolism was carried out.[61] Disappointingly, cyclooctyl and cycloheptyl sulfamates were both metabolized to a greater extent than cyclamate. Recently, 2-norbornyl sulfamic acid sodium salt was reported to be 100 times as potent as sucrose.[62]



42, R=$CH_2CH_2NHSO_3Na$

A new method of amine sulfamation, compatible with labile functionality, has been reported.[63] The potently [P(350)/R(10)] and cleanly (S/N= 89/11) sweet dihydrochalconesulfamic acid conjugate 42 was thus prepared.

Isothiazolone Dioxides and Oxathiazinone Dioxides - The SAR of these heterocyclic compounds has been reviewed.[64] The most common isothiazolone dioxide is saccharin (43). Although it has minimally acceptable taste quality (S/N=85/15),[8] saccharin is a highly potent [P(300/R(10)][8] sweetener with sucrose-like temporal properties (AT=4s, ET=14s).[13] The thiophene analogs 44 [P(350)/R(3)][67], 45 [P(1000)][65], and 46 [P(1000)][65] have been reported to be pleasantly sweet.[65,66] Recently, the furan analog 47 [P(550)/R(3)] and the dimethyl thiophene analog 48 [P(1050/R(3)] were



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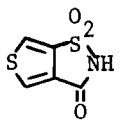

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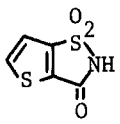

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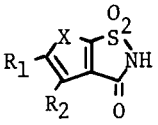
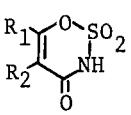


44, X=S, R_1=R_2=H
47, X=O, R_1=R_2=H
48, X=S, R_1=R_2=Me

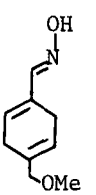
also reported to be potent sweeteners.[67]

Acesulfam (49) is the most studied member of the oxathiazinone sweeteners.[68] In a recently described series of analogs (50-59), increased potency with chloro substitution was observed (cf. 50, 57).[69] The approximate equivalence of Cl and N_3 in enhancing sweetness (cf. 50, 56, 57) is noteworthy since smilarly modified sucrose derivatives were not sweet.



Cpd	R_1	R_2	P(X)/R(4)
49	Me	H	130
50	$ClCH_2$	H	150
51	$BrCH_2$	H	150
52	$MeOCH_2$	H	50
53	$EtOCH_2$	H	0
54	$AcOCH_2$	H	0
55	$HOCH_2$	H	50
56	N_3CH_2	H	150
57	Me	Cl	200
58	Me	Ac	0
59	Me	COOEt	0

Oximes - Since the exhaustive SAR work which culminated in the discovery of oxime 60 [P(225)/R(8.6); S/N=90/10],[6] additional work has been sparse. Oxa-and thia-analogs 61 and 62 were recently described, the latter of which was indicated to be "slightly sweet".[70] QSAR correlations for this class of sweeteners have been described.[71] Molecular connectivity methods were ineffectively applied to a small sample of oximes to attempt qualitative discernment of compounds which are primarily sweet from those which are primarily bitter.[72]



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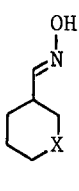
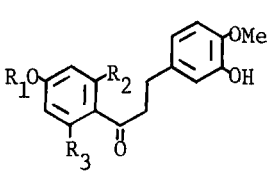


61, X=O
62, X=S

Dihydrochalcones and Isocoumarins - Nearly 200 analogs of the naturally derived dihydrochalcone (DHC) sweetener, neohesperidin DHC (63),[73] have now been prepared. The poor temporal taste properties of 63 (AT=9s, ET=40s) provide the impetus for most of this work.[13] Major recent contributions to the SAR of DHCs include the discovery that the carbohydrate functionality (cf. 64, 65) and the o-hydroxy ketone moiety (cf. 64, 66, 67) are important for activity. It was recently hypothesized that the "atypical" temporal properties of 63 may be due to metabolism, conforma-



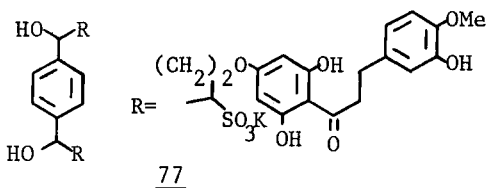
Cpd[a]	R_1	R_2	R_3	P(X)/R(4)	S/N	Ref.
63	β-Neohesperidosyl	OH	OH	340/8.6	77/23	7
64	CH_2COONa	OH	OH	501/8.6	82/18	74
65	$(CH_2)_3SO_3K$	OH	OH	386/8.6	77/23	75
66	CH_2COONa	H	OH	63/8.6	42/58	74
67	CH_2COONa	H	H	0	---	74
68	$(CH_2)_3PO(OH)(OK)$	OH	OH	280/8.6	80/20	7
69	CH_2COCH_2COONa	OH	OH	310/8.6	85/15	7
70	$(CH_2)_2CHOHCOONa$	OH	OH	440/8.6	92/8	7
71	$(CH_2)_2CHNH_2COOH \cdot HCl$	OH	OH	400/8.6	85/15	76
72	βDGa2(α1LR)	H	OH	2.0[b]	---	77
73	βDGa2(α1LR)	H	H	0.002[b]	---	77
74	βDGu	OH	OH	0	---	77
75	$CH(COOH)CH_2CH_2COOH$	OH	OH	0	---	74
76	$(CH_2)_3SO_3Na$	H	OH	178/8.6	81/19	75

(a) Ga=galactopyranosyl, R=rhamnopyranosyl, Gu=glucopyranuronosyl; (b) times saccharin

tional effects, chelation, or hydrophobic effects.[7] Four new potently sweet compounds (42, 68-70) further illustrate the general nature of the groups which may be substituted for the carbohydrate moiety of 63. None of these compounds showed improved temporal sensory properties, however. It has been suggested that DHC sweeteners interact with two receptor systems, one similar to the sucrose receptor and another which results in atypical temporal properties.[7,10] A homoserine-DHC conjugate (71) was recently reported to exhibit potent, high-quality sweet taste, with significantly improved temporal properties (AT=8s, ET=29s).[13,76] An improved preparation of D,L-71 has been described.[78] D and L isomers of 71 exhibit similar sensory properties.[79] The conclusion that a minimum of one A-ring hydroxyl is required for sweet taste in DHCs was supported by similar studies on carbohydrate substituted DHCs (cf. 72, 73). Inspection of the potently sweet compounds 63-66 and 68-72 suggests that the DHC R_1 moiety may be any polar group. This is generally true, as long as polarity does not exceed a certain limit (cf. 74, 75).

The simplified sulfopropyl DHC 76 has recently been strongly advocated for use in food systems.[80] However, the finding that it is less than half as potent as 65, without improvement in quality or temporal properties, places its utility in doubt.[75]

Safety of nonnutritive sweeteners has received much publicity in recent years. A unique approach to this problem is to render the sweetener nonabsorbable through the GI tract wall, as is exemplified by the sweet "dimeric" DHC 77.[81,82] Although unusually long taste AT and ET prevented detailed sensory evaluation of 77, the low absorption (1.2%) illustrates the principle of this approach.



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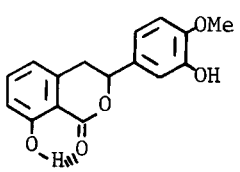

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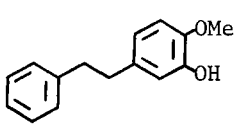

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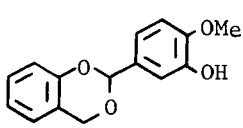
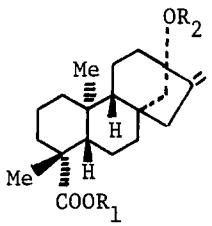


80

A great number of analogs of the dihydroisocoumarin, phyllodulcin (78), the sweet principle of Hydrangea macrophylla, have been evaluated. An efficient synthesis of 78 has been described.[83] The results of SAR studies on isocoumarin type sweeteners have been reviewed.[84,85] Dihydrostilbene 79 [P(300)/R(3)] appears to contain the minimum functionality for potent sweet taste. The most potent dihydroisocoumarin reported is acetal 80 [P(3000)/R(6)].[86] Utility is limited, however, due to poor solubility and stability [$t_{1/2}$(100°C)=3 min].

Terpenoids - Stevioside (81), a diterpenoid glycoside, is the major sweet component of the Paraguayan shrub Stevia rebaudiana (Bertoni). It exhibits a sucrose-like taste onset, but a significant sweet aftertaste (AT=4s, ET=22s).[13] Several new sweet glycosides, including rebaudioside-A(82),[87] -C(83),[88] -D(84),[88] -E(85),[88] and dulcoside A(86),[89] have been isolated from the same plant. The sweet principle of the leaves of Rubus chingii (Hu) has been identified and called rubososide (87).[90] The recently reported potently sweet synthetic glycosides 88-90 further illustrate the absence of defined structural requirements for the carbohydrate portions of 81. The exocyclic methylene moiety appears to be essential for potent sweetness, since hydrogenation of 81 yields a dihydro compound of much reduced activity



Cpd[a]	R_1	R_2	P(X)/R(Y)	S/N	Ref.
81	βDG	βDG2(β1DG)	190/10	62/38	8
82	βDG	βDG2(β1DG)3(β1DG)	170/10	85/15	30
83	βDG	βDG2(β1LR)3(β1DG)	30	---	89
84	βDG2(β1DG)	βDG2(β1DG)3(β1DG)	221/5.5	---	92
85	βDG2(β1DG)	βDG2(β1DG)	174/4.4	---	92
86	βDG	βDG2(β1LR)	30	---	89
87	βDG	βDG	150/10	69/31	30
88	βDG2(α1LR)	βDG2(β1DG)	300/0.6	---	91
89	βDGa2(α1LR)	βDG2(β1DG)	300/0.6	---	91
90	βDG2(αLQ)	βDG2(β1DG)	225/0.6	---	91
91	$(CH_2)_3SO_3Na$	βDG2(β1DG)	160/10	92/8	8
92	$(CH_2)_3SO_3Na$	$(CH_2)_3SO_3Na$	110/10	18/82	8

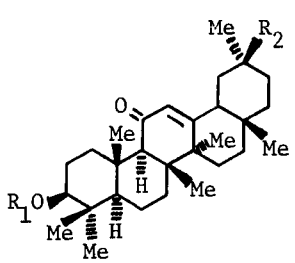
(a) G=glucopyranosyl, Ga=galactopyranosyl, R=rhamnopyranosyl, Q=quinovopyranosyl

[P(30)/R(0.6)].[91]

A process has been described for preparing oligomeric glucose conjugates of 81 by incubation with starch and an α-glucosyl transferase enzyme.[93] This material is reported to be free of the bitter taste of 81, although temporal sensory properties (AT=6s, ET=29s)[13] include a significant sweet aftertaste. Oligomerization proceeds by exclusive attachment to the 4-OH moieties of the terminal Glu units of the carbohydrate sidechain.[92] Interestingly, the three oligomer mixture resulting from conjugation of two Glu units [P(138)/R(6.9)] and the four oligomer mixture resulting from conjugation of three Glu units [P(131)/R(6.6)] retain high sweetness potency.

It has been determined that 81 is completely broken down to its aglycone, steviol, when incubated with rat cecal contents, conditions which simulate those of the human GI tract.[94] The aglycone thus formed is rapidly absorbed. Since steviol toxicity is in doubt, stable stevioside analog 91 was prepared.[8] This compound exhibited high sweetness potency and surprisingly high quality sweet taste, with a sucrose-like taste onset and only a slightly prolonged aftertaste (AT=6s, ET=25s).[13] Thus 91 appears to possess the sensory properties required for an acceptable sucrose substitute. It is of interest that the carbohydrate functionality of 81 is apparently unnecessary for sweet taste, since the bis-sulfopropyl analog 92 also exhibits some sweet taste.

Glycyrrhizic acid (93), the sweet triterpenoid glycosidic constituent of licorice root (Glycyrrhiza glabra), is currently in use as a sweetener and sweetness enhancer as the water soluble monoammonium salt [P(33)/R(10); S/N=84/16].[30] This compound exhibits a taste very slow in onset and with a very long aftertaste (AT=16s, ET=69s).[13] Attempts to improve its temporal



Cpd[a]	R_1	R_2
93	βDGu2(β1DGu)	COOH
94	βDG2(β1DG)	COOH
95	βDG2(α1DG)	COOH
96	βDGa2(α1LR)	COOH
97	βDG	COOH
98	βDGu	COOH
99	βL	COOH
100	αDGu	COOH
101	βDG	COOMe
102	H	CH_2OβDG

(a) Gu=glucopyranuronosyl, G=glucopyranosyl, Ga=galactopyranosyl, R=rhamnopyranosyl, L=lactosyl

properties by addition of guanosine 5'-monophosphate, inosine 5'-monophosphate and arabinogalactan, all of which have been reported to reduce sweet aftertaste, were unsuccessful. Preparations of 94-96 have been reported, all of which were described as "slightly sweet" relative to 93.[95] Analogs 97-102 have also been reported.[96] Qualitative sensory data indicates 97, 98, 100, 102, and 94 to be roughly equivalent in potency with 93, while 99 was weakly sweet and 101, tasteless. The 11-deoxo analog of 97 was reported to be equivalent to 93 in potency.

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CHAPTER 33. DRUG METABOLISM

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Introduction - The drug metabolism chemist is usually concerned with only three major reaction pathways; these are oxidative, conjugative and hydrolytic processes. The vagaries of Mother Nature determine the sequence and combinations of these three reactions which are responsible for the vast majority of biotransformations involving drug substances. These biological reactions, in conjunction with the kinetics of the processes involved and the development of the technology needed to measure these products, have expanded the literature of drug metabolism at an increasing rate.

The relationship between toxicity and drug metabolism is of continuing interest,[1] with the role of the liver[2] and the effect on the fetus[3] receiving attention. A two-part text[4] and the fifth volume of the series Progress in Drug Metabolism[5] have been published. Two recent books relate drug metabolism and disposition with pharmacokinetics[6] and drug absorption.[7] Volumes on conjugation reactions with glucuronic[8] and sulfuric[9] acid have been published.

Pharmacokinetics - Two recent books of general interest have appeared; one is clinically oriented[10] and the other is a small, useful handbook.[11] Alternative methods for estimating pharmacokinetic parameters based on direct linear plotting,[12] linear system analysis[13] and zero-order absorption with first-order elimination processes[14] have been described. Simplified equations can determine the steady state volume of distribution[15] and can use the steady state pharmacological response to constant rate i.v. infusion to estimate the pharmacokinetic parameters.[16] Michaelis-Menten kinetics have been examined in relation to both nonlinear equations for first-pass effects[17] and the difficulties of fitting this model to observed plasma concentrations.[18] Model-independent approaches to define the total volume of distribution after i.v. administration of drugs[19] and to deal with variations in both size and interval of dose in a variable dosing regimen[20] have been reported. There are new methods to evaluate bioequivalence of drugs with changing half-lives[21] and to assess the acceptability of two comparable drug formulations.[22] Multiple blood sampling influenced the pharmacodynamics of dicumarol in small animals[23] and the use of the cut end of the rat tail for blood sampling may introduce considerable errors in the estimation of pharmacokinetic parameters because of the low tail blood flow.[24] In addition, the bioavailability of indomethacin in humans[25] and plasma salicylate levels in the rat[26] were shown to undergo circadian changes. The effects of antacids on the bioavailability of diflunisal[27] and theophylline[28] have been reported. Urinary pH dramatically affects the excretion of both meperidine and its metabolite, normeperidine, but has a negligible effect on the blood concentration.[29] The elimination half-life of phenylbutazone was significantly shorter in malnourished males, and the apparent volume of distribution was higher, as compared to normal male subjects.[30]

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The absorption rate constant for intramuscular amikacin appeared inversely proportional to dose as a result of changes in the volume at the injection site.[31] After i.v. infusion, ceforanide, a semisynthetic cephalosporin, gave peak plasma levels and apparent volumes of distribution which were independent of creatinine clearance; mean half-life was 3 hr in normal subjects and increased to 25 hr in patients with severe renal deficiency.[32] Orally administered nalidixic acid was rapidly absorbed and oxidized to 7-hydroxynalidixic acid which was excreted in urine with an apparent elimination half-life of 6-7 hr; another metabolite of nalidixic acid, 7-carboxynalidixic acid, was not detected in plasma, but accounted for 25% of urinary recovery.[33]

Cimetidine was 76% bioavailable after oral administration, while the volume of distribution, total body clearance and renal clearance were 1.39 L/kg, and 655 and 475 ml/min, respectively, after i.v. administration.[34] Plasma half-life of cimetidine was lengthened in renal failure and the effective dose was dependent on the creatinine clearance values.[35]

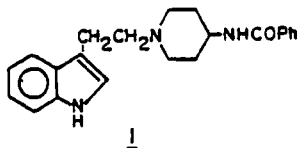
After an infusion, the plasma concentration of nitrofurantoin was described by a two-compartment body model with a terminal half-life of 58 min; bioavailability of a tablet form was not affected by feeding.[36] The intravenous pharmacokinetics of nitroglycerin were variable with a terminal elimination half-life from plasma of about 3 min and an apparent volume of distribution which exceeded the body mass.[37] Mexiletine, an antiarrhythmic agent, was eliminated from the bloodstream in a tri-exponential mode with a terminal half-life of about 6 hr.[38] In bioavailability studies of 11 commercial quinidine products, the terminal half-life was 7.5 hr, and the total body clearance was estimated at 4.2 ml/min/kg.[39] After i.v. administration to a group of hypertensive patients, prazosin had a β-half-life of 3 hr and an apparent volume of distribution of 0.6 L/kg.[40]

A one-compartment body model with first-order absorption described the kinetics of desipramine in plasma over the range of 75-150 mg.[41] After i.v. administration of chlorpheniramine, the elimination half-life in children was 9.6 hr, shorter than in adults, probably due to higher body clearance.[42] There was no correlation between the area under the plasma concentration vs. time curve and the amount excreted in the urine for a group of thiazide diuretics.[43]

Analytical Methods - Two recently published books[44,45] present general discussions of modern analytical techniques and their application to the study of drug metabolism. A renewed interest in whole body autoradiography has emerged in a recent book[46] and the ability of the technique to discern differences in tissue radioactivity compared favorably with that of quantitative scintillation methods.[47]

The technique most broadly applicable to drug analyses remains high pressure liquid chromatography (HPLC), with hundreds of specific applications appearing in the literature each year. The need for time-consuming sample extraction is eliminated in both manual[48] and automated[49] techniques involving direct injection of plasma, without compromising column integrity. Both pre-[50] and post-column,[51] on-line hydrolytic techniques have also been described. Column switching,[52] the use of ternary mobile phases,[53] and the application of gradient HPLC, both to routine analysis[54] and the rapid selection of optimal isocratic conditions,[55] have resulted in dramatic improvements in selectivity.

Although the use of ultraviolet detectors in HPLC is ubiquitous, the continuing search for greater sensitivity and selectivity has fostered many new developments. Fluorometric techniques have yielded sensitivities in the low ng/ml range in plasma assays for verapamil,[56] indoramin (1),[57] and, with post-column base treatment for enhancement of fluorescence, for warfarin.[58] The high sensitivity and specificity of electrochemical detectors has led to their expanding popularity; recent examples include determination of sulfinalol,[59] imipramine,[60] and a large number of phenothiazines.[61] The novel use of a dropping mercury electrode as a reductive amperometric detector could have broad applicability.[62] Considerable effort continues to be expended on various techniques for interfacing a liquid chromatograph with a mass spectrometer. Several moving-band devices have been described.[63 64] The optimal situation, however, would allow for direct introduction of the chromatographic effluent without stream splitting, a condition which is currently being approached through application of micro-HPLC,[65 66] with flow rates of <20 µl/min. The routine use of HPLC radioactivity monitors for metabolite isolation and interspecies metabolic profiling has appeared in studies of fluproquazone (2)[67] and purine metabolism;[68] this application could undergo explosive growth within the next few years.



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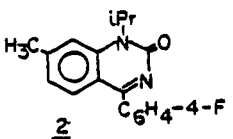

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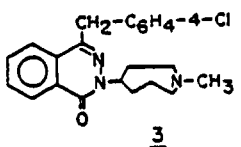

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Gas-liquid chromatography (GLC) continues to be used for the determination of many drugs, as exemplified by lidocaine.[69] Recent reviews have described use of retention indices[70] and a twin-column technique with nitrogen sensitive detectors[71] for identification and quantitation of a large number of pharmaceuticals. The coupling of GLC with mass spectrometry to produce exquisitely sensitive and specific methods has been the subject of two recent books containing discussions of drug metabolism.[72 73] Through use of GC-MS, commonly with select-ion monitoring and deuterated analogs as internal standards, assay sensitivities into the low picogram range are achievable. Recent examples include determination of flurazepam,[74] timolol,[75] and azelastine (3).[76]

In instances where an extremely sensitive assay is required, especially for thermally unstable compounds such as flunisolide,[77] immunoassay represents an alternative to mass spectrometry. The two techniques have been compared in a recent review.[78] The traditional radioimmunoassay (RIA) using ^{125}I- or ^{3}H-labeled substrates remains in broad use; however, considerations of stability, safety, and equipment simplicity have fostered many ingenious variations. The fluorescent immunoassay has been reviewed.[79] Propranolol antiserum was bound to magnetizable (cellulose/iron oxide) particles and used in an immunoassay with fluorescent-labeled propranolol, permitting easy separation of free and bound antigen with a magnet,[80] while coupling of antibody to a solid-phase has been used for analysis of tobramycin in plasma.[81] In a fluorescence enzyme immunoassay, betamethasone was linked to β-galactosidase,[82] and in a similar chemiluminescence assay, dehydroepiandrosterone was bound to horseradish peroxidase;[83] in each example, enzyme activity remaining in solution is a sensitive measure of antigen present.

In a few instances, the selectivity or sensitivity provided by the techniques described above are not necessary. Solution fluorometry has been applied to analysis of fluproquazone[84] and various tetracyclines,[85] without chromatographic separation. High performance thin-layer chromatography has recently been reviewed[86] and, while lacking sensitivity, applied to the analysis of metronidazole in serum.[87] Centrifugal TLC has been used for preparative scale separation of complex biological mixtures.[88]

Drug Disposition - The consequences of polymorphic drug oxidation on drug usage and the implications for new drug development have been discussed.[89] An animal model of oxidative drug metabolism for the human extensive and poor metabolizing phenotypes was developed.[90] The technique of feeding rats 20% aqueous glucose depressed the activities of hepatic mixed function oxidases and inhibited the *in vivo* O-demethylation of indomethacin.[91] The induction of necrosis by various hepatotoxins permitted study of the regional distribution of drug metabolizing enzyme systems.[92] An electrochemical approach for the N-dealkylation of lisuride, diazepam, methysergide and imipramine gave the same N-dealkylation pattern as liver microsomal biotransformation.[93] Immobilized cytochrome P-450 and glucuronyltransferase produced a useful system for small scale syntheses of drug metabolites; i.e., N-demethylation of ethylmorphine and O-demethylation of 4-nitroanisole, followed by conjugation of 4-nitrophenol.[94]

Both triacetyloleandomycin[95] and erythromycin[96] induce their own transformation to metabolites which form stable inactive cytochrome P-450 complexes. Propranolol pretreatment inhibits its own metabolism, presumably by the covalent binding of a reactive metabolite to P-450.[97] Interferon inducing agents depress P-450 dependent drug metabolism via an interferon-mediated interaction.[98] The suicide inactivation of P-450 by choramphenicol resulted from the covalent binding to a lysine residue as N-ε-chloramphenicol oxamyl lysine.[99] Inhibitors of cytochrome P-450 and their mechanisms of action have been reviewed.[100] Adriamycin[101] and a number of 6-thiopurines[102,103] were also covalently bound to proteins. Neither clofibrate nor fenofibrate bound to liver DNA.[104] The cardiotoxicity of doxorubicin was linked to interaction with the cell nuclei.[105]

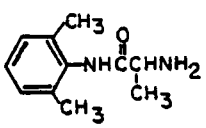
The mechanism of acetaminophen hepatotoxicity was reviewed[106] and evidence presented that N-acetyl-p-benzoquinonimine, and not N-hydroxyacetaminophen, was the toxic metabolite.[107] A HPLC method was developed for acetaminophen and its conjugated metabolites; the glutathione conjugate was concentrated in liver, whereas the cysteine conjugate was concentrated in kidney.[108] Horseradish peroxidase converted acetaminophen to a reactive species that binds to protein.[109] In kidney microsomes, the co-oxidation of acetaminophen during prostaglandin biosynthesis may be related to the renal toxicity of this compound,[110] although deacetylation to the nephrotoxic 4-aminophenol may also be involved.[111] The hepatotoxicity of acetaminophen can be blocked by ascorbic acid[112] and N-acetylcysteine.[113] The effects of a number of thiol compounds on the formation of a reactive species were of lesser importance than their effects on its subsequent covalent binding.[114] Mouse liver post-mitochondrial fractions bound 65% of acetaminophen radioactivity and the major component co-chromatographed with glutathione S-transferase.[115] The depletion of free sulfate following acetaminophen administration can be overcome with N-acetylcysteine, which acts as a sulfate donor, or by i.v. sodium sulfate.[116] The kinetics of the for-

mation of the various conjugates of acetaminophen and the implications of cosubstrate depletion were reported.[117]

Lien has reviewed the relationship between a drug's physicochemical properties and its disposition.[118] Radiolabeled gitoxin, digoxin and digitoxin were administered to guinea pigs and levels of the various metabolites in cardiac tissue were determined.[119] Nonsteroidal anti-inflammatory agents accumulated in inflamed tissues.[120] In rats receiving equal doses of either erythromycin propionate or stearate, the tissue:plasma ratios at 2 hr were greater for the stearate, while at 12 hr the ratios were higher for the propionate.[121] The use of an isolated perfused lung preparation[122] and the effects of hypoxia[123] were reviewed. The first-pass uptake of propranolol by the lung was greater in dogs under general anesthesia compared to conscious animals.[124]

Drug metabolism by the gastrointestinal mucosa was reviewed.[125] Metabolizing enzyme levels were greater in guinea pig than in rat intestinal microsomes, while arylesterase activity was twice as high in rats.[126] The intestinal microflora was responsible for enterohepatic circulation of warfarin[127] and the transformation of sulfur containing metabolites of 2-acetamido-4- (chloromethyl)thiazole.[128] Antibiotic pretreatment blocked the formation of the methylsulfonyl metabolite of propachlor in the rat, without affecting the mercapturic acid pathway, indicating the role of gut microflora in this process.[129] Niridazole was metabolized to 1-thiocarbamyl-2-imidazolidinone by the intestinal microflora and not hepatic enzymes,[130] and ethinylestradiol was extensively metabolized by the gut wall.[131] Microorganisms were capable of metabolizing imipramine[132] and phencylidine[133] to known mammalian metabolites in vitro.

Several reports of novel transformations or new metabolites of drugs have appeared. Two unusual imine metabolites and an oxime were formed by oxidation of the primary amino group of dezocine.[134] A 2-carbon fragment, presumably acetic acid, was added to the carboxyl group of benzoic acid, producing benzoylacetic acid, which was then reduced to β-hydroxyphenylpropionic acid.[135] The first catecholamine-related metabolite, 3-methoxy-α-methyldopamine, of an amphetamine was identified in dog and monkey urine.[136] The desethyl-metabolite of procainamide was found in human urine.[137] Tocainide (4) forms a carbamic acid derivative which, following O-glucuronidation, forms (5).[138] Eprazinone (6) was metabolized by man to benzoylcyclopropane.[139] Two interesting pathways of metabolism of piroxicam (7) were observed, involving cyclodehydration (8) and a ring contraction (9).[140] Two unusual minor products were formed from methylphenidate: a taurine conjugate and a carbamoyl derivative.[141] Bifluranol, a fluorinated dibenzyl compound, formed two double conjugates: glucuronide-sulfate and glucuronide-phosphate.[142]



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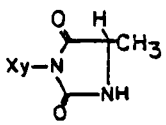

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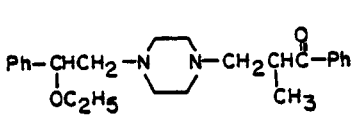

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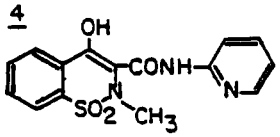

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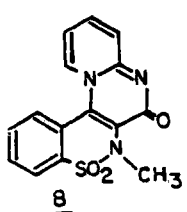

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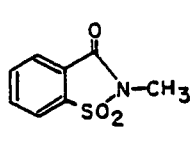

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Propranolol and 4'-hydroxypropranolol were conjugated by different glucuronyltransferases. Propranolol glucuronidation was stereoselective with the R-(+)-form favored;[143] all seven ring hydroxylated metabolites were synthesized.[144] Presystemic hepatic extraction of orally administered propranolol in man was not stereospecific.[145]

Mephenytoin metabolism in man was stereoselective, with 46% of the dose eliminated as a 4-hydroxy-metabolite derived almost entirely from S-mephenytoin, while the demethylation of the R-form leads to accumulation of that metabolite, nirvanol, in plasma.[146] The further oxidative metabolism of nirvanol was also stereoselective in the dog.[147] The hydroxylation of N-benzoyl-and N-benzenesulfonylamides occurred predominantly adjacent to the nitrogen atom.[148] Rats dosed with cyclohexene oxide excreted the two diastereoisomers of N-acetyl-S-(trans-2-hydroxycyclohexyl)-L-cysteine. The ratio of the isomers was dose-dependent, and the corresponding cis isomers were not detected.[149]

The location of drug binding sites on human serum albumin was reviewed.[150] The degree of plasma binding and urinary excretion of an oxacephem derivative were stereoselective in humans, with a direct correlation between the unbound fraction and the renal clearance; no differences were observed in rats.[151] Sulfinpyrazone and phenylbutazone stereoselectively altered the clearance of warfarin in man and augmented its effects; phenylbutazone also displaced albumin-bound warfarin.[152]

Neonates exposed in utero to methyldopa or lorazepam eliminated these drugs at slower rates than adults; the ratio of conjugated to free drug in urine was higher for methyldopa sulfate and lower for lorazepam glucuronide in neonatal urine.[153] The rate of glucuronide conjugation of naphthol was greater than sulfate conjugation in human lung tumor tissue, whereas in normal lung tissue the reverse was true.[154]

Both acetaminophen administration and a low protein diet decreased serum sulfate levels, while only the former decreased the extent of sulfation of a tracer dose of phenol.[155] Saturation of sulfation capacity was more important than sulfate depletion on the ratio between sulfate and glucuronide conjugates.[156] Similar findings were reported for perfused liver, with harmol as the substrate.[157] At low doses, salicylamide is preferentially consumed by sulfotransferase in the cytosol of rat hepatocytes, thus lowering the concentration available for microsomal glucuronyltransferase.[158] Rat hepatic nuclear glucuronyltransferase, localized in the nuclear membrane, responds differently to inducers and inhibitors than the microsomal enzyme.[159] The rate of glucuronyltransferase activity was substrate dependent and species specific.[160] The enzyme catalyzing the deacetylation of mercapturic acids was purified and characterized.[161]

The formation of methylthio metabolites, mainly from glutathione and cysteine derivatives, was reviewed.[162] Rat liver forms two isomeric glutathione conjugates of styrene oxide, a mutagenic metabolite of styrene.[163] The amounts of mecapturic acid metabolites of methyl acrylate, methyl methacrylate and methyl crotonate were increased by pretreatment with a carboxylesterase inhibitor.[164] The reductive metabolism of sulindac to the active sulfide was studied in rat liver preparations[165] and the involvement of thioredoxin in renal sulfoxide reduction demonstrated.[166]

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