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Volume 18

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

Editor-in-Chief: ***HANS-JÜRGEN HESS***

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CONTRIBUTORS

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PREFACE

This volume of Annual Reports in Medicinal Chemistry contains 32 chapters organized in the format of previous volumes. In selecting the chapter topics, the editors again sought to obtain a suitable balance between updates of the literature in areas of current active drug research, and developing areas of science that may provide a basis for future drug discoveries. Included in this volume are in-depth reviews of the neurotransmitter functions of adenosine, bacterial penicillin-binding proteins, immunotherapy for infectious diseases, pharmacological developments in dermatology, calmodulin antagonists, and mechanisms of action of insulin, glucagon, and somatostatin. Chapters on substance P and calcium antagonists extend reviews of these topics that appeared in the previous volume. Similarly, a chapter in volume 17 that reviewed the techniques employed in recombinant DNA research is followed this year with two chapters concerning the applications of this technology. One deals with the study of structure–function relationships of proteins, and another discusses other applications of potential interest to medicinal chemists. A review of the biochemical events involved in IgE-mediated degranulation of mast cells and basophils complements the annual chapter on pulmonary and antiallergy agents, while scientific advances discussed in chapters on oncogenes, tyrosine-specific protein kinases, plasminogen activators, and natural killer cells suggest entirely new approaches to the development of anticancer drugs.

A cumulative author index has been included in this volume to complement the cumulative title keyword index.

In concluding my five-year term as editor-in-chief, I would like to extend my sincere gratitude and appreciation to the many authors for their excellent contributions, to the section editors for their conscientious assistance and advice, and especially to Mary Heinold, whose help was invaluable in preparing the manuscripts for photoreproduction. The opportunity of serving in this capacity has been to me both intellectually stimulating and personally gratifying.

Hans-Jürgen Hess
Groton, Connecticut
May 1983

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Section I - CNS Agents

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Chapter 1. Adenosine as a Neuromodulator in the Mammalian Central Nervous System

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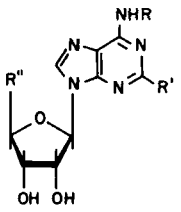
While the cardiovascular effects of adenosine have been known for over half a century,[1] the neuromodulatory potential of this purine has, until the last decade, received little attention.[2] This hiatus has resulted largely from skepticism about a discrete modulatory rôle for so ubiquitous a compound which is, with its nucleotides, involved in all aspects of cellular metabolism.[3] Despite this, the seminal studies of Burnstock[4] on non-cholinergic, non-adrenergic neurotransmission in peripheral tissues, those of Sattin and Rall on adenosine-elicited increases in brain tissue cyclic AMP levels,[5] and other biochemical[6,7] and electrophysiological studies,[8] have indicated that adenosine, by interacting with extracellular recognition sites, can modulate cell function in a physiologically relevant manner.[8]

Adenosine and its N^6-substituted cyclohexyl (CHA, 1) and ℓ-phenylisopropyl (ℓ-PIA, 2) analogs are potent central depressants.[9] Conversely, the central stimulatory properties of the alkylxanthines, caffeine and theophylline, have been ascribed to antagonism of endogenous adenosine.[5,10] Adenosine and its analogs also have profound effects on peripheral cardiovascular function,[11] and it is important to distinguish between direct effects of the compound on nerve tissue excitability and secondary effects resulting from changes in blood flow.[12]

Biochemical Effects of Adenosine - Both cyclic AMP and cyclic GMP have been described as intracellular second messengers.[13] Thus changes in the levels of these nucleotides by various effectors can be related to nerve cell activity. Adenosine can either stimulate or inhibit cyclic AMP formation in mammalian tissues, depending on the type of receptor activated.[14,15] Guanylate cyclase activity can also be stimulated by adenosine[16,17] at 5-50 fold higher concentrations. Increased c-AMP formation is mediated by extracellular recognition sites rather than by adenosine uptake and incorporation into precursor pools.[13,18] While alkylxanthines such as caffeine, theophylline, and



1: R=  ; R'=H; R"=CH_2OH

2: R=(ℓ)-$CHCH_2Ph$ (CH_3); R'=H; R"=CH_2OH

3: R=H; R'=Cl; R"=CH_2OH

4: R=R'=H; R"=$CONHC_2H_5$

5: R=R'=H; R"=CH_2SCH_3

6: R=  ; R'=H; R"=CH_2OH

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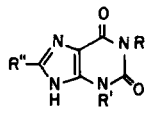


7: R = CH_3; R' = *i*-Bu; R" = H
8: R = R' = C_2H_5; R" = Ph
9: R = R' = CH_3; R" = Ph

isobutylmethylxanthine (IBMX, 7) are generally considered phosphodiesterase inhibitors, more recent evidence[19,20] suggests that the physiological effects of these compounds are probably due to antagonism of endogenous adenosine.

In fat cells,[23] mouse fetal brain cells,[14] rat striatal homogenates[24] and other tissues,[25] evidence has been obtained for three adenosine recognition sites (Table 1), designated A-1, A-2 and P in brain tissue[2,14] and Ri, Ra and P in other tissues.[7,15,100] The A and R sites (activation dependent on an intact ribose moiety) are extracellular and susceptible to alkylxanthine blockade while the P (purine requiring) site is intracellular and insensitive to alkylxanthine blockade. A-1 or Ri receptors are activated at low purine concentrations (10^{-9}M) and inhibit adenylate cyclase activity. At higher concentrations (10^{-6}M), adenosine stimulates cyclic AMP formation by activating A-2 or Ra receptors.[14,15] Activation of the P site causes an inhibition of cyclase activity possibly via a GTP-dependent mechanism.[25] These receptor subtypes are discussed further below in the section on radioligand binding.

Table 1. Adenosine Receptor Subtypes

Receptor	Location	Agonist Pharmacology	Affinity (M)	Biochemical Response
P-1	Extracellular	Ado > AMP > ADP > ATP	10^{-6}	↑ c-AMP formation[4]
P-2	Extracellular	ATP > ADP > AMP ≥ Ado	-	↑ Prostaglandin formation[4]
Ra	Extracellular	NECA > Ado ≥ PIA	10^{-6}	↑ c-AMP formation[15]
Ri	Extracellular	CHA > *l*-PIA > Phado > 2-CADO, Ado > N^6-methyl ado > N^6-benzyl ado > d-PIA, NECA	10^{-9}	↓ c AMP formation[15,100]
P	Intracellular	2'5'-dideoxyadenosine >> Ado	10^{-6}	↓ c-AMP formation[2,15,86]
A-1	Extracellular	CHA = *l*-PIA > 2-CADO > *d*-PIA	10^{-9}	↓ c-AMP formation[2,14,7]
A-2	Extracellular	NECA > 2-CADO > Ado > PIA	10^{-6}	↑ c-AMP formation[14,98]

Ado = adenosine
Phado = phenyladenosine

Adenosine can depress spontaneous and evoked release of neurotransmitters both *in vivo* and *in vitro*. In prelabeled brain slice preparations, the purine inhibits the release of norepinephrine, acetylcholine, dopamine, serotonin and GABA.[26-28] Adenosine also inhibits spontaneous acetylcholine release from guinea pig neocortex *in situ*[29] via a process which is theophylline sensitive. In contrast, the alkylxanthines enhance resting acetylcholine release from neocortical slices[30] and rat cortex *in situ*.[31] 2-Chloroadenosine (2-CADO, 3) potently inhibits dopamine release from striatal synaptosomes[27] (IC_{50} = 10 nM) and *in vivo* decreases acetylcholine turnover in rat hippocampus and parietal cortex.[32,33] The ethylcarboxamide analog of adenosine (NECA, 4) also modulates striatal dopaminergic function when directly injected.[40] Adenosine may affect transmitter release by reducing uptake of Ca^{2+}.[34] In olfactory cortex slices[35] adenosine inhibition of postsynaptic potential generation is antagonized by elevated (56 mM) Ca^{2+} concentrations. K^+-depolarized synaptosomal calcium uptake is theophylline sensitive, and is probably mediated by the A-2 receptor subtype.[36] Adenosine also antagonizes the effects of calcium in peripheral tissue.[37,38] Evidence has been presented for interactions between adenosine and dihydropyridine calcium entry blockers.[39]

The activity of tyrosine hydroxylase is modulated by adenosine.[41-43] In pheochromocytoma cells,[43] adenosine deaminase (ADA)

decreases basal tyrosine hydroxylase activity, while in ADA-pretreated cells, 2-CADO elicits a 2-5 fold increase in enzyme activity which is associated with an increase in cyclic AMP levels. Tyrosine hydroxylase may be activated by a cyclic AMP-dependent protein kinase mechanism,[51] supporting the hypothesis of a positive feedback system for catecholamine neurotransmission.[41] Studies on the presynaptic effects of adenosine are, however, complicated by high affinity uptake systems for the purine, which cause an underestimate of the nucleoside's efficacy.[44] IBMX stimulates norepinephrine metabolism in rat brain via a clonidine sensitive mechanism.[45]

Electrophysiological Effects of Adenosine - Iontophoretic application of adenosine or adenosine nucleotides depresses spontaneous brain cell firing.[8] This response is antagonized by alkylxanthines and enhanced by dipyridamole and ADA inhibitors, such as deoxycoformycin. ADA and alkylxanthines increase spontaneous electrophysiological activity in brain slices,[46] presumably by antagonizing the effects of endogenous adenosine. This observation and the central stimulant properties of alkylxanthines suggest that central neurons are under a purinergic inhibitory tone.[47,48] While it is generally accepted that the effects of adenosine on cell firing result from presynaptic inhibition of transmitter release, more recent studies using intracellular recording techniques also indicate a postsynaptic action on K^+ conductance.[49] A poor correlation between the effects of a series of adenosine analogs on cyclic AMP accumulation and their depression of cell firing in hippocampal slices suggest that cyclic AMP is not involved as a second messenger in the electrophysiological actions of adenosine.[50] Furthermore, the dibutyryl, monobutyryl and 8-bromo analogs of cyclic AMP[51,52] enhance rather than decrease postsynaptic potentials in olfactory cortex and hippocampal slice preparation. Phillis[8] has suggested a third type of extracellular recognition site not linked to adenylate cyclase which mediates the electrophysiological actions of adenosine. However, an excellent correlation has been found in rat hippocampal slices between the potencies of a series of adenosine analogs in displacing radioligands from A-1 receptors and in depressing evoked potentials.[53]

Behavioral Effects of Adenosine - When administered either centrally or peripherally, adenosine, several of its stable analogs and ATP have sedative and anticonvulsant effects.[54-56] Central administration of the purine causes hypnogenic and sedative effects in dog[55] and induces behavioral and electrocortical sleep in chicks.[56] Peripheral administration of the enantiomers of PIA depresses mouse locomotor activity, the *l*-enantiomer being more potent than the *d*-enantiomer.[57] CHA, *l*-PIA and 2-CADO cause sedation and hypothermia and increase seizure latency when given prior to the convulsants metrazole, strychnine and kainic acid.[58] While the sedative, hypothermic and anticonvulsant actions of CHA and 2-CADO are blocked by theophylline, the anticonvulsant action of *l*-PIA is not, suggesting that *l*-PIA may interact with nonpurinergic receptors.[58] Theophylline increases locomotor activity[59] and sensitivity to nociceptive stimuli,[60] reinforcing the purinergic tone concept. A close correlation between the hypotensive effects of a number of adenosine analogs and their action on rat cortical cell firing has been reported.[12]

Endogenous Adenosine in the Mammalian CNS - The effects of adenosine documented so far refer to the action of exogenously applied compound. A physiological role for the purine may, however, be inferred from increased cellular activity resulting from removal or antagonism of endogenous adenosine by ADA and alkylxanthines.[47,48] Further evidence

for a physiological role for adenosine and related nucleotides depends, however, on demonstrating some of the criteria required of a neurotransmitter.[61] While adenosine is present in the CNS at concentrations of up to 2 nmol/g wet weight,[62] no regional variations in content have been observed.[8,63] Similarily, because of its ubiquity, synthesis and degradation have not been demonstrated, nor have discrete neuronal pathways been mapped. Nonetheless, A-1 adenosine receptors have a differential distribution,[64] and adenosine release has been extensively studied.

i) Release - Since release of adenosine is often expressed in terms of the radioactivity derived from tissues prelabeled with adenine or adenosine, there is some degree of controversy as to the identity and origin of the purines released.[2,65]

Electrical stimulation, K^+-depolarization, ouabain and veratridine evoke in vitro release of radioactive purines from prelabeled brain slice and synaptosomal preparations.[66-69] In guinea pig neocortical synaptosomes, K^+-evoked purine release is calcium-dependent,[69] while in hypothalamic synaptosomes it was largely Ca^{2+}-independent.[70] Temporally, K^+-evoked ^{3}H-purine release in rat cortical slices lags behind that of ^{3}H-GABA,[71] suggesting that purine release is secondary to cation elevation.[72]

Cyclic AMP has also been considered as a major source of released adenosine.[65] This is consistent with studies showing that increases in adenosine levels following brain slice stimulation occur subsequent to increases in cyclic AMP.[62] It is also consistent with the finding[73] that adenosine, and not ATP, is released from brain slices despite the high intracellular concentrations of the nucleotide.[65]

In peripheral tissues, ATP undergoes Ca^{2+}-dependent release following electrical stimulation,[74,75] and the co-release of ATP with norepinephrine[76] and acetylcholine[77,78] is well documented. However, it appears unlikely from available evidence that ATP plays any role in purine modulated neurohumoral events in the CNS.[8]

Measurement of endogenous purines by high performance liquid chromatography techniques,[115] rather than prelabeling, may resolve some of the confusion attendant on purine release in vitro. In situ, release of labeled purine from exposed cat and rat cerebral cortex is calcium dependent,[31,79] and over 70% of the purine is released in the form of nucleotides. In the pulmonary artery adenosine autoreceptors have been described.[80]

ii) Inactivation - The effects of adenosine and adenine nucleotides can be terminated either by extracellular catabolism[81] or by reuptake. The latter system is divided into a rapid high affinity system and two slower systems of low and high affinity. All are specific for the nucleoside.[82,83]

Radioligand Approaches to Adenosine Receptor Characterization - Historically, the first classification of purinergic recognition sites was due to Burnstock.[4] Receptors sensitive to adenosine were designated P-1, while those responding to ATP were designated P-2 (Table 1). Independently, Londos and Wolff classified adenosine receptors with respect to effects of various purines on adenylate cyclase activity in adipocytes, hepatocytes and Leydig cell lines.[15] Those receptors termed R required an intact ribose ring for activation, were sensitive to alkylxanthine

blockade, and were located on the external surface of the cell membrane. A second intracellular nucleoside receptor subtype, termed P, was insensitive to alkylxanthine blockade and required an intact purine ring for activity. The R site was further designated Ra and Ri based on whether receptor activation resulted, respectively, in activation or inhibition of cyclase activity. In mouse fetal brain cells,[14] the A-1 and A-2 receptor subtypes correspond to Ra and Ri sites, respectively. In general, CNS studies use the A-1 and A-2 nomenclature.[2,7] P site activation inhibits cyclase activity.[7,15] Pharmacologically, A-1 and A-2 receptors have also been delineated in terms of the efficacy of the diastereoisomers of PIA.[19] It was noted[19] that while A-1 mediated inhibition of evoked potential generation in rat hippocampus showed a 50-100 fold difference in the potency of *d*- and *l*-PIA, only a four-fold difference was observed in the A-2-mediated effects on cyclic AMP production in guinea pig hippocampal slices. A more recent study has suggested that this is a species rather than receptor difference.[84]

Because of the ubiquity of adenosine in mammalian tissues, early studies using ^{3}H-adenosine as ligand were largely unsuccessful. Binding showed low affinity and had subcellular locale and pharmacology inconsistent with that expected of an adenosine recognition site involved in synaptic transmission.[85,86]

Table 2. Radioligands for Central Adenosine Recognition Sites

^{3}H-Ligand	Kd (nM)	Bmax (pmoles/mg protein)	Receptor Subtype
CHA	0.3-5.2	0.34-0.92	A-1
l-PIA	0.9-5.1	0.2-0.8	A-1
2-CADO	1.3-24.0	0.2-0.5	A-1
NECA	2.4	0.1	A-1
	30-40	0.4	A-2
DPX	5.0-70.0	0.5-1.0	A-1 and A-2
Adenosine	50-13000	31-165	?
2'5'-Dideoxyadenosine	80	0.6-1.8	?P
Adenosine Arabinoside	90	1.3	?P

Data from references 86-89,92

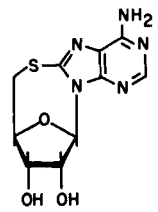
Using ADA in conjunction with the metabolically stable adenosine analogs CHA, 2-CADO and *l*-PIA, high affinity, saturable radioligand binding has been demonstrated.[87-89] In addition, the alkylxanthine 1,3-diethyl-8-phenylxanthine (DPX, 8)[87] also binds to ADA pretreated rat brain membranes. Specific 2-CADO[88] and CHA[90] binding is enhanced in synaptosomal subfractions of mammalian brains, and regional variations in the binding of the former ligand have been described.[88] Binding is highest in caudate and hippocampus and lowest in hypothalamus and spinal cord. Autoradiographic determination of specific ^{3}H-CHA binding has also shown a high density in hippocampus.[64] The cellular localization of A-1 binding sites thus correlates with that of the enzyme 5'-nucleotidase. In cerebellum A-1 receptors are localized on Purkinje cell dendrites and granule cells.[91] The pharmacology of CHA, 2-CADO and *l*-PIA binding is consistent with labeling of an A-1 receptor subtype. 8-Phenyltheophylline (9) is the most potent alkylxanthine, with a Ki of 857 nM against ^{3}H-CHA[87] and 116 nM against ^{3}H-2-CADO.[88] IBMX, caffeine and theophylline had Ki values between 2.6 and 26 μM in all three agonist binding assays. The antagonist DPX shows multiphasic displacement curves

against agonists, but monophasic displacement of antagonists. This suggests that the alkylxanthine binds to both A-1 and A-2 receptors.[87] The carboxamide, NECA (4) may also bind to both A-1 and A-2 receptors.[92] Adenine arabinoside and 2'5'-dideoxyadenosine do not apparently label the P receptor.[86] Since the initial studies with 2-CADO,[88] this ligand has been found for technical reasons to be unsatisfactory for routine use.[85] Species differences in A-1 receptor radioligand have been observed, suggesting that there are A-1 receptor subtypes.[93]

The ontogeny of A-1 receptor binding is consistent with neuronal differentiation.[94] Metrazole-induced convulsions cause a 21% decrease in cerebellar, but not cortical, ^{3}H-CHA binding,[95] while chronic treatment with caffeine[96] and theophylline[97] up-regulate adenosine receptors in rat brain.

Detailed structure-activity relationships (SAR) have been developed for the biochemical effects of adenosine,[15,98,99] with more fragmentary profiles available based on physiological responses.[2] Adenosine analogs with lipophilic N^6 substituents are the most potent agonists at A-1 receptors (Table 1). In general, there is some consistency between these studies and those involving radioligand binding.[87,88]

At A-2 receptors, however, N^6-substitution generally decreases affinity. An extensive SAR study by Bruns[98] indicates that modification of the ribose moeity affects efficacy, while purine modification alters affinity. Furthermore, comformationally rigid analogs support the importance of the anti conformer of adenosine analogs in activating A-2 receptors.[98] Agonist potencies for A-2 receptors in human fibroblasts are NECA > 2-CADO, Ado > N^6-phenyladenosine, N^6-benzyladenosine > $\mathcal{L}$-PIA, CHA > α-PIA.[98]



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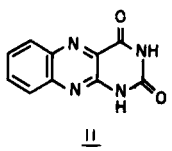

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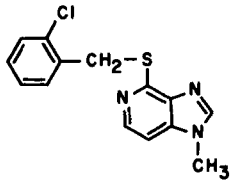
The alkylxanthine antagonists show no selectivity for either A-1 or A-2 receptor subtypes.[98] Other compounds which have been identified as adenosine antagonists are 5'-deoxy-5'-methylthioadenosine (5), 8,5'-S-cycloadenosine (10) and alloxazine (11).[98] In human fibroblasts[99] the SAR for alkylxanthines shows that lipophilic substitution at positions 1 and 8 dramatically enhances affinity for both A-1 and A-2 receptors.

Adenosine and Central Psychotropic Agents - Purinergic mechanisms have been implicated in the actions of several classes of psychotropic agents. In addition to the possibility that the central stimulatory actions of alkylxanthines result from antagonism of endogenous adenosine,[2,7,10] purines have been implicated as potential endogenous ligands for the benzodiazepine receptor.[101,102] A lipophilic adenosine analog, EMD 28422 (6),[103] is a putative anxiolytic, while the imidazopyridines EMD 39593 (12) and EMD 41717 (13)[104] are selective anxiolytic antagonists. It has also been suggested that benzodiazepines may elicit some of their clinical actions by inhibition of adenosine uptake.[8]



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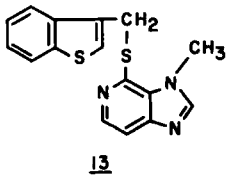

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Because antidepressants and electroconvulsive shock therapy produce marked effects on cyclic AMP, Sattin has suggested[105] that antidepressants may produce their effects by attenuation of adenosine related systems. Chronic antidepressant treatment does not, however, affect A-1 receptor density in rat brain.[105] Alkylxanthines reduce morphine analgesia in mice[107] and increase sensitivity to nocioceptive stimuli.[60] They also elicit a "quasi-morphine withdrawal syndrome" in morphine-dependent rats,[108] which may be related to effects on norepinephrine turnover.[45] Conversely, morphine enhances veratridine-induced purine release *in vitro*[109] and *in situ*.[31] *l*-PIA and CHA block the effects of the psychotic, phencyclidine, in an animal discriminative model,[111] suggesting that adenosine may antagonize the psychotic effects of this substance.

Considering the potential role of adenosine in the actions of several classes of psychotropic agents, the question is raised as to how a purinergic compound might be developed as a therapeutic agent. Apart from compounds such as CV 1808 (2-phenylaminoadenosine),[112] which is a potent coronary vasodilator, there has been little progress toward developing a purinergic drug. Two factors may, however, have discouraged adenosine-related chemistry from being more than an adjunct to basic academic research. One is the potential for cytotoxicity.[113] Adenosine can, for example, interfere with cellular methylation processes, although this does not appear to be a problem in the CNS.[114] The second problem involves the potential for selectivity for so ubiquitous a compound. The question of specificity, especially from a psychotropic viewpoint, is serious. While purines acting at cardiovascular sites may, by exclusion from the blood brain barrier, be considered selective, the converse is not, at the present moment, true. How would one make an alkylxanthine-based central stimulant which would not have profound, and perhaps deleterious, effects on renal, cardiovascular and gastrointestinal function?

Despite these difficulties, the aggregate result of basic research on CNS purinergic systems evidences the potential to develop novel therapeutic agents. The ability to capitalize on this potential will depend on the discovery of novel compounds, structurally dissimilar to the known adenosine agonists and antagonists, which may aid in furthering our understanding of the functional role(s) of central adenosine modulatory systems. The availability of such compounds may turn half a century of dedicated biological research into a therapeutically viable drug candidate. A phencyclidine antagonist,[111] a long acting, non-toxic central stimulant,[2,7] a novel anxiolytic[8,102] or a non-opioid analgesic[108] are all challenging goals.

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

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Introduction - Insight into a rational pharmacotherapy for anxiety continues to evolve along the dual paths of agents and actions. While the benzodiazepines (BZs) continue to be the agents of choice for anxiolysis, new series of non-BZ compounds are emerging which may offer alternatives in the management of anxiety disorders. Continuing efforts, at both the molecular and animal model level, to understand BZ receptor binding, the actions of endogenous ligands, and the properties of BZ antagonists provide new insight into the mechanisms of action of agents with anti-anxiety properties. The dynamic interchanges between preclinical pharmacology and clinical verification of anti-anxiety efficacy continue to define the science of anxiolysis.

Animal Models - The use of animal models continues to increase our understanding of how anti-anxiety agents work, and aids in the rational design of drug candidates. Alterations of stress-induced effects in animals by anxiolytics have been investigated. Neurochemically, anti-anxiety drugs antagonize stress-induced increases in dopamine turnover,[1] GABA turnover,[2] and prolactin release[3] in rats. Behaviorally, they reduce hyponeophagia[4] and the burying of objects associated with aversive electroshock.[5] That the impact of noxious stimuli upon behavior is reduced by anti-anxiety agents is also reflected in reduced attempts to escape from electrical stimulation of brain sites such as the periaqueductal gray,[6,7] and increased latency to terminate aversive brain stimulation.[8] The effect of anxiolytics upon intracerebral self-stimulation depends upon the stimulation site; diazepam increases self-stimulation in the lateral hypothalamus, but suppresses it in the dorsolateral hippocampus.[9] The most commonly used tests for anxiolytic potential continue to be anti-conflict paradigms,[10-12] with more attention being focused on inter-subject variability.[13] While BZs have been reported to induce a hyperdipsia in water-deprived rats[14] which is blocked by opiate antagonists,[15] anticonflict activity reflected in licking for water can be dissociated from non-conflict related increases in water consumption.[16] Anticonflict activity may be related to GABA[17] mechanisms in the amygdala[18,19] and does not appear to reflect direct serotonergic activity.[20,21] While intracerebral administration of chlordiazepoxide to the largely serotonergic dorsal raphe nucleus reduces shock-induced suppression of behavior, this may be due to its effects upon GABAergic neurons found in this and neighboring brain sites.[22] BZs are observed to exert anti-conflict effects in rats which have been subjected to neonatal forebrain norepinephrine depletion.[23] Other whole animal tests for examining properties of anti-anxiety agents include discrimination learning,[24,25] drug discrimination,[26,27] locomotor,[28-30] and rotational[31] models. At the cellular level, changes in the activity of noradrenergic,[32] dopaminergic,[33,34] serotonergic,[35] and GABAergic[36] nerve cells have been reported. Interactions of antianxiety agents with caffeine,[37] morphine,[38] and selective BZ antagonists have been described. BZ effects upon conflict behavior,[39] drinking,[40] drug discrimination,[41] visually evoked

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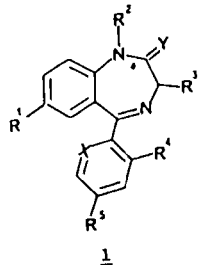
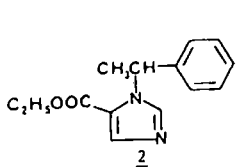
potentials,[42] and kindled seizures,[43] as well as anxiogenic compounds' effects upon social interaction[44] can be reversed with selective BZ antagonists. Tolerance develops to the sedative[45] and motor impairment[46] component of some anti-anxiety drugs' actions. Dependence resulting in withdrawl syndromes[47] upon cessation of treatment has also been reported.

Benzodiazepine Receptor Dynamics - Studies pursuing the mechanism by which the BZs elicit their biological responses continue to focus on a supramolecular complex which displays saturable, stereospecific, binding dynamics for this class of compounds. The proposed linking between the GABA and BZ binding sites in this complex has led to an *in vitro* binding assay which discriminates between BZ agonists and antagonists.[48] In the presence of 10 μM GABA the potency of BZ agonists to displace [^{3}H]carboethoxy-β-carboline (β-CCE) from BZ receptor sites is considerably enhanced, while no effect is observed with BZ antagonists. Similarly, GABA causes a moderate increase in the binding affinity of flunitrazepam (FLU,1a) in ligand/[^{3}H]FLU competition experiments, but has no effect on binding of the BZ antagonists propyl β-carboline-3-carboxylate (β-CCP) or RO 15-1788 (3). It was also confirmed that pyrazolopyridines, certain barbiturates, and etomidate (2) stimulate the binding of BZs in this system in a chloride-dependent manner.[49] The ability to define diverse groups of BZ receptor ligands, based on the different factors which modulate their binding affinity, has further strengthened the postulate that heterogeneity exists among BZ receptor types.[50,51] The existence of multiple areas or "domains" of BZ receptors is inferred from studies with irazepine (1b), an alkylating BZ, which reduces the B_{max} of [^{3}H]diazepam (DZ,1c) binding by 25-33% in competitive binding experiments; a small reduction (< 8.5%) is observed in the B_{max} of [^{3}H]β-CCE.[52] Photoaffinity labelling of BZ receptors with FLU alters BZ agonist but not antagonist binding.[53,54] A portion of the apparent heterogeneity observed in the BZ receptors may be a reflection of the differential interaction existing between BZ agonists, and antagonists, with irreversibly photolabelled receptors. The cooperativity proposed to exist among the BZ receptor sites may reduce [^{3}H]FLU binding by 80%, in a receptor population that has had only 25% of its sites irreversibly photolabelled with FLU; the binding characteristics of BZ antagonists are virtually unchanged under these conditions.



1

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



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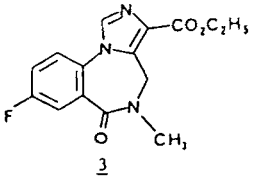

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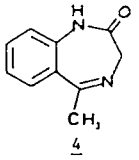

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Quantitative autoradiography has been used to support the existence of multiple BZ receptor types,[55] as well as the functional coupling between a portion of these sites and a particular subpopulation of GABA receptors.[56] The barbiturates are proposed to have a binding site on the GABA-BZ ionophore receptor complex through which they enhance [^{3}H]DZ binding in a picrotoxin-sensitive and chloride-dependent manner. It is suggested they may be divided into different groups accorded by their

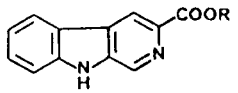
pharmacological actions, based on their type of interaction with BZ receptor binding.[57] GABA and ethylenediamine (EDA) potentiate the binding of [^{3}H]DZ to BZ receptors in a nonadditive manner through mechanisms that are probably unrelated.[58]

Further biochemical and electrophysiological evidence supports the notion that BZs exert their primary action in the CNS by facilitating GABAergic transmission. A dimension of increasing importance is the role of anions and cations in this mechanism. BZ receptors were protected against heat inactivation by a combination of one of several possible divalent cations, plus GABA, but by neither substance alone.[59] The anion-dependent picrotoxin inhibition of GABA potentiated [^{3}H]FLU binding was also observed. This was formulated as interference with the GABA receptor effector system, which might involve an allosteric perturbation of GABA recognition sites.[60] The stimulatory effects of GABA on [^{3}H]FLU binding derive from not only an increase in the rate of binding, but also from an increase in the stability of the ligand-receptor complex.[61,62] These experiments indicate GABA may provoke a disaggregation of the native dimeric BZ binding complex which abolishes the cooperativity among the respective units.[62]

The complex correlation between *in vivo* BZ receptor binding and the resultant pharmacological responses effected through this process has been the subject of several papers.[63-68] The ligand [^{3}H]RO 5-4864 (11) has been used to characterize the peripheral type of BZ receptor believed to be localized predominantly on glial elements.[69] The physiological role of this binding site is unresolved but probably excludes mediation of the anticonvulsant or anxiolytic effects of the BZ class. Virtually all BZ receptor ligands are inactive at the inhibition of [^{3}H]RO 5-4864 binding to this peripheral receptor, which apparently is not coupled to the GABA system.[70] RO 15-1788 lacks major pharmacological activity outside its role as a BZ antagonist, but does display specific proconvulsant activity on isoniazid induced convulsions.[71] Autoradiographical localization of the receptors interacting with this ligand revealed they were indistinguishable from those labelled by [^{3}H]FLU.[72] The pharmacological utility of the BZ antagonist properties of RO 15-1788 is disclosed in several reports.[43,73-77] The convulsant BZ, RO 5-3663 (4), is reported to have anxiolytic properties, suggesting a dissociation between the anticonflict and anticonvulsant properties of the BZs may be possible.[78] Characterization of type 1 and type 2 BZ receptors, distinguished in part by their affinity for the ligand CL 218,872 (14), and sensitivity to GABA, has been further pursued.[79] A distance geometry approach was employed to propose a BZ binding site model which could accommodate a structurally diverse group of ligands.[80]

Endogenous Ligands - The list of putative endogenous ligands for the GABA-BZ ionophore receptor complex continues to expand, with the β-carboline compounds forming the most extensively researched category.[81-83] The β-carbolines generally antagonize the anxiolytic, anticonvulsant and sedative properties of BZs such as DZ and flurazepam. The *in vivo* potency to inhibit BZ receptor binding for several β-carboline derivatives is much less than their effects when measured *in vitro*.[84] Receptor binding studies on membrane preparations from bovine brain indicated [^{3}H]β-CCP (5b) binds to only a portion of those sites specifically labelled by [^{3}H]FLU.[85] This, and other differences between the binding characteristics of the β-carbolines and BZs, implies that BZ receptor subtypes may exist for each class of ligand.[86,87] Studying the anxiogenic action of the β-carbolines in various animal models could possibly lead

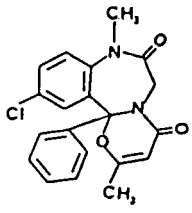
to the development of a reproducible model of human anxiety.[39,45,88] The ability of BZ antagonists RO 15-1788 and CGS 8216 to block β-CCM (5c) induced seizures in mice suggests different mechanisms for BZ antagonism are available.[89] Structure-activity relationship studies have been initiated in the β-carboline class.[90,91] The inhibition of adenosine uptake has been linked to the anxiolytic action of the BZs, encouraging the study of purine derivatives as BZ antagonists. Presently, there appears to be little high affinity, specific binding of purine analogues at the BZ receptor,[92] or of BZs at sites of adenosine uptake.[93]



5a, R=C_2H_5
5b, R=nC_3H_7
5c, R=CH_3

Benzodiazepines

Anxiolytic Agents - The neuropharmacology of the BZs with their applications and performance in various anxiety disorders has been the subject of several reports.[94-100] A double-blind study indicated ketazolam (6) had an anxiolytic efficacy equivalent to clorazepate (1d) with slightly fewer side effects.[101] The relationship of DZ serum levels to its anti-anxiety effects,[102] the combination of DZ and propranolol in anxiety therapy,[103] and the anti-tardive dyskinesia effects of DZ were studied.[104] The central effects of DZ in man were antagonized by RO 15-1788 without alteration of its bioavailability.[73] Low dose DZ may offer an alternative treatment for extrapyramidal symptoms in some patients.[105] RO 15-1788 was also found to antagonize the sedative effects of acute lorazepam (1e) in rats, but chronic treatment concomitantly with lorazepam did not prevent the development of tolerance to this drug.[106] The prolactin lowering effects of centrally active BZs were prevented by the simultaneous administration of RO 15-1788.[107] A reassessment of ethanol-BZ interactions was reported.[108] Studies evaluating DZ's performance versus clobazam (7),[109] cloxazolam (8),[110] and tofisopam (9),[111] and its efficacy in several clinical models of anxiety were reported.[112,113] A comprehensive review of clobazam's pharmacology has been issued.[114] Halazepam (1f), contrary to chlordiazepoxide and diazepam, did not increase hostility or aggression in man but was equally effective in producing sedative or anti-anxiety effects.[115] Other clinical studies have addressed the efficacy of bromazepam (1g),[116,117] ketazolam (6)[101] and alprazolam (10a)[118] in the treatment of anxiety.



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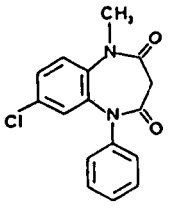

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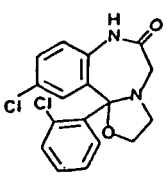

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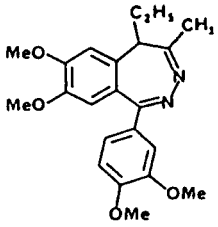
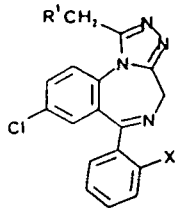
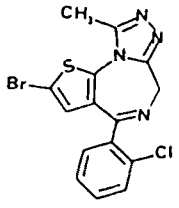


9

Sedative-Hypnotics - The hypnotic effects of different BZs and their related pharmacokinetics have been the subject of two reviews.[119,120] A model of insomnia induced by methylphenidate and caffeine has been used in the evaluation of temazepam (1h).[121] The thienodiazepine, brotizolam (11), proved superior to flurazepam (1i) in the treatment of chronic insomnia[122] and several reports continue to document the hypnotic efficacy of quazepam (1j).[123-125] Three studies have evaluated the



10a, R^1=H, X=H
10b, R^1=H, X=Cl



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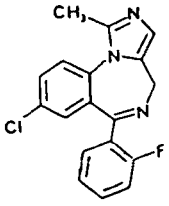
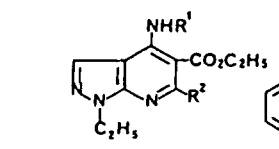


12

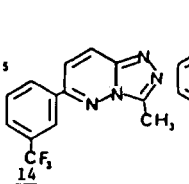
safety and efficacy of triazolam (10b) versus nitrazepam (1k). While triazolam usually appeared to be the subjective preference, no significant differences existed between the two in their side effect profiles.[126-128] Midazolam (12) is less effective than DZ in anticonflict models in animals,[129] but it may prove to be an effective sleep inducer or i.v. anaesthetic with rapid onset and short duration in man.[130,131] An extensive series of reports on the pharmacodynamics of this drug have appeared.[132]

Non-Benzodiazepines

Anxiolytic Agents - Tracazolate (13a) enhances both BZ and GABA binding.[133] It is less potent than chlorodiazepoxide in conflict models but is also less sedating.[134] Etazolate (13b), cartazolate (13c), and tracazolate show in vitro inhibition of adenosine-stimulated [^{3}H]cAMP formation in guinea pig cortical preparations.[135] CL 218,872 (14)



13a, R^1=n-C_4H_9, R^2=CH_3
13b, R^1=N=C$(CH_3)_2$, R^2=H
13c, R^1=n-C_4H_9, R^2=H



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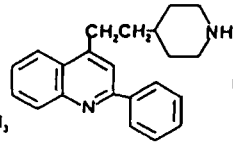

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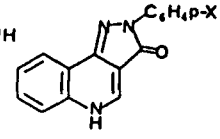
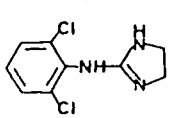


16a, X=H
16b, X=Cl

labels two distinct BZ receptor sites in rat cerebral cortex with K_D values of 10-30 and 200-600 nM.[136] A study in rats failed to confirm earlier reports of the drug's lack of sedation.[137] Both PK 8165 (15)[138] and CGS 9896(16b)[139] showed GABA-enhanced inhibition of β-CCP binding in rat brain. CGS 9896 has been described as having anticonflict activity comparable to that of DZ but without sedation,[140,141] while CGS 8216 (16a) is a potent BZ antagonist which inhibited [^{3}H]FLU binding and blocked the pharmacological effects of DZ.[142]

The anti-anxiety effects of β-adrenergic blockers have been reviewed,[143] as has the role of dopamine and dopaminergic agents in anxiety.[14] A single 5 or 10 mg dose of the β-blocker mepindol failed to reduce examination anxiety in students.[144] The α-agonist clonidine (17) was found superior to placebo in a double-blind study in patients having either generalized anxiety or panic disorders.[145] Buspirone (18a)



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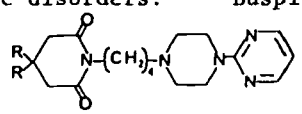
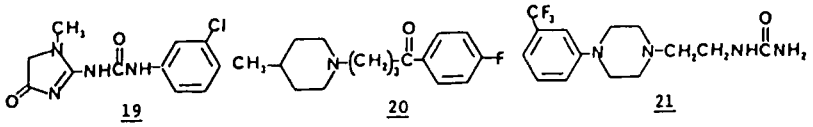


18a, R,R=-$(CH_2)_4$-
18b, R=CH_3

demonstrates clinical anxiolytic efficacy equivalent to DZ without sedation, anticonvulsant or muscle relaxant effects and in various

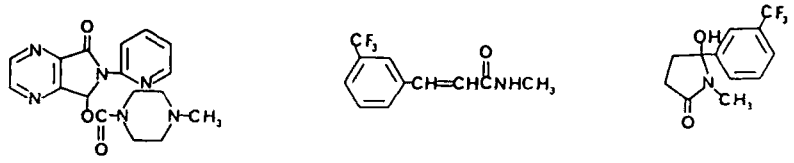
binding studies shows affinity only for dopamine receptors.[146] In biochemical studies in rats, the drug increased whole brain DOPA formation while decreasing 5-HTP formation.[147] It also caused a dose-dependent decrease in striatal acetylcholine levels,[148] and at high doses raised plasma prolactin levels.[149] The drug reversed drug-induced catalepsy in both unaltered and dopamine-depleted rats.[150] In a double-blind crossover comparison with lorazepam in healthy volunteers, buspirone, unlike the BZ, did not impair psychomotor function or interact with alcohol.[151] The analogue MJ 13805 (18b) showed good activity in the Vogel conflict model (M.E.D. = 1.0 mg/kg) without dopaminergic activity.[152]

Clinical assessment of fenobam (19) vs. placebo showed it to be an effective anxiolytic with minimal side effects[153] and the drug was found to be as effective as DZ in a double-blind clinical study.[154] A 50 mg dose of melperone (20) abolished experimental anxiety in normal subjects.[112] The phenylpiperazine, DU 27716 (21), inhibited hypothalamically-induced aggression in rats.[155] The electrocorticogram/operant task profiles of various non-BZ anxiolytics differ from those of BZs in monkeys.[156]



19 20 21

Sedative-Hypnotics - The in vivo effects and potential biochemical mechanisms,[157] structure-activity relationships,[158] and GABA receptor interactions[159] of barbiturates have been reviewed. The interactions of various barbiturates with BZ receptors have been studied;[160,161] barbiturate enhancement of GABA binding correlated with the anesthetic activity of the studied compounds.[162] A clinical study of the hypnotic activity of zopiclone (22) showed it to have favorable effects on sleep

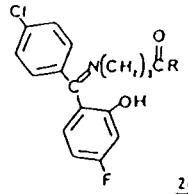


22 23 24

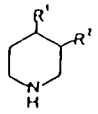
with neither impairment of performance nor interaction with alcohol.[163] The significant sedative effects of adenosine analogues appear to be mediated by mechanisms different than that of the BZs.[164] Compounds 23 and 24 both induce sleep in monkeys at 15-30 mg/kg, p.o.[165] Cerebral infusion of 5 pmol/kg of Factor S, a small glycoprotein isolated from human urine, induced a 50% increase in slow wave sleep in rabbits.[166]

Anticonvulsants - Anticonvulsant structure-activity relationships have been reviewed.[167] Several clinical evaluations of valproic acid have been reported.[168-170] Studies in rodents suggest a possible role for both prostaglandins[171] and opioid peptides[172] as endogenous anticonvulsants. Clonidine (17) and related selective α_2 adrenergic agonists showed anticonvulsant activity in rats.[173] The neuropharmacology and anticonvulsant profiles of the GABA agonist, progabide (25a) and its major metabolite SL 75102 (25b) have been described.[174] Other GABA-mimetic agents which exhibit protection against seizures in rodents are EDA[175] and the p-nitrophenyl esters of nipecotic (26a) and isonipecotic (26b) acid.[176] Additional compounds reported to have anticonvulsant activity in various animal models are the α-substituted γ-butyrolactones

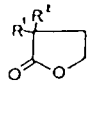
(27),[177] 1,2,4-triazoles (28),[178] 4-quinazolones (29),[179] AHR-8559 (30)[180] and MK-801 (31) which also shows anticonflict activity.[16] Neither the anticonvulsant nor anticonflict activities of MK-801 are blocked by RO 15-1788.[181] β-Substituted analogues of 27 are proconvulsant.[177,182]



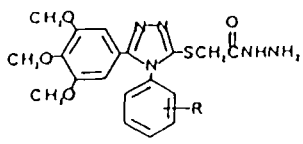
25a, R=NH$_2$
25b, R=ONa



26a, R^1=H, R^2=$CO_2C_6H_4$-p-NO_2
26b, R^1=$CO_2C_6H_4$-p-NO_2, R^2=H



27a R^1:R^2=CH_3
27b R^1=CH_3;R^2=C_2H_5



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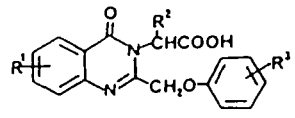

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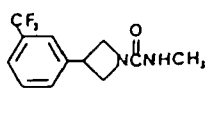

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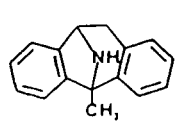

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Chapter 3. Antipsychotic Agents and Dopamine Agonists

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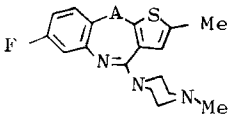
The past two years have produced substantial progress in medicinal chemistry and neuropharmacology related to psychosis. Several symposia and reviews have appeared on neuropharmacological aspects of schizophrenia.[1] New insight has been gained in the function and biochemistry of dopamine (DA) receptors,[2] which circumstantially are involved in the pathophysiology of this and other diseases.[3] A large number of new compounds are being developed as antipsychotics, many inspired by the atypical drugs clozapine and sulpiride. The emergence of selective presynaptic DA agonists instead of conventional DA blockers has increased strongly.[4] Their clinical usefulness, however, is still unproven.

The unique profile of clozapine is suggested to depend on its anticholinergic effect,[5] or on its specific DA-blockade in the mesolimbic system.[6] Studies of drug-induced elevation of acetylcholine have indicated that only the D-2 receptor is involved in the regulation of striatal cholinergic transmission and subsequently involved in the extrapyramidal function.[7] The ability of neuroleptics to antagonize hypothermia in rats induced by sub-stereotypic doses of apomorphine is found to correlate to antipsychotic activity in man.[8]

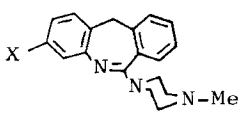
Antipsychotic Drugs

Dibenzepines - Isosteric permutation of various heteroatoms in the 11H-dibenzo[b,e]1,4-diazepine structure has produced a number of active compounds.[9] Fluorine is often being introduced in order to improve the pharmacological profile and to protect against metabolism.[10]

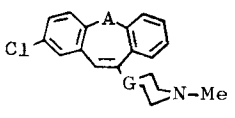
Flumezapine (LY-120363, 1a) has been selected for clinical trials.[11] Its ratio for antagonizing DA over acetylcholine is 5 times that of clozapine.[12] The corresponding methylene bridge compound 1b is one of the most potent in the antiapomorphine and haloperidol displacement tests.[9]



1a A = NH
1b A = CH_2



2a X = F
2b X = H



3a G = CH, A = CH_2
3b G = N, A = O

Fluperazine (NB 106-689, 2a) is another drug currently undergoing clinical evaluation in schizophrenic patients.[13] It is active at less than 1 mg/kg in the hyperactivity blockade test, and is without the sedative properties of perlapine (2b).[14] The all-carbon analogue 3a is twice as potent as clozapine in displacing ^{3}H-clozapine from nonmuscarinic binding sites, demonstrating the significance of the distal nitrogen atom in the dibenzepine series.[15] The piperazine substituted dibenzoxepine 3b has a 12-fold greater affinity for the spiperone receptor than clozapine, while being 164 times less anticholinergic.[16]

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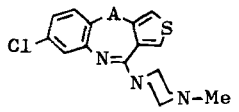
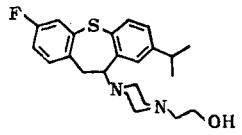
The effect of heteroatom substitution in the seven-membered ring was examined in a series of thieno[3,4-b]benzazepines 4. The presence of an oxygen bridge (4b) increases protection against amphetamine lethality 2-fold,[17] compared to having an NH-bridge (4a),[18] while the sulfur analogue 4c is inactive.[17]



4a A = NH
4b A = O
4c A = S



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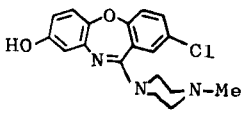
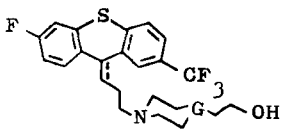


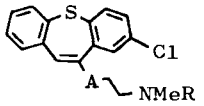
6

Isofloxythepin (VUFB 10662, 5) is a long-acting neuroleptic which raises prolactin levels and induces DA supersensitivity in rats.[19] In comparison to octoclothepin, 5 is a potent neuroleptic in the locomotor activity model.[20] Loxapine, which generates a 6 times higher steady-state level in human plasma of its active 8-hydroxy metabolite 6 than the parent compound,[21] has demonstrated dose-related efficacy in five therapy resistant patients.[22] The importance of protecting these tricyclic agents against metabolic degradation is further demonstrated in the long acting teflutixol (LU 10-022, 7a) and piflutixol (LU 13-013, 7b), which are shown to be potent and selective D-1 antagonists.[23]

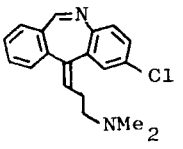
QSAR calculations on phenothiazines show that inhibition of apomorphine-induced emesis in dogs correlates positively with the electron-withdrawing properties of the 2-substituent and negatively with their pKa values.[24] Attempts to restrict the conformational freedom of the amine chain of chlorpromazine by incorporation into a five,[25] six[25] or seven[26] membered ring have removed neuroleptic activity, at least in the non-halogen substituted analogues.



7a G = N , sat
7b G = CH , unsat



8a A = O , R = Me
8b A = S , R = H

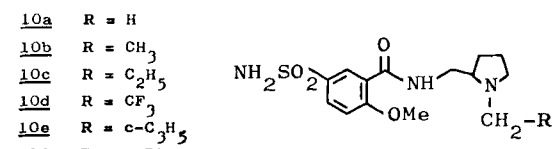


9

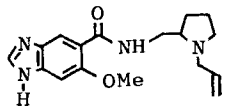
Chronic administration of zotepine (8a) reduces striatal ^{3}H-spiperone binding in the rat brain but does not cause enhancement in apomorphine-induced stereotypy.[27] The corresponding thia analogue 8b is twice as active as imipramine in tetrabenazine antagonism, revealing antidepressant activity.[28] Its fluoro analogue, fluradoline, is a potent analgetic.[29] The clomipramine isostere (RMI 81582) (9) shows a clozapine-like pharmacological profile. Its ability to potentiate ^{3}H-flunitrazepam binding in vivo suggests the involvement of GABA-ergic mechanisms in non-classical antipsychotic action.[30]

Substituted Benzamides (Orthopramides) - With the increasing number of reports of successful antischizophrenic therapy with sulpiride (10b),[31] a variety of structural modifications have emerged. The N-methyl analogue sulmepride (TER 1546, 10a) displays post- and possibly presynaptic D-2 antagonism,[32] a property it shares with sulpiride. The N-propyl analogue prosulpride (GRI 1665, 10c) is an antipsychotic with psychostimulant properties.[33] The trifluoroethyl derivative 10d is an appetite stimulant,[34] illustrating a close structural relationship of these CNS-activities.

Cipropride (10e), equipped with an N-cyclopropylmethyl group, is a potent antiemetic.[35] In flubepride (SL 74205, 10f), the anti-DA activity resides in the R-(+)-enantiomer.[36] The benzimidazole derivative alizapride (11) is marketed as an antiemetic.[37]



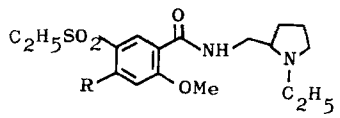
10a R = H
10b R = CH_3
10c R = C_2H_5
10d R = CF_3
10e R = c-C_3H_5
10f R = p-FC_6H_4



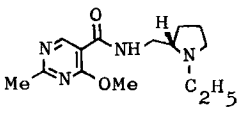
11

Replacement of the sulfonamide group in sulpiride with more lipophilic substituents such as an ethylsulfone in sultopride (12a) increases potency[8], but causes extrapyramidal side effects.[38] Amisulpride (DAN 2163, 12b) reverses hypothermia in low doses in the rat[8].

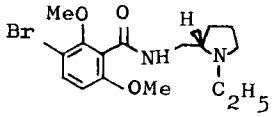
Increased potency is also demonstrated in a series of pyrimidines. The levorotatory 2-ethoxypyrimidinecarboxamide 13 is 13 times more potent orally than (-)-sulpiride.[39] This effect is further improved in remoxipride (FLA 731, 14).[40] The separation in 14 between the inhibition of apomorphine-induced hyperactivity and drug-induced catalepsy in rats is 12 times that of sulpiride or haloperidol. Racemic 14 shows only 10% of this separation. In fact, the S-(-)-enantiomer is 15 times more active than the racemate in the hyper-activity assay.[40]



12a R = H
12b R = NH_2



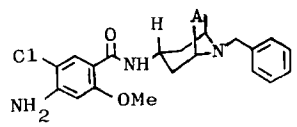
13



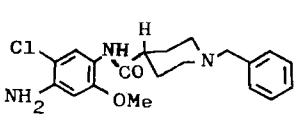
14

Attempts to increase potency in the sulpiride series by incorporating the pyrrolidine in a rigid azabrendane ring system were unsuccessful.[41]

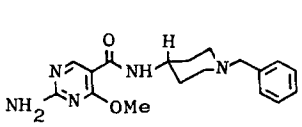
Only preclinical data on clebopride (15a) as a potential antipsychotic agent seem to be available.[42] Further characterization of this drug shows it to be a very selective and potent antagonist of peripheral presynaptic DA receptors in the dog (inhibition of dipropyl-DA-induced reduction of tachycardia).[43] The effect of restricting the conformational freedom of the piperidine ring of clebopride has been investigated. The tropane derivative BRL 25594 (15b), with an equatorial trans-chair conformation, displays a 20-fold increase in antiemetic activity compared to 15a in dogs and 2-fold in blocking apomorphine-induced climbing in mice.[44] Reversal of the amido group to the anilide BRL 20596 (16) retains anti-DA activity.[44] The deschloro-pyrimidine analogue 17 shows a variety of neuroleptic properties, such as inhibition of apomorphine-induced climbing, conditioned avoidance, and hypothermia.[39]



15a A = nil
15b A = CH_2CH_2

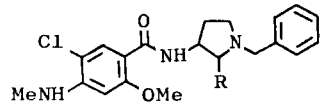


16

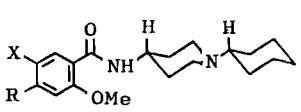


17

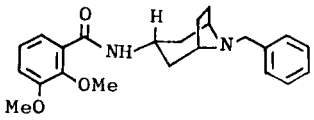
Considerably enhanced potency is found in the ring-contracted structural isomer YM 09151-2 (18a) compared to clebopride.[45] The ability of 18a in blocking apomorphine-induced stereotypy in rats after subcutaneous administration is 13 times that of haloperidol and twice that of the earlier reported YM 08050 (18b).[46,47] The separation for 18a between the stereotypy-blocking dose and the dose that induces catalepsy is 28 times that of chlorpromazine and 6 times that of haloperidol.[45] It is also an extremely potent elevator of prolactin serum levels in the rat.[48]



18a R = Me
18b R = H



19a R = H, X = SO_2NH_2
19b R = NH_2, X = Cl

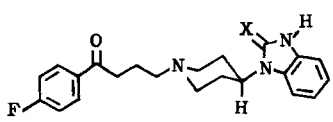


20

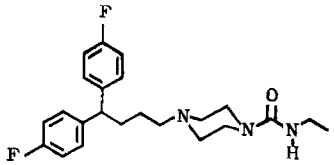
Replacement of the benzyl group in 18b with cyclohexyl produces a 10-fold decrease in activity.[45] On the other hand, replacement of the sulfonamido group of AHR-6092 (19a)[49] with 4-amino-5-chloro substituents to AHR 5859 (19b) increases antiemetic potency 50-fold,[50] while retaining low activity on the peripheral D-1 receptors.[51]

Tropapride (MD 790501, 20) induces catalepsy in rats at a 61-fold higher dose than that which inhibits apomorphine-induced stereotypy,[52] indicating a very selective action.

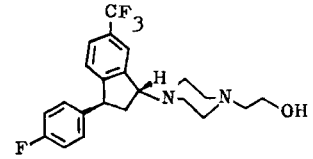
Butyrophenones and Diphenylbutylamines - Timiperone (21a), a thioxobenzimidazole derivative of benperidol 21b, is 8 times more potent than haloperidol on apomorphine antagonism in rats,[53] and it is superior to perphenazine in antipsychotic effects in schizophrenic patients.[54]



21a X = S
21b X = O

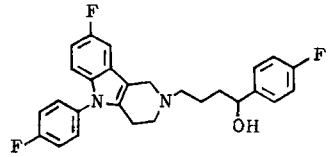


22



23

A cyclic analogue Y-12112 of pimozide, in which the butylamine chain is incorporated into a cyclohexylpiperidine moiety, is equipotent with pimozide.[55] Amperozide (FG 5606, 22) inhibits conditioned avoidance in rats but produces no catalepsy.[56] It has pronounced antiaggressive properties. Structural modification of flupenthixol has produced tefludazine (LU 18-012, 23), which has a long duration.[57] Compared to flupenthixol it is 3 times less cataleptogenic in relation to its antiamphetamine activity. From a pilot study on schizophrenic patients, flutroline (CP-36584, 24) seems to begin exerting an antipsychotic effect from the third day of treatment.[58]



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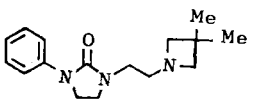
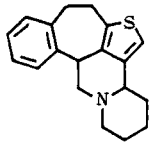


26

Miscellaneous Structures - Mezilamine (25 R=Me) shows a lower propensity than haloperidol or tiapride to elicit acute dyskinesias in squirrel monkeys.[59] The N-benzyl analogue UK-177 is 8-fold more active in the spiperone binding assay[60] and about half as active as haloperidol. Studies with zetidoline (DL 308-IT, 26) against schizophrenia have shown clinical improvement in 50% of the patients.[61] The pharmacological profile of 26 includes anticholinergic properties.[62] QM-7184 (27), a thiophene isostere of the antidepressant taclamine, is equipotent with chlorpromazine in classical tests for neuroleptic activity.[63]



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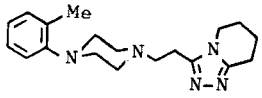

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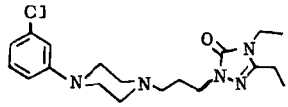
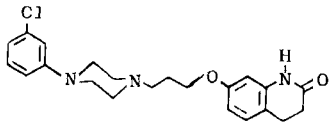


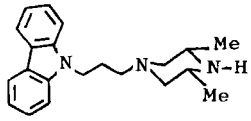
29a

A new trazodone derivative, dapiprazole (AF 2139, 28), displays both central and peripheral adrenolytic properties.[64] It is 4 times more active than chlorpromazine in protection against lethal doses of amphetamine but inactive against apomorphine-induced stereotypy.[64] Preliminary clinical experiences show improvement of delirium, delusions and interpersonal relationships.[65] A ring-opened hydrotrazodone analogue, etoperidone (29a), is showing particular improvement in such items as cooperation and relief of tension in a double blind evaluation versus chlorpromazine.[66] Another m-chlorophenylpiperazine derivative, OPC-4139 (29b), is a potent suppressor of DA activities in rats without catalepsy-inducing properties.[67]

The carbazole BW 234U (30) blocks apomorphine-induced climbing in mice and aggression in rats but fails to block stereotypy.[68] Significant improvements and few side effects were observed with 30 in acute schizophrenic patients.[69]



29b



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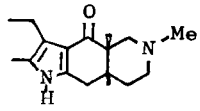


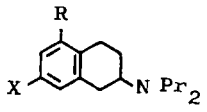
31

The rigid molindone analogue RO 22-1319 (31) was selected for its separation between the ability to inhibit conditioned avoidance and escape response.[70] All activity resides in the 4aR, 8Ar-(-)-enantiomer.[71]

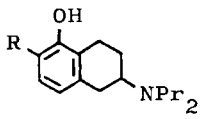
Des-tyrosine-γ-endorphine (DTγE) displays neuroleptic-like activity in a variety of behaviour tests.[72] Its active metabolite has been identified as β-endorphin-(6-17), which, like DTγE, is active only in vivo.[73] Peripherally administered cholesystokinin-octapeptide (CCK-8) in rats diminishes apomorphine-induced stereotypy at 0.64 mg/kg i.p. and shows neuroleptic-like effects on behaviour.[74]

Dopamine Agonists - The continued interest in DA receptor agonists in the CNS seems to focus on two aspects. One is to find selective agents which stimulate only the presynaptic DA receptor, thereby giving the possibility of treating mental disorders in which neuronal DA hyperactivity is the pathophysiological condition, as an alternative to neuroleptic

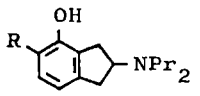
therapy.[75] The other is to find agents which stimulate only the post-synaptic DA receptor, thereby overcoming DA deficiency diseases such as parkinsonism.[4] Additional pharmacological and chemical objectives are to find agonists which are able to induce specific behavioural effects for use in animal models,[8] and to gain topographical knowledge of the DA receptor through these agonists, in view of the mounting evidence that the structural requirements for agonist receptor affinity may be different from those of the DA antagonists.[4] Recent commentaries on the nomenclature of central and peripheral DA receptors have appeared.[78,79]



32a R = H , X = OH
32b R = OH , X = H



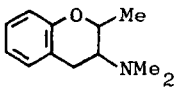
33a R = H
33b R = Me



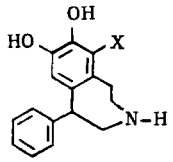
34a R = OH
34b R = H

In the aminotetraline series, maximum potency on both D-1 and D-2 receptors is obtained with the enantiomer of 32a having (+)-2R configuration and with the enantiomer of 32b having (-)-2S configuration.[80] This inversion of stereochemistry is further evidence that these agonists bind via a single aromatic hydroxyl group to the DA receptor site.[81] The monohydroxyl aminotetraline 33a is as active as the 6,7-dihydroxy analogue TL-99,[82] and the methyl substituted derivative DK 118 (33b) appears to selectively stimulate presynaptic DA receptors.[83]

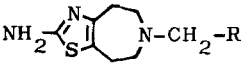
The indanylamine congeners are weak in agonist and antagonist receptor binding, but 34a inhibits postganglioic cardioaccelerator nerve stimulation,[84] and is a potent stimulator of DA-mediated behaviour in reserpinized rats.[85] An oxa-analogue of the aminotetralines, trebenzomin (CI-686, 35), which displays both stimulating and blocking effect on DA-mediated behaviour,[86] has failed to show efficacy in schizophrenic patients.[87]



35



36a X = H
36b X = Cl



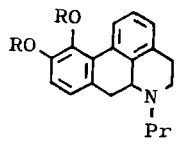
37a R = $CH{=}CH_2$
37b R = CH_3

The more flexible benzazepine SKF 38393 (36a) exerts its stereoselective DA agonistic effect exclusively via the R-(+)-enantiomer.[88] 36a may prove to become a useful tool in psychopharmacology since it is demonstrated to be a specific D-1 agonist.[89] The 6-chloro derivative 36b is a potent renal vasodilator besides having central effects superior to those of DA.[90] Hydroxylation in the phenyl group completely abolishes the central activity,[91] a result also encountered with aromatic hydroxylation of apomorphine.[92]

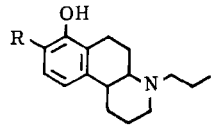
The N-allyl thiazoloazepine B-HT 920 (37a) shows no effect on motor activity in reserpinized mice, but in untreated animals 37a selectively stimulates presynaptic DA receptors.[93] The ethyl analogue B-HT 933 (37b) selectively stimulates adrenoreceptors.[93]

Apomorphine decreases psychotic symptoms when given in doses which activate the presynaptic DA autoreceptor in schizophrenic patients.[94]

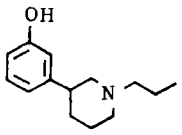
(-)N-Propylapomorphine (NPA, 38a) is 20 times more potent than apomorphine.[95] The duration and bioavailability of NPA is further enhanced by using the 10,11-methylendioxy derivative 38b as a prodrug. Thus, 38b is an orally effective long-acting central DA agonist.[96]



38a R = H
38b R = $-CH_2-$



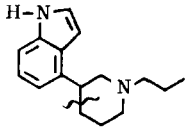
39a R = OH
39b R = H



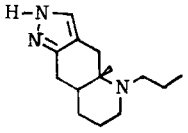
40

From studies of the behavioural effects of the rigid octahydrobenzo[f]quinolines it was concluded that DA exerts its functional action in the α-trans rotameric configuration.[97] Thus, 39a is more potent in reducing motor activity in mice than the 7,8-dihydroxy analogue corresponding to the β-rotamer of DA.[97] The monohydroxyl analogue retains this activity,[98] and removal of the ethano bridge in the 5,6-position results in the presynaptic agonist 3-PPP, 40.[99]

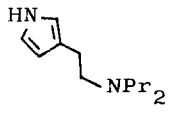
The blocking effects of various neuroleptic agents on 40 show that it is selective on the D-2 receptor.[75] The unique profile of 3-PPP is explained by its stereoselectivity. While the (+)-form is a mixed pre- and postsynaptic agonist like apomorphine, the (-)-form is a selective presynaptic agonist without effect on the postsynaptic receptor.[100] Restriction of the conformational mobility of 40 by incorporation of the propyl group into an azabicyclo[2.2.2]octane system abolished apomorphine binding activity.[101]



41a bond
41b no bond

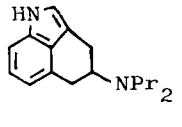


42



43

Replacement of the hydroxyl group with indole nitrogen (RU 27251, 41a) retains agonist activity.[102] Further approaches towards the ergoline structure have produced several interesting compounds based upon Cannon's proposed ergoline pharmacophore 41b.[103] The effects of LY 141865 (42), however, stand out in support of Bach and Kornfeld's pyrrolethylamine moiety 43 as the ergoline pharmacophore.[104] Compound 42 is a selective D-2 agonist as evidenced by potentiating striatal acetylcholine transmission[105] and inhibition of α-MSH release without effect on the D-1 receptor.[106] It is also a potent H-2 agonist,[107] besides being an orally active antihypertensive agent in the rat.[108]



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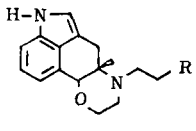
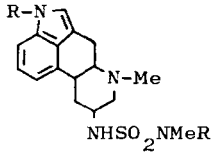


45a R = Me
45b R = H

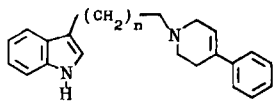


46a R = Me
46b R = H

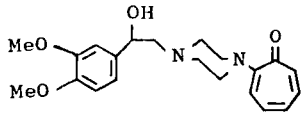
Another ergoline analogue, RU 28251 (44), is a potent agonist in the Ungerstedt rotation model.[102] The oxa-ergoline RU 29717 (45a) is equipotent with pergolide in causing circling behaviour of unilateral

nigrostriatal 6-OHDA-lesioned rats.[109] The corresponding N-ethyl derivative, (-)-EOE (45b), although more potent orally than apomorphine,[110] is equally active on both pre- and postsynaptic DA receptors and thus not useful therapeutically.[111]

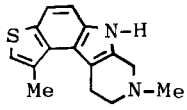
In a series of 8-amino-6-methylergolines, CU 32-085 (46a) was one of the most active compounds.[112] A time-dependent biphasic effect on DA synthesis and turnover is observed with 46a,[113] indicating first an antagonistic activity which later becomes agonistic by formation of the 1,20-di-desmethyl metabolite 46b.[114] The corresponding diethylaminosulfonylamide ergoline CQ 32-084 is a multiple D-1/D-2 agonist, besides being a blocker of presynaptic α-adrenergic receptors.[115]



47a n = 3
47b n = 1



48



49

Presynaptic DA-agonistic properties with no marked effects on the postsynaptic receptors in striatum are shown with EMD 23448 (47a).[116] In addition, 47a is lacking antiapomorphine activity on locomotion,[117] in contrast to the two-carbon shorter analogue AHR 1709 (47b).[49] The troponylpiperazine AY 27110 (48) represents a novel class of DA agonists. All of its behavioural effects on 6-OHDA-lesioned rats reside in the S-(-)-enantiomer.[117] A new rigid heterocyclic compound, TVX 4148 (49), displays multiple actions such as antiagressive, anticataleptic, and serotonin-potentiating properties, besides being a DA agonist in the lesion model.[118]

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Chapter 4. Cholecystokinin and Substance P in the Central Nervous System

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Introduction - An increasing number of neuronally localized bioactive peptides have been found in the mammalian CNS, and several recent volumes[1,2] give an overview of the neurobiology of many of these peptides which are usually considered to be either neurohormones (e.g., the hypothalamic releasing factors[3,4]) or slow acting neurotransmitters. Apart from the enkephalins, the two neuropeptides that have attracted most interest amongst pharmacologists are cholecystokinin (CCK) and substance P. The reason for this interest in CCK is due primarily to the co-existence of a CCK-like immunoreactivity with dopamine in neurones in the mesolimbic dopaminergic neurones,[5] perhaps providing a clue to novel approaches to the therapy of Parkinsons disease and schizophrenia in which dopaminergic underactivity[6] or hyperactivity,[7] respectively, are implicated. In the case of substance P, the localization of this neuropeptide in small diameter primary afferent sensory fibres suggests that substance P and other peptides may be responsible for the transmission of "painful" sensory information through the spinal cord to higher centres in the CNS[8], so that development of substance P antagonists might provide a new class of non-opioid analgesic drugs.

Cholecystokinin

The cholecystokinin octapeptide (CCK-8) (Table 1) is the major CCK-like peptide found in the CNS[9] (although an unknown CCK-like peptide is present in primary afferents in the spinal cord of the rat[10]), and it belongs to the cerulein family of peptides. The ceruleins, like the tachykinins, are a family of amphibian skin peptides.[11] The carboxy-terminal pentapeptide sequence of CCK-8 is identical to that of gastrin, and both peptides are unusual amongst CNS peptides in being sulfated. The sequence similarities between gastrin and cholecystokinin led to the original description of the CCK immunoreactivity in the brain as "gastrin",[12] a confusion that was soon resolved by the sequencing of ovine brain CCK-8.[13] Development of suitable radioimmunoassays and improved immunocytochemical techniques have localized CCK immunoreactivity in particular to the cerebral cortex, basal ganglia, hypothalamus and spinal cord in all mammalian species, including man.[9] CCK immunoreactivity is concentrated in synaptic vesicle/membrane fractions[9] and can be released by depolarizing stimuli,[14,15] consistent with a physiological role for the peptide. Electrophysiological experiments show that in the hippocampus,[16] spinal cord[17] and mesencephalon,[18] CCK is potently excitatory when applied to neurones. In neurological illness a depletion of CCK immunoreactivity has been reported in Huntington's disease[19] and in Parkinsons disease.[20] The majority of CCK in human brain is found in the cerebral cortex where it has been suggested to have a role in stimulating local metabolic activity and enhancing local blood flow.[21]

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Apart from a neurotransmitter/modulator role, CCK has been implicated as a central satiety factor,[22-26] and this would be in keeping with the documented role of plasma CCK as a peripheral satiety signal.[27] Intraventricular injections of CCK into sheep produce satiety.[23] Intraventricular injections of CCK also reduce plasma luteinizing hormone and

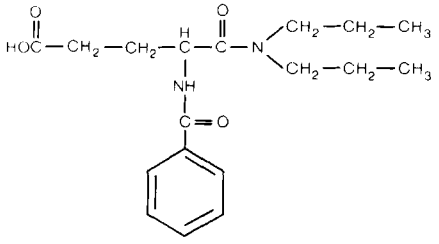
TABLE I. Ceruleins

Glp-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2	Cerulein
Glp-Asn-Asp-Tyr(SO_3H)-Leu-Gly-Trp-Met-Asp-Phe-NH_2	Asn^2, Leu^5-cerulein
Glp-Glu-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2	Phyllocerulein
Asp.Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	CCK-8
Tyr-(SO_3H)-Gly-Trp-Met-Asp-Phe-NH_2	Hexagastrin II

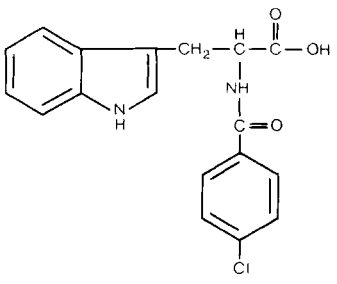
thyroid stimulating hormone content, whilst only 40 ng CCK-8 elevate prolactin and growth hormone levels, an effect probably mediated through a hypothalamic mechanism.[29]

Structure-activity Relationships - A number of early studies compared gastrin, CCK, cerulein and analogues for their effects on peripheral systems, including the stomach and pancreas.[30-32]. Studies on acid secretion showed that the C-terminal tetrapeptide of gastrin (Trp-Met-Asp-$PheNH_2$, "tetrin") retains full activity but is some 10-20 fold less potent than gastrin-17 The sulfated and non-sulfated gastrin-17 were approximately equipotent,[33,34] and within the C-terminal tetrin sequence the aspartate residue was critical for activity.[32] In contrast to gastrin, the position of the tyrosyl-O-sulfate in CCK is of particular importance. Moving the tyrosyl-O-sulfate to left or right results in loss of the characteristic cholecystokinin effects on smooth muscle and pancreas and leaves only gastrin like activity.[35-39] Similarly, replacement of the sulfate group by a phosphate group or removal of the sulfate dramatically reduces the ability to enhance amylase secretion. Studies using the ^{125}I-Bolton-Hunter labelled CCK-33 or ^{3}H-cerulein have shown that on the pancreatic receptor sulfation of CCK increased receptor activity some 100-1000 fold consistent with its effect on amylase secretion.[40] There are two binding sites for CCK-33 on the pancreatic

acinar cell, a high affinity (K_d=64 pM) and a low affinity (K_d=21nM) site.[41] The high affinity site is that involved with amylase secretion, stimulation of cGMP formation and increased calcium efflux and is likely to be the physiological receptor responding to circulating CCK levels found after a meal.[41] In contrast, although the brain CCK binding site demonstrated using ^{125}I-CCK-33 has the highest affinity for the sulfated CCK-8, it resembles the "gastrin" receptors in the stomach more closely in that both the desulfated CCK-8, CCK-4 and gastrin are relatively potent at displacing ^{125}I-CCK-33 from the brain binding site.[42-47] The distribution of CCK binding sites in the brain correlates reasonably with distribution of CCK-like immunoreactivity (Table 2). The majority of the CCK binding sites develop postnatally in the rat.[47] The differences in receptor affinity and specificity between pancreas and brain are of particular interest in relation to the pancreatic antagonists, dibutryryl cyclic GMP, proglumide (1) and benzotript (2).[48a]



Proglumide (1)



Benzotript (2)

Dibutyryl-cGMP displaces ^{125}I-CCK-33 binding from mouse brain sites,[45] but no evidence is yet available on proglumide and benzotript, nor is it known whether these compounds are antagonists or agonists at CNS CCK receptors. There is a clear need for a suitable *in vitro* test system for CNS CCK receptors.

TABLE II Regional distribution of CCK-33 binding in rat and guinea pig brain

Region	Rat*[a]	Guinea pig*[a]	Rat*[b]
Cerebral cortex	100	40	94
Olfactory bulb	87	100	100
Hypothalamus	85	18	46
Pons/medulla oblongata	70	15	13
Caudate/putamen	66	23	80
Hippocampus	40	24	46
Spinal cord (cervical/thoracic)	25	11	N.D.
Cerebellum	10	54	0

*Percentage of region with highest binding.
[a]ref. 42
[b]ref. 44. N.D. = Not Determined.

Using lesion experiments to interrupt CNS pathways that might carry CCK binding sites, Hays et al.[48b] investigated the localization of CCK binding sites in the rat basal ganglia. They concluded that most basal ganglia sites were on intrinsic neurones, as neurotoxin lesions using kainic acid depleted the striatum of some 75% of its CCK-binding sites, whilst a further 25% of binding sites were on ascending afferents to the striatum. Parallelling these animal data, a similar reduction (75%) was found in the number of CCK binding sites in patients dying with a diagnosis of Huntington's disease. The localisation of CCK receptors on ascending afferents to the striatum suggests that some of these receptors may be present on the axons of the mesolimbic dopamine neurones, which contain a CCK-like peptide.[5] Consistent with this suggestion, intracerebral injections of CCK enhanced dopamine turnover,[50] enhanced DA release from striatal slices has also been reported. In addition to these observations, several groups have reported effects of CCK-8 on binding of dopamine agonists and antagonists to striatal membranes.[51,52] Finally, a very recent report has shown that endogenous dopamine apparently also enhances the release of CCK from rat striatum.[53]

Substance P

Substance P (SP) is one of the most extensively studied of the peptide messengers in the nervous system. Insight into the mode of action of SP may, therefore, give some general understanding of peptides as neurotransmitters. SP has particular significance in that it has been proposed as the "pain transmitter" used by a sub-population of sensory nerve fibres at their first relay point in the dorsal horn of the spinal cord.[54-56] In the brain, SP is present within a number of intrinsic neuronal pathways. Although the function of SP in these pathways is unknown at present, the finding that some of these pathways terminate in areas containing catecholamine neurones, e.g., substantia nigra (dopamine), locus coeruleus (norepinephrine), suggests that SP may be involved in the regulation of catecholamine turnover.[57] Thus, the study of the physiology of SP may offer important insights into pain, analgesia, neuropathology and the design of novel centrally acting analgesic or neuroleptic drugs. There have, however, until recently been several key elements missing from our basic understanding of SP as a neurotransmitter, such as the biochemical characterization of its receptors and the mechanism of its synaptic inactivation.

Receptor Recognition and Ca^{2+} Mobilisation - A number of different SP receptor binding assays in brain tissue, using either ^{125}I-Tyr^{8}-SP, ^{125}I-Bolton-Hunter labelled SP or ^{3}H-SP as the radiolabelled ligand, have been reported.[58-62] Because SP is rapidly degraded by several enzymes present in the brain special precautions have to be taken in order to prevent degradation of the radiolabelled ligand. Receptor binding to brain membrane preparation has, therefore, been carried out at low temperature or in the presence of peptidase inhibitors or both. In spite of the technical problems involved, SP binding sites have been demonstrated to be specific, saturable, reversible and linearly dependent upon protein concentration and are thus consistent with expectation for peptide receptor sites. At least one of these "binding assays" exhibits a good correlation between the potencies of C-terminal fragments of SP and the biological activity of the same fragments on guinea pig ileum contraction or their ability to excite rat spinal cord neurones.[62]

A number of cell surface receptors, including SP receptors, which raise intracellular Ca^{2+}, are linked to the hydrolysis of inositol

phospholipids in the cell membrane.[63] Although the exact role is largely unknown, there is now evidence that the "phosphatidylinositol response" is closely linked to the occupation of a class of receptors which are not coupled to cAMP, and it has been suggested that it may be a reaction essential to the coupling between the activation of receptors and the mobilization of Ca^{2+} within the cell.[64]

SP has been shown to cause a phosphatidylinositol response in rat parotid[65] gland, but similar studies in brain tissues have been hampered by the low sensitivity of the assay. Recently a significant improvement in the assay amplifies the SP-induced response to the extent that it is now also suitable for studies in brain tissue.[66] Recently, results from work with hypothalamic[67] slices indicate that this could be an important method in the studies of the receptor mediated events induced by SP.

Metabolic Inactivation - If SP can be released from nerve-terminals and interact with specific receptors sites, there is presumably a mechanism by which the action of substance P is terminated. In principle this could occur either by re-uptake, diffusion, or by enzymatic degradation. Since no uptake system has ever been demonstrated for substance P[68-70], enzymatic inactivation or diffusion are the more likely means of terminating substance P action. There are a number of enzymes reported to degrade SP.[71-86] However, most of the enzymes are either intracellular, or SP is not their preferred substrate. Recently, a plasma membrane bound enzyme[87] from human brain was isolated which cleaves SP between one of the following amino acid residues: Gln^6-Phe^7, Phe^7-Phe^8 or Phe^8-Gly^9. It is a neutral, metallo-endopeptidase with a molecular weight of 40-50,000. The close similarities between the cleavage pattern of ^{3}H-SP by the purified enzyme from human brain and by a synaptic membrane preparation from rat brain,[87] as well as from hypothalamic slice perfusate, suggests a similar enzyme may be involved in SP activation in the CNS. A number of SP analogues exhibiting different degrees of resistance towards the enzyme were designed. One of these $(Glp^5,MePhe^8,MeGly^9)$-$SP_{(5-11)}$ was resistant not only towards the purified enzyme,[89] but more importantly, completely stable towards digestion, both by crude rat brain synaptic membrane preparations[89] and hypothalamic slices. The analogue retains the full biological activity of SP in the CNS and has a number of physical properties which makes it a very attractive tool. When the analogue was injected into a specific region of the rat brain, it was approximately equipotent with SP in causing increased locomotor activity.[90,91] However, in contrast to the brief effects of SP, the behavioural response to the analogue was considerably prolonged. The prolonged action of the analogue is supported by the observations that although SP was almost completely degraded after 5 min in the brain, the enzyme-resistant analogue was virtually intact 50 min after the injection. Biochemical evidence supports the claim that diffusion rather than enzymatic inactivation determines the time-course of the behavioural response to the analogue.[89]

Mammalian Tachykinins - A class of naturally occurring SP-related peptides called tachykinins has been frequently used in SP research.[92] Tachykinins can be subdivided into the SP-physalaemin family and eledoisin-kassinin family. For all these peptides, the C-terminal sequence Phe-X-Gly-Leu-Met-NH_2 is a common feature. However, SP-physalaemin peptides have an aromatic amino acid in position X, whereas eledoisin-kassinin peptides have a hydrophobic non-aromatic amino acid in this position. The N-terminal sequences of these peptides do not show

any obvious similarities. The eledoisin-kassinin family shows a different spectrum of activity compared with SP and SP-like peptides. These differences have led to a postulated sub-division of SP receptors.[93-96]

The high selectivity of some tissues for eledoisin and kassinin, rather than SP, suggests that SP itself may not necessarily be the best candidate for mediating physiological action at these sites. Centrally administered eledoisin has been reported to be substantially more potent than SP in inducing antidipsogenic activity[97] in rat and eliciting scratching behaviour in mice,[98] suggesting the possibility of an eledoisin-kassinin like ligand in the mammalian CNS. Consequently, a search for a novel tachykinin in mammals has begun in several laboratories. Recently, a peptide was isolated from a human lung tumour which is identical in a number of properties to the amphibian peptide physalaemin.[99] There is also strong evidence for the presence of another tachykinin, similar but not identical to kassinin, in the bovine spinal cord.[100] It is therefore possible that in the near future we will have to consider that there may be a number of naturally occurring mammalian tachykinins and that some of the physiological events assumed to be mediated by SP might in fact be mediated by other mammalian tachykinins.

TABLE III. Tachykinins

Molluscan	
Glp-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2	Eledoisin
Amphibian	
Glp-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2	Physalaemin
Glp-Ala-Asp-Pro-Lys-Thr-Phe-Tyr-Gly-Leu-Met-NH_2	[Lys^5,Thr^6]Physalaemin
Glp-Pro-Asp-Pro-Asn-Ala Phe-Tyr-Gly-Leu-Met-NH_2	Uperolein
Glp-Asn-Pro-Asn-Arg-Phe-Ile-Gly-Leu-Met-NH_2	Phyllomedusin
Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2	Kassinin
Asp-Glu-Pro-Lys-Pro-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2	[Glu^2,Pro^5]Kassinin
Asp-Pro-Pro-Asp-Pro-Asp-Arg-Phe-Tyr-Gly-Leu-Met-NH_2	Hylambatin
Mammalian	
Arg-Pro-Lys-Pro-Gln-Gln Phe-Phe-Gly-Leu-Met-NH_2	Substance P
Not Determined	Physalaemin-like peptide
Not Determined	Kassinin-like peptide

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Chapter 5. Antidepressants

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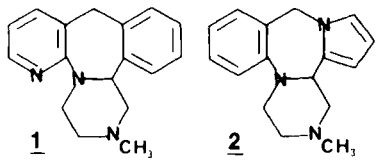
Introduction - The role of neurotransmitter amines in depression has remained a central theme.[1-5] The proceedings of symposia on neurotransmitters and receptors,[6] lithium,[7] and antidepressants (ADs)[8-10] have been published, as have monographs on affective disorders[11] and atypical ADs.[12] The pharmaco-EEG profiles of ADs have been studied.[13] A report of a genetic linkage of depressive disorders to human leukocyte antigen (HLA) markers[14] has elicited both support[15] and criticism.[16] Although numerous new chemical entities were predicted to be ADs based upon findings in animals and man, the final verdict as to whether such agents are truly advances must await results from extensive, carefully controlled clinical trials.

Tricyclic Compounds with Antidepressant Activity - The clinical use,[17] toxicity,[18] and continuation and maintenance therapy[19] of TCAs were reviewed. Some established TCAs were reviewed (amoxapine,[20] trimipramine,[21] maprotiline,[22] mianserin[23] and dimexiptiline[24]) and/or extensively studied (lofepramine,[25] clomipramine,[26] desmethylclomipramine,[26] cianopramine[27] and oxaprotiline[28]). Amitriptyline-N-oxide was shown in clinical trial to give a more rapid improvement with less severe side effects than amitriptyline and imipramine.[29] The solution conformation of the alkylamino side chain in a series of polycyclic amitriptyline analogs was found to be folded toward the polycyclic rings with the positively charged nitrogen situated above an aromatic ring.[30]

Several mianserin analogs were studied whose clinical evaluation might clarify the role of α_2 antagonism and concomitant NE uptake inhibition in mianserin's mode of AD activity. They could also provide a test of the AD predictive value of human EEG studies. Both the mianserin metabolite 8-hydroxymianserin and 6-azamianserin (1, ORG 3770) retain the α_2 antagonist property of mianserin but are only 1/300 and 1/60 as active as NE uptake inhibitors. As with mianserin, the α_2 antagonist property of ORG 3770 is stereoselective and resides in the (+) isomer.[31] However, human EEG studies predict AD activity not only for (±)-ORG 3770 (at 1/3 mianserin dose) and (+)-ORG 3770 but also for the (-)-isomer.[32] CGS 7525 A (2) is a considerably more potent α_2 antagonist in vivo than mianserin and retains the weak uptake inhibition of mianserin on 5-HT and NE.[33]



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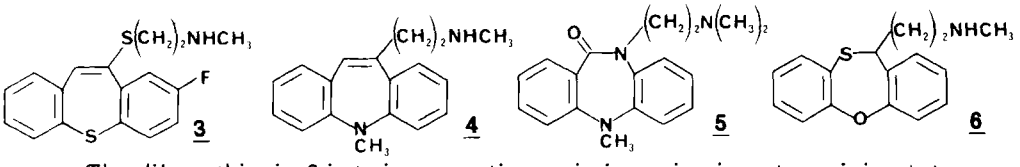

3 4 5 6

The dibenzthiepin 3 is twice as active as imipramine in antagonizing tetrabenazine ptosis and has analgesic activity comparable to pentazocine.[34] The pharmacological and biological profile of RU 5031 (4) is similar to desipramine.[35] Noveril (5) was reported in an open study to be effective in several classes of depression.[36] The dibenzoxathiepin 6 was the best of a series in the antagonism of

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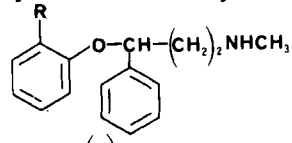
reserpine ptosis and hypothermia in mice and in the antagonism of the gastric ulcerogenic effect of reserpine in rats.[37]

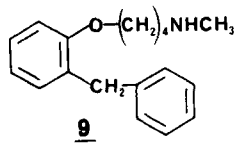
Non-tricyclic Compounds with Antidepressant Activity - Many non-TCAs, particularly nomifensine,[38] trazodone,[39] citralopram,[40] bupropion,[41] iprindole,[42] viloxazine,[43] zimelidine,[44] alprazolam,[45] diclofensine,[46] perafensine,[47] binodaline,[48] ciclazindol,[49] clovaxamine[50] and BRL 14342[51] have been the subjects of reviews and/or clinical trials.

LY 139603 (7), a specfic competitive NE uptake inhibitor is nine times more potent in vitro than the (+)-isomer and in vivo is both more effective as a NE uptake inhibitor and longer acting than nisoxetine (8).[52] MCI-2016 (9) potentiates NE by reuptake blockade and is devoid of α_2 antagonist properties.[53] Adinazolam (10, U 43,465F) was shown to exert simultaneous but independent anxiolytic and antidepressant effects in animal testing.[54] The best of a series, HRP 543 (11), is equipotent to amitriptyline in antagonism of tetrabenazine ptosis and potentiation of yohimbine toxicity and is without MAO inhibitory or anticholinergic properties.[55]



7 (−), R=CH$_3$

8 R=OCH$_3$



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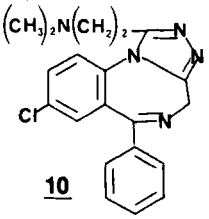

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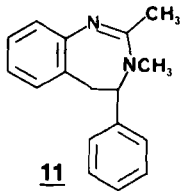
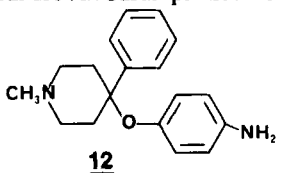


11

B 777-81 (12) reverses tetrabenazine or reserpine ptosis with a potency similar to imipramine and nomifensine. In vitro, B 777-81 inhibits 5-HT uptake in platelets, potentiates the effect of NE on isolated vas deferens, and has less anticholinergic activity than imipramine (ileum).[56] RS-84353 (13) and RS-99707 (14) are more effective than TCAs in reversing reserpine-induced hypothermia. RS-84353 may act as a prodrug for RS-99707, which is two orders of magnitude more potent as an in vitro inhibitor of NE, dopamine (DA) and 5-HT uptake but is less active in vivo.[57] DEAP (15) was reported to be a potential AD with less propensity toward cardiovascular problems than the TCAs.[58]



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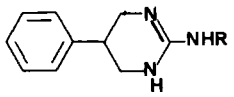
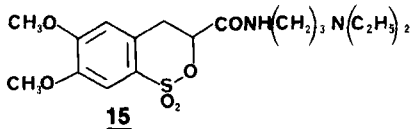


13 R=CO$_2$CH$_3$

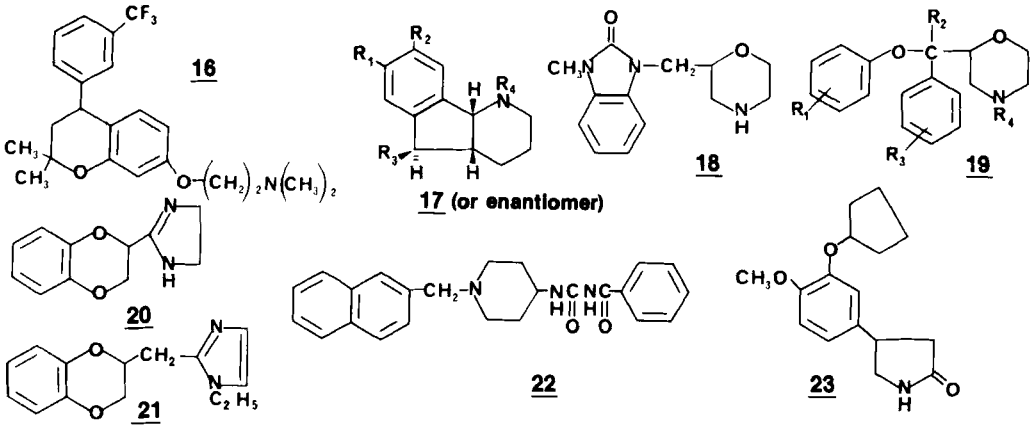
14 R=H



15

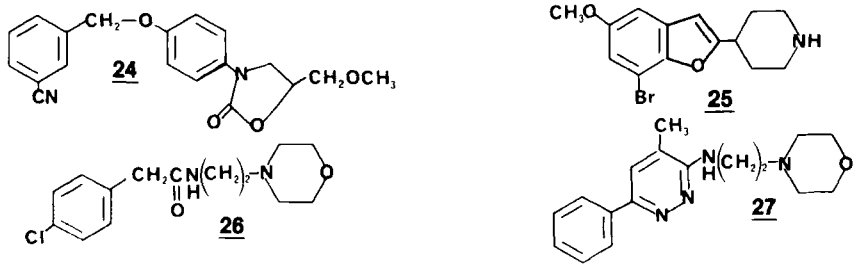
BRL 16644 (16) is a potent in vitro inhibitor of DA, NE, and 5-HT reuptake as well as a potent inhibitor of reserpine-induced hypothermia.[59] The most active of a series of hexahydro-1H-indeno[1,2-b]pyridines (17) are potent inhibitors of tetrabenazine ptosis and uptake (rat synaptosomes) of NE and DA; the activity resides almost exclusively in one enantiomer.[60] UP 614-04 (18), structurally similar to viloxazine, is equipotent to imipramine in antagonizing tetrabenazine ptosis and is active in several other tests indicative of AD activity; it exhibits central anticholinergic and MAO inhibitory properties and is less cardiotoxic than the TCAs.[61] Another series of viloxazine-like compounds (19) possesses both NE uptake inhibitory and α_2 adrenergic antagonist properties.[62] SARs have appeared on congeners of the AD clinical candidate α_2 antagonists RX 781094 (20)[63] and RS 21361 (21).[64] Wy 26002 (22) is a more selective 5-HT uptake inhibitor in vitro and in vivo than fluoxetine or zimelidine.[65] Sertraline [(1S,4S)-N-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-napthylamine] is a selective, competitive 5-HT uptake inhibitor, active in the behavioral despair model, which on chronic administration gave diminished NE-stimulated cAMP formation in rat limbic forebrain.[66] Rolipram (23, ZK 62711), a potent phosphodiesterase inhibitor,

is equipotent to imipramine in potentiating yohimbine toxicity and 15 times more potent in antagonizing reserpine hypothermia. Preliminary results in endogenously depressed patients indicate that rolipram has a rapid onset of action.[67]



Reviews on neuropeptides,[68] neurotransmitter precursors[69] and hormones[70] as antidepressants have appeared. RX 77368 [pyroGlu-His-(3,3-Me_2)ProNH_2], a stabilized TRH analog, is active in reserpine hypothermia, behavioral despair, and olfactory bulbectomy models.[71,72] Subsequent to a report of AD activity in a study in retarded adult and geriatric patients, ORG 2766 [4-Met(O_2),8-D-Lys,9-Phe-$ACTH_{4-9}$] was tested and found active in the bulbectomized rat and apomorphine-induced hypomotility models.[73] SAMe (S-adenosyl-L-methionine) was effective as an AD in two small clinical trials[74,75] and abbreviated the time required for clinical improvement with the β_2 agonist phenoterol.[76] The first controlled study confirming the effectiveness of L-triiodothyronine (T_3) in converting TCA non-responders to responders was published.[77] In a study with 12 patients refractory to imipramine or amitriptyline, eight patients showed marked improvement in one to three days with a combination of the TCA and T_3.[77] In another report two patients refractory to electroconvulsive therapy (ECT) or TCAs improved dramatically with a combination of desipramine and T_3.[78] The effect with T_3 is reminiscent of rapid (within 48 hours) recovery of TCA non-responders when Li was added to the TCA treatment.[79]

Monoamine Oxidase Inhibitors (MAOIs) - Reviews on MAOIs[80] and on the "one molecular entity" hypothesis for MAO-A and MAO-B[81] were published. As the clinical AD efficacy of type B selective MAOIs remains equivocal most research appears directed toward discovery of reversible, selective type A MAOIs free of the "cheese effect." Several compounds which meet the first two criteria (i.e. cimoxatone, 24,[82,83] CGP 11 305 A, 25,[84] Ro 11-1163, 26[85] and (+)-FLA 336 [2-CH_3-4-$(CH_3)_2$N-$C_6H_3CH_2CH(CH_3)NH_2$][86,87]) have received attention. However, no type A MAOI has emerged free of the "cheese effect." Combinations of specific tricyclics (amitriptyline,[88,89] imipramine[89] and desipramine[89]) with MAOIs have been found to be effective in preventing the uptake of tyramine into NE storage sites, thus



preventing the release of NE and therefore the indirect pressor effect of tyramine. K 1349 (N-propargyl desipramine), an irreversible selective MAO-A inhibitor, blocks tyramine effects on the vas deferens but blocks NE effects only at high concentrations.[90]

Miscellaneous Antidepressants - The β_2 adrenergic agonist phenoterol was effective in a study of eight patients with endogenous depression.[76] The authors of a review on the clinical trials of the β_2 agonist, salbutamol, concluded that further salbutamol-type AD studies should await the development of a β_2 agonist that will cross the blood-brain barrier more readily.[91] Minaprin (27), a new psychotropic drug, demonstrated potential in certain depressive states, particularly "masked depressions".[92] (+)-Sulpiride, suggested to be "disinhibitory" in clinical evaluation, is active in the behavioral despair model.[93] The AD effect of exercise was reviewed.[94] ECT was found to be more efficacious than drug treatment in unipolar depressed patients.[95]

Diagnosis and Classification - The search for biological markers to aid in the classification of depression and for prediction of treatment outcome continues to be intense. Two of the most widely studied markers, the use of urinary MHPG to classify subtypes of unipolar depression[96] and the dexamethasone suppression test (DST),[97] have been reviewed. The use of an abnormal DST result as an alert to an increased suicidal potential was also reviewed.[98] Some other markers receiving attention were sleep disorders (particularly REM sleep),[99] growth hormone response to clonidine[100] or to insulin-induced hypoglycemia,[101] TRH-induced release of TSH,[102] physostigmine stimulated release of β endorphin[103] and the diazepam test (used to predict the effectiveness of benzodiazepines versus TCAs in anxiodepressive states).[104] In addition, the platelets of depressed patients have continued to be studied for abnormalities in α_2 receptor binding (decreased[105] and unchanged[106] β_{max}), 5-HT uptake (reduced[107]), ^{3}H-imipramine binding (increased β_{max}[108] and a state dependent marker[109]) and MAO activity (increased[110]).

Screening Methods - Animal models which clearly mimic the features of human depression are still lacking, thus hindering the search for novel ADs. In rats, classical learned helplessness, which mimics certain features of depression, is reversed by subacute TCAs, MAOIs, atypicals and ECT.[111] The Porsolt "behavioral despair" test was found to detect convulsants as false positives.[112] Amine depletors (e.g., reserpine and tetrabenazine) also reduce tonic immobility in this procedure.[113] Since these agents are believed to precipitate depression rather than alleviate it, serious questions about the specificity of the procedure were raised. Chronic restraint stress, which produces β-subsensitivity similar to ADs, reduces tonic immobility in the behavioral despair test.[114] A water wheel modification of this procedure was claimed to be more objective and selective.[115] Chronic administration of atypical antidepressants reverses the behavioral deficits produced by multiple stressors in a new animal model.[116] Repeated forced running stress in female rats results in a syndrome characterized by complete inactivity and constant diestrus in some subjects; 10 days of imipramine causes recovery of spontaneous activity and cyclicity.[117] The kindling model was re-evaluated as a method for assessing antidepressant potential, and earlier results could only be partially confirmed.[118]

Since mianserin exhibits α_2 receptor blocking activity and since chronic treatment with many ADs leads to α_2 subsensitivity, models have been developed to assess the ability to antagonize clonidine-induced responses. Chronic desipramine, but not amitriptyline or iprindole, attenuates the clonidine inhibition of the acoustic startle reflex.[119] Chronic ADs enhance clonidine-induced aggression in mice.[120] Similarly, chronic AD treatment in rats results in facilitation of shock-induced aggression.[121] Chronic administration of ADs abolishes muricidal behavior 24 hours after the last injection and induces a significant decline in the number of

β-adrenergic, but not 5-HT_2, receptors in cortex that is correlated with the time of onset for behavioral change. [122]

Mechanism(s) of Action - The major thrust in this area continues to focus on the effects observed only after chronic administration[5, 123-125] since the onset of clinical efficacy is typically delayed by a week or more. The most consistently reported alteration after chronic AD treatment is β-receptor subsensitivity. Significant decreases in the number of β-receptors, especially in the cortex, have been consistently reported with the majority of ADs, including imipramine,[126-130, 132-134] desipramine, [126-131] clomipramine,[126, 130] amitriptyline,[128, 129] iprindole,[129] nortriptyline,[126, 129, 130] nialamide,[126, 130] phenelzine,[134] clorgyline[134, 135] and pargyline.[129] However, a few ADs failed to produce β-subsensitivity: mianserin,[128-130] nisoxetine,[129, 130] bupropion,[128] salbutamol[136] and lithium.[131] It was claimed that previous ECT β-subsensitivity studies had not utilized clinically relevant treatment regimens; however, when appropriate treatment schedules were utilized (3 treatments per week for 4 weeks), β-subsensitivity still occurred.[137] In rats with unilateral locus coeruleus lesions, desipramine and iprindole reduced the cAMP response to NE on the non-lesioned side although no significant change occurred on the lesioned side.[138] β-Receptor loss after chronic ADs only occurs when 5-HT neurons are intact since lesions with 5,7-dihydroxytryptamine blocked this effect;[139, 140] however, the decreased responsiveness of adenylate cyclase to NE still occurred. [140]

Alterations in the sensitivity of α-adrenergic receptors have been observed. Treatment with ECT,[132] TCAs[133, 141-143] and MAOIs[134, 135] resulted in decreased numbers of α_2 receptors; occasionally, increased ^{3}H-clonidine binding was reported.[127-131, 141] Desipramine caused enhanced electrically-evoked NE release,[144] whereas no attenuation of clonidine suppression of release was observed after clorgyline.[135] Mianserin resulted in supersensitive α_2 receptors (enhanced inhibition of release by NE) although K^+-evoked release was unaffected.[145] In electrophysiological studies, desipramine reduced and lithium enhanced responsivity to iontophoretically applied NE in cerebellum.[131] Many of the actions of clonidine have been attenuated after chronic AD treatment, e.g., hypothermia,[132, 133] hypomotility;[142, 146] EEG synchronization[146] and decreased brain MHPG.[129] Both increases (imipramine and clorgyline)[147] and decreases (MAOIs)[134] in α_1-receptor density have been observed after 3 weeks of AD treatment.

Subsensitivity at 5-HT_2 sites was consistently observed following chronic ADs.[148-151] Although variable results have previously been reported, only decreased receptor densities at 5-HT_1 sites were reported.[148, 152] ADs caused the induction of two binding sites for ^{3}H-5-HT.[149] Both raphe lesions[153] and lesions with p-chloroamphetamine[154] failed to prevent the decrease in 5-HT_2 receptors following TCAs. Decreased behavioral responses (e.g., head twitches) to 5-HTP[150, 155] or 5-MeODMT[151, 156] were observed after chronic ADs. Since head-twitching is a 5-HT_2 receptor-mediated response, functional 5-HT_2 down-regulation occurs after chronic AD treatment.

Adaptive changes of DA, histamine, and opiate receptors may also play a role in the mechanism(s) of action of ADs. Acutely, few ADs alter DA release, reuptake or metabolism.[157] However, upon chronic administration ECT,[158] lithium,[159] and TCAs[160] caused DA autoreceptor subsensitivity although down-regulation has not been observed in other studies.[161, 162] Treatment with desipramine (14 days) caused a decrease in cortical opiate receptors as measured by ^{3}H-naloxone binding;[127] however, chronic treatment with several ADs failed to alter the responsiveness of locus coeruleus neurons to iontophoretically applied morphine.[163] Naloxone attenuated the effects of clomipramine in the Porsolt behavioral despair test in mice.[164] Chronic AD caused hippocampal neurons to become supersensitive to histamine, although the response of adenylate cyclase to histamine was

unaltered.[165] Histamine receptor subsensitivity was reported in guinea pig brain following chronic AD treatment.[166]

Several agents have been studied for their ability to accelerate the adaptive responses of receptors to ADs. A more rapid decline in β-receptor numbers occurred when the α_2 antagonist yohimbine was combined with desipramine or imipramine; however, this potentiation did not occur with several other ADs.[167] Yohimbine caused a more rapid subsensitivity at 5-HT_2 sites when combined with most ADs.[167] ACTH caused a more rapid decline in β but not 5-HT_2 receptor density when combined with imipramine; ACTH did not accelerate the effect of other ADs on β-receptors, but a more rapid decline in 5-HT_2 receptors occurred when ACTH was combined with iprindole or mianserin.[167] Methiothepin, a presynaptic 5-HT antagonist which enhances 5-HT release, precipitated rapid 5-HT_1 receptor down regulation when coadministered with ADs.[168]

With one exception,[169] there is agreement that high-affinity ^{3}H-imipramine binding sites are associated with, but not identical to, the recognition sites for 5-HT reuptake into nerve terminals,[170-177] while ^{3}H-desipramine binding sites are analogously associated with NE reuptake sites.[178-181] It has been suggested that these ligands might be labelling the binding sites for endogenous neuromodulators or cotransmitters.[181,182] The frontal cortex of suicides has been found to contain a reduced number of ^{3}H-imipramine binding sites.[183]

The relationship between REM sleep and depression has been reviewed,[184,185] and additional studies have appeared confirming the suppression of REM sleep by antidepressants in depressed patients,[186-188] normal volunteers,[189] and cats.[190] In support of the cholinergic-adrenergic hypothesis of depression and mania, bipolar affective patients in remission were shown to be hyper-responsive to the induction of REM sleep by the cholinergic agonist arecoline.[191]

The evidence for a circadian rhythm phase-advance hypothesis of depression has been presented.[192-194] Neurons of the suprachiasmatic nucleus (SCN) of the hypothalamus, identified as critical to the generation and entrainment of mammalian circadian rhythms,[195] have been shown in electrophysiological studies to be responsive to iontophoretic 5-HT, imipramine and clorgyline.[196] Electrical stimulation of the SCN of blinded rats and hamsters has been reported to cause phase shifts and period changes in circadian feeding and activity rhythms.[197] Chronic imipramine treatment enhanced the responsiveness of rat SCN neurons to 5-HT.[198]

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Chapter 6. Analgesics, Opioids, and Opioid Receptors

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Introduction - Vigorous research efforts continue to be focused on the endogenous opioids and their biosynthesis and metabolism, on the multiplicity of opioid receptor sub-types, and on the development of receptor-specific ligands. During the past year reviews were provided on endogenous opioids and opioid receptors,[1,2] endorphins and pain modulation,[3-6] opiate and non-opiate analgesia,[7] neural mechanisms of pain transmission,[8] the behavioral pharmacology of the endorphins,[9,10] CNS effects of neuropeptides,[11] endorphins and enkephalins,[12] and stress and endogenous opioids.[13] The proceedings of a conference on opioids in mental illness[14] and the proceedings of the 1982 International Narcotic Research Conference[15] were provided. The search for superior analgesics with improved side-effect profiles continues in the clinic through additional evaluations of non-steroidal antiinflammatory peripheral analgesics, and through continued study of oral administration of centrally acting analgesics. Proceedings of symposia on pain[16] and on narcotic analgesics in treatment of postoperative and cancer pain[17] were published, as was a brief overview of methods of postoperative pain relief.[18]

CLINICAL HIGHLIGHTS

Peripheral Analgesics - Non-steroidal antiinflammatory drugs (NSAIDs) evaluated during 1982 as oral analgesics for postoperative pain included fenoprofen,[19] etodolac,[20] isoxepac,[21] zomepirac,[22] and diflunisal.[23-25] For relief of pain following outpatient surgery, oral fenoprofen (200 mg) compared favorably with parenteral morphine (8 mg) after an initial two hour period during which morphine was more effective.[19] Diflunisal (Dolobid®), approved by FDA in 1982, appears to be a well-tolerated NSAID. Diflunisal was an efficacious and long-acting (up to 12 hours) analgesic for oral surgery patients,[23-25] and was effective against pain encountered in general practice.[26] In treatment of chronic back pain, diflunisal (500 mg twice daily) appears superior to paracetamol (1000 mg four times a day),[27] although less effective than naproxen sodium (550 mg twice a day).[28] Studies with diflunisal,[29] flurbiprofen,[30] ibuprofen,[31] naproxen sodium,[31] and zomepirac sodium[32] provided additional evidence of the effectiveness of oral NSAIDs for treatment of symptoms of primary dysmenorrhea.[33] In treatment of renal colic, intramuscular injection of the NSAID diclofenac (50 mg) was reported to be more effective than injection of a narcotic-spasmolytic combination drug.[34] In an experimental pain model in man, carprofen (200 mg p.o.) and aspirin (1200 mg p.o.) provided similar analgesic effects.[35] Reviews on the pharmacology and efficacy of zomepirac[36] and on the metabolic disposition of etodolac[37] and tolmetin[38] were provided. In animal studies, the non-acidic NSAIDs GP 650,[39] nabumetone (BRL 14777),[40] and KB-1043[41] were reported to have analgesic effects and to have only weak ulcerogenic properties. Nabumetone was reported to be clinically effective against pain and symptoms of rheumatoid arthritis.[42]

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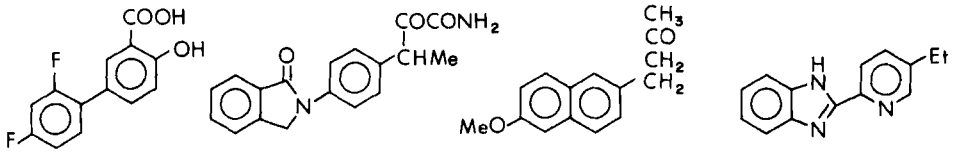


Diflunisal GP 650 Nabumetone KB 1043

Centrally Acting Analgesics - The opiate agonist-antagonists ciramadol,[43] meptazinol,[44-48] and nalbuphine[49] received additional clinical evaluation as orally active strong analgesics. Several additional studies on the sublingual administration of buprenorphine[50-52] and the oral use of morphine[53-55] were reported. Meptazinol (200 mg p.o.) had efficacy comparable to a combined preparation of dextropropoxyphene (32.5 mg) and paracetamol (325 mg)[44] and appears to have a generally acceptable side-effect profile.[44-47] Additional efficacy studies of oral bicifidine[56] (a non-opiate analgesic) and parenteral dezocine[57] and butorphanol[58] were reported. In a comparative study of epidurally administered strong analgesics, morphine had the longest duration of action.[59] The pharmacokinetics in man of the potent short-acting opiate analgesic alfentanil have been described.[60-63] Anesthesia with alfentanil,[64,65] or with sufentanil,[66] provided satisfactory cardiovascular stability for cardiac surgery. Curiously, alfentanil appeared to reduce the analgesic effect of morphine in mice.[67] A brief review of alfentanil and other analgesics related to fentanyl was presented.[68]

Epidural β-endorphin produced analgesia of slower onset and shorter duration than intrathecal β-endorphin.[69] Several additional clinical studies with enkephalin analogs were described. In treatment of post-surgical pain, metkephamid (H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2) acetate (70 mg i.m.) was at least as effective as pethidine (100 mg i.m.); temporary side effects included nasal congestion, drowsiness, and a heavy sensation in the extremities.[70] Epidural administration of the Met^5-enkephalin analog FK 33-824 (H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol) produced an unpredictable, dose-independent, and clinically insufficient analgesia,[71] although FK 33-824 was reported to facilitate detoxification of hard-core heroin abusers.[72] The enkephalinase (carboxypeptidase) inhibitor D-phenylalanine was reported to alleviate acute or incident pain in terminal cancer patients whose chronic pain had been controlled by other treatments.[73]

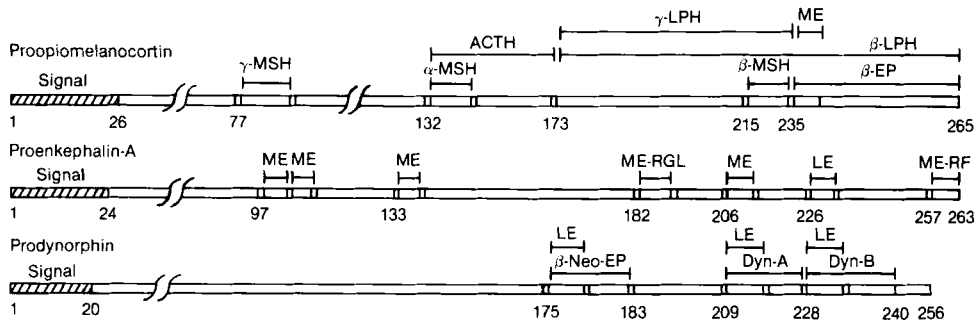
BIOSYNTHESIS AND METABOLISM OF OPIOID PEPTIDES

Enkephalin Metabolism - Enzymatic inactivation of the natural enkephalins continues to be studied.[74-78] Inhibitors of the peptidases which degrade the enkephalins are of particular interest. Thiorphan (d,l-3-mercapto-2-benzylpropanoylglycine), a potent inhibitor of enkephalin dipeptidyl carboxypeptidase ("enkephalinase A"), was reported to potentiate the analgesic effects of D-Ala^2-Met^5-enkephalinamide,[79,80] D-Ala^2-Met^5-enkephalin,[81] and Met^5-enkephalin[82,83] *in vivo*; enhanced potentiation was observed with combinations of thiorphan and the aminopeptidase inhibitor bestatin.[82,83] A combination of four peptidase inhibitors (thiorphan, bestatin, captopril and Leu-Leu) provided pronounced potentiation of Met^5-enkephalin effects *in vitro*.[84] Thiorphan was also reported to potentiate stress-induced analgesia.[85] Structure-activity studies were reported on amino acid hydroxamates[86] and β-endorphin analogs[87] as inhibitors of enkephalin-degrading aminopeptidases. Compounds related to bestatin,[88]

hydroxamates,[89] phosphoryl-Leu-Phe,[90] and carboxyalkyl derivatives of Phe-Leu and Phe-Ala,[91] were studied as inhibitors of enkephalinase-A.

Biosynthesis of Endogenous Opioid Peptides - Three major groups of endogenous opioid peptides have now been identified: enkephalins, β-endorphins, and dynorphins. Their corresponding precursor peptides are proopiomelanocortin (POMC), prodynorphin (also called proenkephalin-B) and proenkephalin-A (fig. 1).[92-99] These three peptides are similar in length and share a number of common features. Each contains at its N-terminus a signal sequence of 20-26 amino acids, predominantly hydrophobic. Each precursor contains more than one copy of at least one of the biologically active peptide products, and active peptide sequences are flanked by paired basic amino acids which provide signals for post-translational cleavage.

Figure 1. Opioid Precursors



Abbreviations: LPH = lipotropin; ME = Met-enkephalin; EP = endorphin; RGL = Arg-Gly-Leu; LE = Leu-enkephalin; RF = Arg-Phe

β-Endorphin - POMC is the precursor of several pituitary peptides, including ACTH, β-endorphin, and α- and β-melanocyte-stimulating hormone (MSH).[92,93] Research is now concentrated in post-translational regulation. The co- and post-translational modifications involved in the biosynthetic pathway include proteolytic cleavages, glycosylation, phosphorylation, amidation and N-terminal acetylation.[100-106] Proteolysis and N-terminal acetylation have profound effects on the biological activities of the product peptides, and these processes are tissue and species specific.[101] Various acetylated, phosphorylated and/or glycosylated products have been isolated from rat intermediate pituitary.[102-106]

Enkephalins - Various sizes of precursor peptides which contain enkephalin sequences were isolated from bovine adrenal medulla, pituitary and hypothalamus.[107-109] The nucleotide sequences of complete cDNA copies of enkephalin precursor mRNA from human pheochromocytoma[96] and bovine[94,95] and human[97] adrenal medulla were reported. The corresponding amino acid sequences show that the precursors are about 265 amino acids long and contain one Leu-enkephalin and six Met-enkephalin sequences.[110]

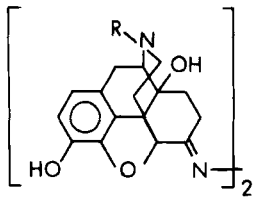
Dynorphin (DYN) - Since the identification of the tridecapeptide DYN-13,[111] several DYN-related peptides have been characterized. These are DYN-(1-8),[114,115,116] a 4000 dalton DYN-32 which contains heptadecapeptide DYN-A[117] and another tridecapeptide DYN-B[117] (which has been independently called rimorphin),[118] and α- and β-neoendorphin.[112-114] All of these peptides contain a Leu-enkephalin sequence at the N-terminus followed by two basic amino acid residues. Immunocytochemical studies suggested a close relationship between distribution of DYN-A, DYN-(1-8)

and α-neoendorphin,[115,116] which is supported by the structure, deduced by the cloning method, of a precursor peptide from porcine hypothalmus.[98] This pro-DYN contains all three DYN-related peptides (fig. 1). β-Neoendorphin, DYN-A and DYN-B are each bounded by Lys-Arg. This protein, like proenkephalin-A and POMC, comprises multiple repetitive units.[98]

OPIATE RECEPTORS

Four subtypes of opiate receptors which now have been well characterized are μ-, δ-, κ- and ε-receptors. σ-Receptors, originally proposed to account for the psychomimetic activity of SKF10047 (N-allylnormetazocine) and related compounds, are now believed to be non-opiate and perhaps related to putative phencyclidine receptors.[119,120]

μ-Receptors - The distribution of μ-opiate receptors in monkey cerebral cortex was determined by autoradiographic visualization of ^{3}H-naloxone binding.[121] In the rat, the so-called μ_1-receptor sites appear to mediate the morphine-induced release of prolactin, but not growth hormone.[122] Evidence supporting the concept that μ_1-receptors mediate antinociceptive activity continued to accumulate.[123,124] The irreversible antagonist activity of naloxazone is now believed to be attributed to the divalent azine naloxonazine, 1; more potent azines naltrexonazine (2) and oxymorphonazine (3) have also been described.[124]



1 R=allyl
2 R=CH_2◁
3 R=Me

Several μ-receptor selective ligands were reported. An octapeptide somatostatin analog, SMS 201-995, is μ-selective *in vitro* and has antagonist properties *in vivo* (vs. morphine, mouse tail flick).[125] Syndaphalin (SD-25), Tyr-D-Met(O)-Gly-MePhe-ol, has an IC_{50} value of 0.29 nM in displacing the binding of the μ-agonist ^{3}H-dihydromorphine.[126,127] Its potency in displacing δ- and κ-agonists is at least 3000-fold lower, and it is thus as selective as morphiceptin[128] and DAGO[129] for μ-receptors. SD-25 is 23,000 times as active as morphine in the guinea pig ileum assay, and nine times as active in the tail flick analgesic test (s.c.). The cyclic enkephalin analog, Tyr-cyclo-(NY-D-A_2bu-Gly-Phe-Leu), was shown to be selective for μ-receptors, both in isolated tissues and receptor binding studies.[130] The analgesic activity of morphiceptin and β-casomorphin correlated with their μ-receptor binding affinity.[131]

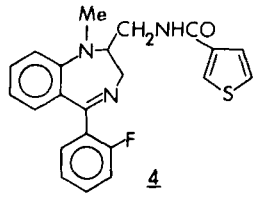
δ-Receptors - Many neuroblastoma cell lines contain homogenous δ-receptors and this system has been extensively used to study the mechanism of opioid action. Down-regulation of these receptors was produced by opioid peptides but not by μ-, κ- or σ-agonists.[132-134] Rather, down-regulation was actually blocked by classical agonists and antagonists.[132,133] This process evidently involves receptor-mediated endocytosis.[135,136] Opioid peptides appear to reduce adenylate cyclase activity in neuroblastoma-glioma hybrid cells by stimulation of GTP hydrolysis.[137,138] Such models serve well to distinguish agonist/antagonist drug profiles.[139]

Two conformationally constrained cyclic enkephalin analogs, [2-D-penicillamine, 5-L-cysteine]- and [2-D-penicillamine, 5-D-cysteine]enkephalinamide, were shown to be δ-selective ligands in mouse vas deferens and rat brain membranes.[140] The novel compound N,N-bisallyl-Tyr-Gly-Gly-ψ(CH_2S)-Phe-Leu-OH (ICI 154129) was shown to be a selective δ-receptor antagonist in the mouse vas deferens.[141] Furthermore, ICI 154129 antag-

onized the slowing of the etorphine-induced head turn in rats,[142] which appears to be an in vivo model of δ-receptor activation.[142,143] Certain strongly δ-selective enkephalin dimers were reported (vide infra).

κ-Receptors - The existence of κ-receptors is now well established by: (1) the selectivity of dynorphins and α-neoendorphin for receptors in isolated tissues;[144,145] (2) the remaining κ-binding sites in guinea pig brain and spinal cord after suppression of the μ- and δ-binding sites with highly selective μ- and δ-agonists;[146,147] (3) the unique distribution of κ-binding sites in the cerebral cortex in guinea pig brain utilizing autoradiographical localization;[148] (4) κ-specific receptors on human placental membranes[149] and rabbit vas deferens;[145] and finally (5) the discovery of several new κ-specific compounds.[150-155] κ-Agonists have diuretic activity in the rat;[156] their analgesic activity is mainly at the spinal level.[157]

The benzodiazepine analog, tifluadom (4), which is devoid of benzodiazepine activity, was shown to be an orally effective κ-opioid analgesic which produced no respiratory depression in rats, was not self-applied by rhesus monkeys, and did not elicit opiate withdrawal symptoms.[150,151] Another recently discovered κ-selective agonist is U-50488H (5),[152-155] which displays analgesic actions in a variety of antinociceptive assays in rodents, and causes opioid receptor mediated sedation, diuresis and corticosteroid elevation. U-50488H does not produce physical dependence, but produces tolerance upon chronic administration. Receptor binding, cross tolerance, and behavioral models distinguish its effects from those of morphine, a μ-agonist.



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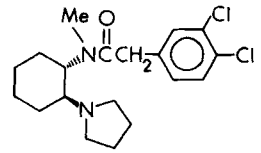


5

The irreversible μ-antagonist β-flunaltrexamine blocked the activity of normorphine and Leu-enkephalin, but not of dynorphin or ethylketazocine, in the guinea pig ileum, which further suggests that DYN acts predominantly at κ-receptors.[158] A guinea pig brain membrane preparation containing only κ-receptor sites can be obtained by treating membranes with β-chlornaltrexamine in the presence of dynorphin.[159]

ε-Receptors - Detailed studies of the activities of β-endorphin and fragments on ε-receptors of rat vas deferens were reported.[160,161]

NEW LIGANDS, STRUCTURAL STUDIES, SAR

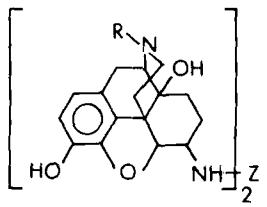
Quaternary Narcotics - There is increasing interest in development of opiate agonists and antagonists which are excluded from the CNS. Quaternization of classical narcotics provides peripherally active opiate agonists such as N-methylmorphine and antagonists such as N-methylnalorphine and N-methylnaloxone.[162-166] N-Methylmorphine was an active analgesic in the acetic acid-induced writhing test in mice, but was inactive in the hot plate test.[163] Its activity in the writhing test was antagonized by both naloxone and N-methylnalorphine, which indicates a peripheral component to antinociception in this model. N-Methylnalorphine was ineffective against the central antinociceptive action of morphine[163] but effectively antagonized morphine-reduced GI tract transit of a charcoal meal.[164] How-

ever, such peripheral selectivity is transient, possibly due to biological N-demethylation.[164] Levallorphan allyl bromide (CN32191) was found to be more peripherally selective than naloxone methyl bromide.[165] The N-methylnalorphine isomer with the greatest affinity for μ- and δ-receptors was shown to have the N-allyl group in an equatorial configuration.[166]

Opioid Dimers - Certain dimeric (bivalent) ligands, formed by covalent linkage of two opioids through a bridging chain, have been reported to have enhanced potency and receptor sub-type selectivity compared with the corresponding monovalent ligands. These effects may arise from receptor cross-linking. Dimers derived from β-endorphin,[167] enkephalins,[168-170] tetrapeptide enkephalin analogs,[171] naltrexamine,[172,173] and oxymorphamine[168,173] have been studied. The optimal bridging distance appears to vary with receptor sub-type,[169-171] and with the agonist or antagonist nature of the monomeric units.[173] The most potent and selective δ-ligand from a series of enkephalin dimers was DPE_2 (6).[170] A selectivity inversion was found with DTE_{12} (7), a dimer of the μ-selective ligand H-Tyr-D-Ala-Gly-Phe-NH_2. DTE_{12} was a δ-selective ligand which had nearly 1000-fold greater δ/μ selectivity than the μ-selective monomer. Disulfide 8 was 7-fold more potent in the ileum than the corresponding thiol.[168] Dimers 9 and 10 appear to be selective antagonists for κ- and δ-receptors, respectively.[172] In the ileum, the most potent μ-antagonist was 11 and the most potent agonist was 12.[173]

$[H\text{-}Tyr\text{-}D\text{-}Ala\text{-}Gly\text{-}Phe\text{-}Leu\text{-}NH\text{-}CH_2]_2$ $[H\text{-}Tyr\text{-}D\text{-}Ala\text{-}Gly\text{-}Phe\text{-}NH\text{-}(CH_2)_6]_2$

6 DPE_2 7 DTE_{12}



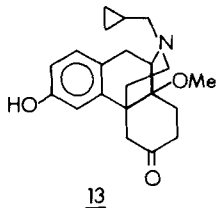
8 R=CH_3; Z=CH_2CH_2-S-S-CH_2CH_2-
9 R=CH_2-◁; Z=$(CH_2CH_2O)_2CH_2CH_2$-
10 R=CH_2-◁; Z=$(CH_2CH_2O)_5CH_2CH_2$-
11 R=CH_2-◁; Z=CO-CH_2CH_2-CO
12 R=CH_3; Z=$[(COCH_2NH)_2COCH_2]_2$-

Enkephalin Analogs - Numerous studies have examined the structures of the enkephalins and their analogs to identify those elements important for their activity. Low energy conformations were sought by computational[174-176] and spectroscopic[177] methods. The in vitro opiate activities of a group of analogs variously substituted in the 4- and 5-residues were studied by QSAR, and good correlations were found with electronic, hydrophobic and steric parameters.[178] Several studies with conformationally restrained enkephalin analogs were reported.[130,140,179,180] Replacements of the Gly-Gly amide bond of Met-enkephalin by CH_2-CH_2,[181] and the Tyr-Gly bond of Leu-enkephalin by CH=CH,[182] result in complete retention of binding activity. The Phe residue was also successfully replaced by α,β-dehydro-Phe, to give analogs with enhanced affinity for δ-receptors.[183] It was reported that replacement of the Tyr residue by Trp at the 27-position of human β-endorphin improves potency fourfold (i.c.v., mouse tail flick), though binding affinity is slightly reduced.[184] The inactivity of a metazocine/Met-enkephalin hybrid was interpreted as evidence that the tyramine moiety in opiate drugs does not play the same functional role as the Tyr residue in opioid peptides.[185]

Other Analgesics - A series of codeinones and morphinanones containing a 14-OCH_3 group was reported.[186] Compound 13 was regarded as the most interesting, because of its good balance of agonist and antagonist effects

in antinociception in rodents. Placement of acyl or carbethoxy groups at the 7-position of a similar series resulted in generally reduced agonist activity (mouse writhing).[187,188] Both cis and trans 2-aminotetralins 14 produced significant opiate agonist (hot plate) and antagonist (tail flick) effects, though they lack either phenolic hydroxyls or quaternary carbons normally associated with potent analgesic activity.[189] A series of 14 conformationally restricted analogs of profadol proved to have generally reduced analgesic activity (p.o., mouse writhing).[190]

Portoghese, et al., synthesized the enantiomers of erythro-5-methylmethadone (15) and studied their opiate properties and X-ray structures.[191] They concluded that μ-receptors and δ-receptors have slightly different stereochemical requirements, and/or that different compounds in the methadone series interact with opiate receptors in different conformations. The stereochemical properties of opioid receptors were further studied with the enantiomers of 4-phenylpiperidine analgesics.[192] The favored conformations of methadone (16) and several analogs, which were inferred using an empirical computational method,[193] were apparently consistent with Portoghese's hypotheses and with other SAR. Of a large



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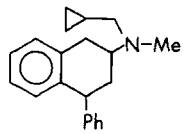

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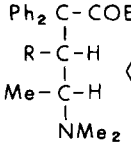
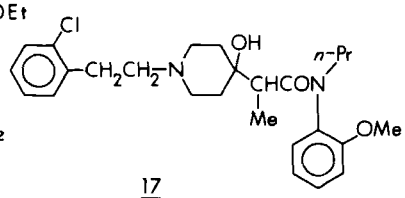


15 R=Me
16 R=H



17

series of analgesic 4-substituted 4-piperidinols, 17 was the most potent in rodents (30 x morphine, s.c.; blocked by naloxone).[194] A previous series of aromatic esters of N-methyl-4-piperidinol was expanded by 16 compounds and the effects of aromatic substitution on analgesia (mouse hot plate) were analyzed by QSAR.[195] Low-energy conformations of various analgesic 4-phenylpiperidines were predicted by computational methods and compared to conformations seen by X-ray cyrstallography.[196]

Other Topics - The analgesic properties of the peptides Tyr-Arg (kyotorphin),[74,197,198] Tyr-D-Arg,[199] cyclo(-Tyr-Arg) and analogs,[200] and Thr-Ser-Lys-Tyr-Arg (neo-kyotorphin)[201] were studied. Reviews on GABA-ergic analgesia,[202] the possible roles of substance P in nociception and neuronal function,[203-206] and the role of endogeneous opioids in appetite regulation[207] were provided.

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Section II - Pharmacodynamic Agents

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Chapter 7. Pulmonary and Antiallergy Agents

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Introduction - Compounds of specific interest with respect to allergy and asthma are discussed in some of the following sections. Research on arachidonic acid and its various metabolites proceeded at a brisk pace in 1982. Several general reviews dealing with cyclooxygenase and/or lipoxygenase products have appeared.[1-5] However, until clinical data appear on agents affecting arachidonic acid metabolites, whether in inhibiting synthesis of leukotrienes, blocking release or effects of SRS, or inhibiting lipoxygenase products, the clinical benefit of this type of agent remains unclear.

Slow Reacting Substance (SRS) and Leukotrienes (LTs) - Several significant papers have appeared which describe new synthetic preparations of leukotrienes. A stereospecific synthesis of leukotrienes from 2-deoxy-D-ribose has been described,[6] and LTC_4, LTD_4, and LTE_4 have been prepared from 5-HPETE by a stereoselective, biomimetic route.[7] Eight synthetic isomers of LTD_4 have been synthesized and compared. Only the 5S,6R,7,9-trans,11,14-cis isomer was identical to biologically-generated guinea pig LTD_4 in all respects.[8] In another study involving analogs of LTC_4 and LTD_4, it was found that the 5-OH and carboxyl groups were essential for SRS activity but variation of the peptide moiety only partially decreased activity.[9] Sulfones of LTC_4, LTD_4 and LTE_4 have been prepared from the corresponding LTs.[10] They were biologically similar to the LTs with about half the potency.[11]

LTC_4 administered by aerosol was shown to cause bronchoconstriction in normal human volunteers,[12] while in cultures of human lung cells both LTC_4 and LTD_4[13] were found to increase mucus production. In monkeys, LTC_4 caused a decrease in pulmonary compliance, contracted isolated tracheal spirals and reduced circulating leukocytes.[14] Studies in guinea pigs have attempted to elucidate the mechanisms of actions of the LTs. In guinea pig tracheal preparations, LTC_4 caused a decrease in cAMP but did not alter cGMP. The contractions caused by LTC_4 in this study were not altered by indomethacin.[15] In another study, LTC_4 and LTD_4 were shown to release TXA_2 and prostaglandin-like material from guinea pig lung and induce contraction in parenchymal strips. Both these actions were inhibited by imidazole, a thromboxane synthetase inhibitor.[16]

SRS Antagonists - Several compounds other than FPL 55712 and its analogs antagonize the actions of SRS. The bromo derivative 1 selectively inhibited the SRS-induced contractions of guinea pig ileum[17] (IC_{50} = 50 μM), while the fused ring analog 2 inhibited with an IC_{50} of 5 μM.[18] BL5255 (3) antagonized SRS and LTD_4-induced contraction

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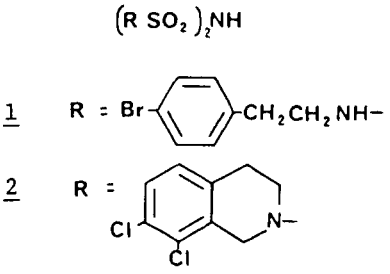
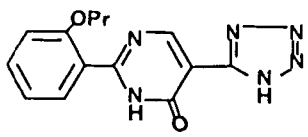


1 R = Br-⟨⟩-CH_2CH_2NH-

2 R =



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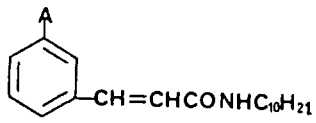
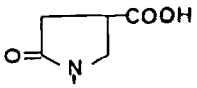
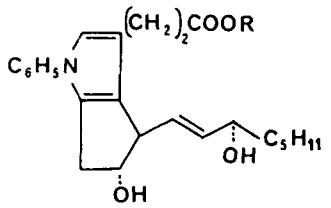
of guinea pig tracheal rings over the concentration range 10^{-6}-10^{-4}M.[19] At the higher concentrations, histamine- and methacholine-induced contractions were also inhibited. The propenamides 4 and 5 are representative of a series that was shown to antagonize SRS actions on guinea pig ileum at 10^{-4}M.[20,21]



4 A = -NHCOCH=CHCOOH



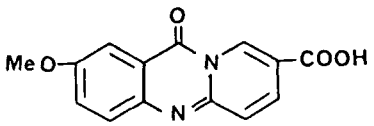
5 A =



6 R = H

7 R = Me

Leukotriene Synthesis Inhibitors - The pyrrole prostacyclin analog U-60,257 (6) and its methyl ester U-56,467 (7) inhibited LTC_4 and LTD_4 synthesis induced by calcium ionophore in human polymorphonuclear leukocytes.[22] U-60,257 did not inhibit formation of 12-HETE in human platelets but did inhibit antigen-induced bronchopulmonary changes in monkey and guinea pig in vivo.[23] Ro 21-7634 (8) inhibited the synthesis



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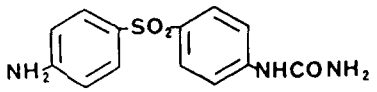

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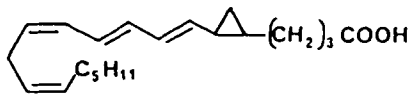
of SRS in rat peritoneal mast cells and TXA_2 synthesis in perfused guinea pig lung at similar concentrations but did not inhibit TXA_2 production induced by direct perfusion of SRS into the lung.[24] A preliminary report indicated that the urea 9 is a selective inhibitor of LTC_4 synthesis.[25] Diethylcarbamazine inhibited LTB_4 and C_4 biosynthesis in calcium ionophore stimulated mouse mastocytoma cells, while cyclooxygenase and lipoxygenase activity were unaffected.[26]

Lipoxygenase Inhibitors - The non-steroidal anti-inflammatory agent benoxaprofen, previously reported to selectively inhibit lipoxygenase at therapeutic levels, failed to be efficacious in aspirin-sensitive patients.[27] In in vitro studies, benoxaprofen inhibited SRS release from human and guinea pig lung following antigen challenge and reduced SRS release in vivo but did not antagonize the actions of SRS.[28] Nafazatrom (Bay g 6575) inhibited tumor cell lipoxygenase activity but not cyclooxygenase product formation at μM concentrations.[29] The 5,6-methano-leukotriene A_4, (KCN-TEI-6173, 10), a stable LT, inhibited 5-lipoxygen-

ase synthesis, (ID_{50} = 18 μM) and did not inhibit prostaglandin (cyclooxygenase) synthesis at concentrations up to 150 μM.[30] The acetylenic



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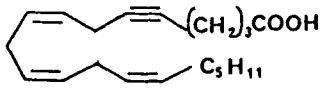

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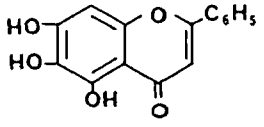
5,6-dehydroarachidonic acid, 11, inhibited 5-lipoxygenase in RBL extracts (K_I = 15 μM) but was much less active in intact cells due to metabolism.[31] The flavonoid baicalein, 12, selectively inhibited platelet lipoxygenase (IC_{50} = 0.12 μM).[32]



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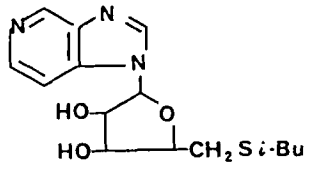

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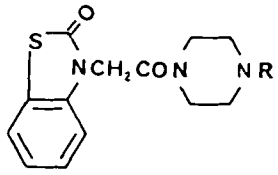
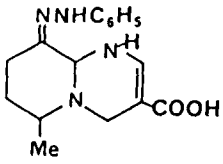
Mediator Release Inhibitors - The imidazopyridine, 3-deaza-SIBA (13), at concentrations of approximately 10^{-4}M, inhibited IgE-mediated histamine release from rat basophilic leukemia cells and histamine release from rat peritoneal mast cells induced by compound 48/80, calcium ionophore, and ATP.[33]



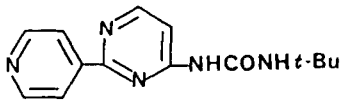
14 R = CH_2CH_2OH
15 R = H



16

In asthmatic patients, tiaramide improved lung function and reduced the need for inhaled beta agonist.[34] Tiaramide (14, RHC 2592-A) and its major metabolite, desethanol tiaramide (15, RHC 3281) inhibited IgE- and IgG-mediated histamine release from rat peritoneal mast cells and guinea pig lung slices, respectively.[35] In other studies, tiaramide inhibited formation of TXA_2 and antagonized bronchoconstrictor mediators with selectivity for $PGF_{2\alpha}$ and SRS in guinea pigs.

The hydrazine derivative 16 had activity 3 x DSCG in the rat PCA test and also inhibited histamine release from rat mast cells.[37,38] The pyrimidine urea 17 inhibited IgE-mediated histamine release from human basophils, as well as the PCA in rats.[39] The oxamate 18 (MTB) inhibited IgE-mediated histamine release from rat peritoneal cells and the PCA reaction in rats and guinea pigs.[40] The orally active antiallergy compound traxanox sodium inhibited histamine release from rat mast cells induced by antigen or compound 48/80 but had only marginal effects on calcium ionophore-induced release.[41] The azaxanthine 19 (M&B 22,948) was 30 x DSCG as a mediator release inhibitor and was



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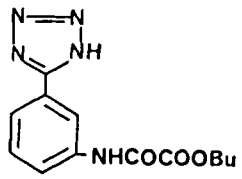

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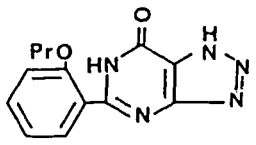


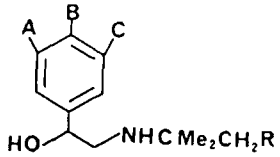
19

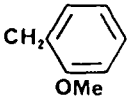
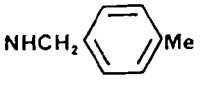
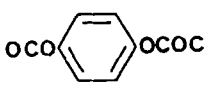
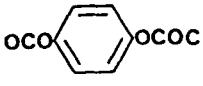
effective against exercise-induced but not histamine-induced bronchoconstriction (fall in FEV_1) in asthmatic patients.[42] TR 2855, a chromone that inhibits antigen-induced release of histamine and SRS from passively sensitized human lung, relaxed human bronchial and guinea pig tracheal muscle and caused bronchodilation in guinea pigs.[43]

β-Adrenoceptor Agonists - Clinical trials in asthmatic patients of the β_2-agonists terbutaline, albuterol, fenoterol and carbuterol failed to differentiate between the group in that all four compounds showed the side effects of tremor and tachycardia.[44,45] Another study with two new highly selective β_2-agonists D-2343 (20) and QH25 (21) also failed to separate bronchodilator and tremorigenic effects and concluded that the β_2-adrenoceptor in bronchial muscle and skeletal muscle are the same.[46] D-2343 and QH25 were found to have a potency of 0.2 x terbutaline and 12 x albuterol, respectively.[47] Although clenbuterol showed a selectivity for β_2-adrenoceptors, its high agonist potency was attributed to high affinity for the β-adrenoceptor rather than efficient adrenoceptor activation.[48] In patients with reversible airway obstruction, procaterol exhibited a dramatic bronchodilator effect with duration up to 12 h.[49]

An FDA study commission has concluded from recent animal studies that cardiac toxicity of β-adrenoceptor agonists was enhanced by concomitant administration of methyl xanthines.[50] There are, however, conflicting reports; in a study of albuterol and theophylline in combination[51] and a second study of theophylline and terbutaline results indicated that low-dose bronchodilator/theophylline combinations gave better bronchodilation with fewer tremors and tachycardia than a high dose of either drug alone.[52] In a different context the combination of fenoterol and ipratropium, an anticholinergic bronchodilator (see below), achieved bronchodilatory efficacy similar to high doses of fenoterol alone but with fewer side effects.[53]

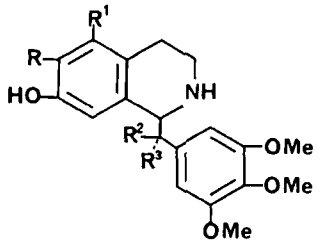
The activity of the terbutaline analog KWD 2131 (22) in bronchial allergen challenge was shown to be due entirely to bronchodilation with



	A	B	C	R
20	H	OH	H	
21		OH	H	H
22	OH	H	OH	OH
23	$OCONMe_2$	H	$OCONMe_2$	H
24		H		H

no antiallergic effect being observed.[54] The terbutaline pro-drugs KWD 2183 (23) and D 2438 (24) were potent bronchodilators in guinea pigs and maintained therapeutic plasma levels in dogs for 23 h and 12 h respectively. These compounds caused a significantly smaller increase in heart rate than did terbutaline.[55]

The bronchodilatory activity of 26 was found to be in the S-isomer as was the case for its parent TMQ (25).[56] The α-methyl derivatives, 27 and 28, were 15- and 4-times more selective for β_2-adrenoceptors over β_1-adrenoceptors than TMQ.[57]



	R	R_1	R_2	R_3	
25	OH	H	H	H	
26	H	OH	H	H	
27	OH	H	Me	H	threo
28	OH	H	H	Me	erythro

A study of the four drugs isoprenaline, terbutaline, albuterol, and soterenol as to the effect of N-aralkyl substitution indicated that this substitution increased β_2- and α_1-adrenoceptor selectivity with the β_2 effect most noticeable with soterenol.[58]

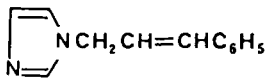
Calcium-Antagonist - Verapamil and nifedipine were found to inhibit exercise-induced bronchoconstriction in asthmatic subjects,[59] while in animal studies nifedipine was found to inhibit bronchoconstriction induced by histamine in both guinea pigs[60] and dogs.[61] In an in vitro study, methoxyverapamil (D-600) inhibited concanavalin A-induced histamine release and calcium ion influx in rat peritoneal mast cells but did not inhibit compound 48/80-induced histamine release.[62] Verapamil inhibited experimental anaphylaxis in guinea pig tracheal smooth muscle but failed to inhibit histamine release from tracheal mast cells.[63] Nifedipine inhibited the PCA reaction in rats with a potency 50 x DSCG but did not inhibit skin reactions induced by histamine or methacholine.[64]

Corticosteroids - The mechanism whereby anti-inflammatory steroids inhibit IgE-mediated histamine release from human leukocytes was explored. It was shown that overnight culture of cells with dexamethasone did not alter either total or occupied F_c receptor number and indirect considerations eliminated the possibility the cAMP levels had changed.[65] Additionally, it was found that dexamethasone failed to inhibit histamine release from human leukocytes induced by TPA, A23187 or F-met-leu-phe[65] or from purified human lung mast cells challenged with anti-IgE.[66]

Budesonide inhibited antigen-induced bronchoconstriction in actively sensitized guinea pigs immunized to produce both IgE and IgG antibodies but failed to affect bronchoconstriction in animals that only produced IgG antibodies.[67] Following overnight preincubation, betamethasone inhibited release of SRS from passively sensitized human lung but did not affect histamine release.[68] In two clinical studies[69,70] dealing with hayfever and chronic asthma, budesonide was found to be

superior to beclomethasone and produced no observable side effects. In a separate study, beclomethasone propionate aerosol produced marked improvement in patients with chronic allergic asthma.[71] Inhaled flunisolide significantly controlled symptoms in patients with perennial rhinitis with no adverse steroidal effects.[72] In a six-month study in patients with chronic airflow obstruction, prednisolone failed to show improvement.[73]

Miscellaneous - The imidazole 29 (SQ 80,338) and the prostaglandin analog 30 (SQ 24775), both inhibited arachidonate-induced bronchoconstriction in the guinea pig but failed to alter histamine or antigen-induced bronchoconstriction. SQ 80,338 inhibited release of TXA_2 from



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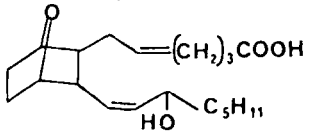

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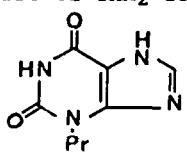
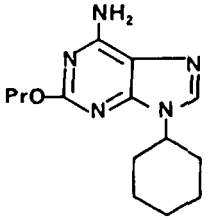


31

isolated guinea pig lung.[74] Astemizole, a histamine H_1-antagonist with high affinity and long duration, was effective for allergic rhinitis with no sedative or cholinergic side effects.[75,76] Clinical studies of terfenadine (RMI 9918) have shown it to be an effective anti-H_1 antihistamine with no anticholinergic, CNS or CV side effects.[77-79] In guinea pigs and monkeys terfenadine produced no adrenergic or histamine H_2-antagonism.[80] Amrinone, a cardiotonic compound, has been shown to inhibit bronchoconstriction in dogs and tracheal contractions in guinea pigs induced by histamine.[81]

The xanthine, enprofylline (31), showed bronchodilator activity 5 x theophylline with no emetic, CNS or renal effects. Lack of CNS effects in asthmatic patients were reportedly due to absence of a 1-nitrogen substituent.[82] BB1502 (32) was reported to be a bronchodilator with long duration in allergic asthma in guinea pigs when administered prior to allergic challenge.[83] The pyrimidobenzothiazole 33 and its 8-chloro analog 34 inhibited allergic bronchospasm in rats with ED_{50}s of 0.38 and 0.11 mg/kg, respectively.[84] Inhaled thiazinamium chloride



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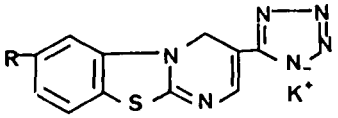
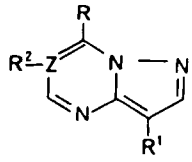


33 R = H

34 R = Cl

showed potent bronchodilator activity with rapid onset and moderate duration and no motor or CV effects in guinea pigs and monkeys.[85]

Inhaled ipratropium bromide, an anticholinergic agent, was found to be a clinically effective bronchodilator with a low incidence of side effects.[86-89] The pyrazolo-pyrimidine 35 and the pyrazolotriazine 36 were PDE inhibitors with potencies 15x and 67x theophylline respectively. 36 was selective for lung PDE over heart PDE and protected against histamine-induced bronchospasm in guinea pigs.[90,91]



35 R = SEt, R¹=Et, R²Z = CCO₂Et

36 R = NHPr, R¹=Br, R²Z = N

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Chapter 8. Antihypertensive Agents

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Introduction - In the past decade, significant advances have been made in the treatment of hypertension,[1,2] offering great potential for prolonging lifetime for large numbers of patients.[3,4] The results of four important mild hypertension trials have been critically analyzed,[5a] and the beneficial trends from lowered blood pressure in fatal myocardial infarcts and cerebrovascular complications continue to be demonstrated. However, a multiple risk factor intervention trial raised questions about the value of primary intervention with antihypertensive drugs. The results of this study suggested that the beneficial effects on blood pressure were obscured by undesirable metabolic consequences.[5b]

Provocative results from basic research which may influence future directions include the role of a circulating sodium-transport inhibitor. This factor has been hypothesized to be involved in the pathogenesis of hypertension.[6] Another new point of focus is based on recent clinical reports that inadequate calcium intake may be a previously unrecognized causative factor;[7] in contrast, other studies find a highly significant positive correlation between serum calcium and blood pressure.[8] The situation with calcium is similar to the relationship between sodium and blood pressure which remains equally complex and incompletely understood.[9] The effectiveness of short term sodium restriction as an alternative to medication continues to be documented.[10] The abnormality of cell membrane sodium handling in hypertensive patients remains confused[11] but has received additional experimental support.[12] It is generally accepted that improved therapeutic agents are needed, and interest in new methods and models for hypertension research remains high.[13] In terms of therapy, a growing concern relates to the adverse effect of certain antihypertensive drugs on lipid metabolism.[14]

Centrally Acting Antihypertensive Agents - It is well established that neuronal systems play a major role in the increased peripheral resistance in hypertension.[15] In the brain, some of the adrenergic pathways are linked to cholinergic mechanisms; this pathway may be involved in the antihypertensive action of certain drugs, e.g. clonidine.[16] Since the CNS regulates sympathetic outflow by a complex interaction of different neuronal centers, the brain remains an important site for blood pressure regulation.[17]

The search for selective α_2-adrenoceptor agonists with affinity for specific sites such as brain stem nuclei has continued. Since most or all of clonidine's therapeutic and undesirable effects are mediated by α_2-receptors,[18,19] more specific agents conceivably could be developed based on a greater affinity for the central "hypotensive" receptors.[20] The cellular events underlying clonidine's action are beginning to be understood. The molecular mechanism involves an α_2-adrenoceptor mediated fall in cellular cAMP levels as a consequence of inhibition of membrane-bound adenylate cyclase.[21] Some of the newer α_2-agonists belong to the

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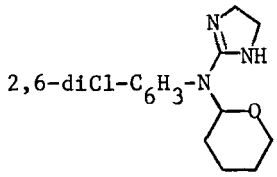
imidazoline,[22] tetrahydropyrroloimidazole[23] and hydrazinotriazole[24] classes. At the clinical level, the sedative liability of these agents continues to be a problem.[25]

Among the newer agents operating by a mechanism other than α_2-stimulation are LR-99853[26] (1) and CP-804-S.[27] The former, although a derivative of clonidine, does not appear to act via α-adrenoceptor stimulation. With the latter, selective blockade of central α_1-receptors may be important. The role of central opioid peptides in blood pressure control continues to be studied, but a coherent concept of the mechanisms involved has not yet emerged.[28] The role of phenylethanolamine N-methyltransferase in central blood pressure control remains attractive, but α-adrenoceptor blocking properties of the PNMT inhibitors need to be taken into account when interpreting the cardiovascular effects.[29]



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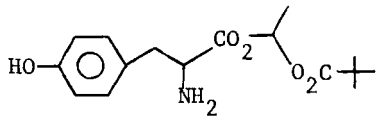
Effecting octopamine,[30] tyrosine and tryptophan levels may provide new approaches to the central regulation of blood pressure. Tryptophan lowers blood pressure by uptake into the brain and conversion to serotonin.[31a] The tyrosine progenitor (2) was more orally effective than tyrosine in reducing blood pressure in SHR. A separation between antihypertensive and locomotor depressive effects suggested that a mechanism other than α_2-adrenergic stimulation was involved.[31b]



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Renin-Angiotensin System - Interest in the renin-angiotensin system (RAS) as an approach to lower elevated blood pressure remains high as new compounds, particularly those which inhibit angiotensin converting enzyme (ACE), continue to prove efficacious in the long-term treatment of essential hypertension.[32,33] Approaches to inhibit angiotensin II formation, as well as its blockade of renin release, continue to be studied.

D-600, a calcium channel blocker, abolished angiotensin II mediated inhibition of renin release without affecting isoproterenol stimulation of renin release.[34] Although renin secretion resulting from low-frequency renal nerve stimulation was not altered by α-adrenoceptor blockade in dogs,[35] phentolamine increased plasma renin activity in patients with essential hypertension.[36] This finding confirmed the inhibitory effect of α-adrenoceptors in the control of renin release. A proposed role for adenosine in the regulation of renin release has been reviewed,[37] but several criteria must be established before an intrarenal role for adenosine can be established.

Tonin, an enzyme capable of forming angiotensin II directly from angiotensinogen, has been shown to activate human amniotic fluid renin.[38] Thus, tonin shares with other enzymes, notably trypsin, pepsin and kallikrein, the ability to activate renin. Its presence in renal parenchyma raises the interesting possibility that it may have an important physiological role in activation of renin.

Potent and specific inhibitors of human renin such as H-77, H-113 and H-142 were designed by modifying the N-terminal sequence of human angiotensinogen at the scissile Leu-Leu bond.[39,40]

Captopril, when used in small doses, has been associated with sufficient modulation of the RAS to reduce arterial pressure.[41] Several proposed mechanisms are under active investigation, including inhibition of the vasopressor action of the RAS[42] and the sympathetic nervous system,[43] increased prostaglandin activity[44] and bradykinin accumulation,[45] and decreased vascular responsiveness to α-adrenergic agonists.[46] Several studies with enalapril (MK-421) demonstrate that its mechanism of action, like captopril, is elusive.[47,48] In addition to these established agents, new ACE inhibitors have been described.[49-55] The strategy used in the synthesis of these agents utilized a peptide-like structure free of a mercapto function, an approach used successfully in the design of enalapril.

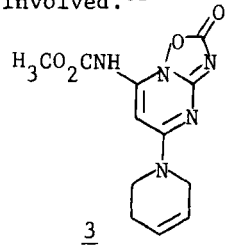
The mechanism by which the RAS system affects aldosterone production is through a direct effect on the adrenal glomerulosa cells via angiotensin II-induced increases in phosphatidyl inositol turnover, which in turn alters cellular calcium distribution.[56]

Vasodilators - Recent studies with vasodilators have involved established agents or structural analogs of such agents. Among these, prazosin has been shown to have favorable effects on blood lipids.[57,58] However, in combination with β-blockers, a favorable lipid profile has not always been achieved.[57] The pharmacology of prazosin and its use in hypertension have been reviewed.[58] The prazosin analog doxazosin (UK-33274) lowered blood pressure in normal volunteers and increased heart rate. The inhibition of α_1-receptors was implicated in the mechanism of action.[59]

Several limited clinical studies with known vasodilators have been reported. In one of these, tolmesoxide lowered blood pressure and increased heart rate in hypertensive patients. As with other vasodilators, it had a short plasma half-life but a long duration of action.[60]

The triple combination of endralazine, a β-blocker, and a diuretic controlled blood pressure in resistant hypertensives. Heart rate increased acutely but returned to base line during chronic administration. Tolerance to the antihypertensive effect did not develop.[61] The pharmacology, toxicology and clinical experience with the structurally related cadralazine have been summarized. This hydralazine analog, either alone or in combination, was effective and well tolerated.[62]

Among the newer agents, the synthesis of RO-124713 (3), a vasodilator related to minoxidil, has been described.[63] In addition, the preparation and pharmacology of the peripheral vasodilator HL-725 (4) have been summarized. Mechanistically, inhibition of phosphodiesterase may be involved.[64] A series of compounds, exemplified by 5, was hypotensive in rats. Receptor studies with 5 using [^{3}H]-prazosin suggested that inhibition of central and peripheral α-receptors was the mechanism involved.[65]



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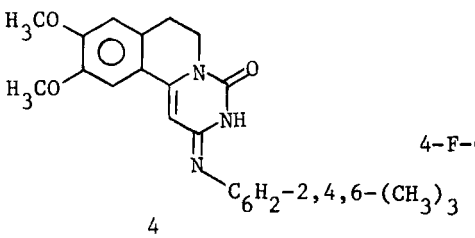

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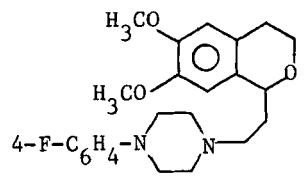

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An in vitro method for comparing the inhibitory effects of vasodilators has been developed. This technique depends on comparing the reduction of phenylephrine versus barium induced tension. Using this ratio, nifedipine, papaverine and dantrolene were distinguishable.[66]

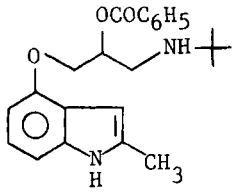
Calcium Entry Blockers - The role of calcium entry blockers (see also Chapter 9) in the therapy of hypertension remains to be defined. They are particularly useful as third step therapy when hypertension and angina are present. In addition to their effects on calcium flux, other mechanisms may be involved, such as a decrease in adrenal responsiveness to angiotensin II[67] and inhibition of calmodulin activation of calcium dependent myosin light chain kinase.[68] In support of this latter mechanism, verapamil and proadifen were shown to interact directly with calmodulin at a different site than the phenothiazines.[69] The role of calcium in the mediation of the vasoconstrictor effects of selective α_1 and α_2-agonists continues to be discussed and debated.[70] Verapamil has been shown to inhibit more readily blood vessel contractions due to α_1 than to α_2-selective agonists.[71] Others suggest that calcium entry blockers inhibit the α_2-receptor mediated component of vasoconstriction.[72]

Both the use of calcium entry blockers in hypertension[73] and the clinical experiences with specific agents, e.g. diltiazem[74] and nifedipine,[75] have been reviewed. The potential of the latter in angina, hypertension, infarction, cardiomyopathy[76] and experimental cardiac hypertrophy[77] has been summarized. Reports continue to appear on the combination of nifedipine with β-adrenergic blocking agents such as metoprolol, timolol and propranolol. The antihypertensive effect was potentiated, but fluid retention occurred if diuretic therapy was not part of the regimen.[78]

β-Adrenoceptor Antagonists - Evidence continues to accumulate in support of potential therapeutic advantages for β-adrenoceptor blocking agents. Whether such advantages, developed around individual agents, can be generalized to the class remains highly controversial. In the area of cardioprotection, sotalol in a multicenter trial lowered the rate of reinfarction. However the mortality rate, although slightly lower, was not significantly different for the treated group.[79] In animal studies, infarct size was significantly reduced by timolol; a combination of hemodynamic and metabolic effects was responsible for protection of the ischemic myocardial tissue.[80] It has been suggested that propranolol's cardioprotective effect may be partly due to a reduction in thromboxane induced vasoconstriction and to inhibition of platelet aggregation.[81]

Further studies demonstrated that nadolol does not decrease glomerular filtration rate or renal blood flow while normalizing blood pressure.[82] The renal effect of nadolol has been reviewed as part of a complete summary on the clinical experience with this agent.[83] However, a renal component may not be specific for nadolol, since it has been hypothesized that there is a direct effect by β-adrenoceptor antagonists on the kidney tubules, which is believed to be exerted via inhibition of the sympathetic control of tubular sodium and water reabsorption.[84]

Extensive clinical studies with pindolol have been described. The compound was effective in reducing blood pressure both alone and in combination therapy with only slight or clinically insignificant reductions in pulse rate.[85] Bopindolol (6), a compound structurally related to pindolol, was effective once-a-day in reducing blood pressure in man without pronounced bradycardia.[86]



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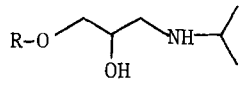
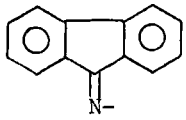


7: R =



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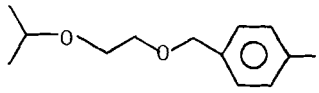
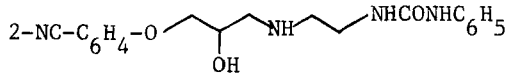


The use of chiral three carbon precursors derived from D-mannitol is being applied with greater frequency in the synthesis of the optically pure enantiomers of β-adrenoceptor antagonists. Both enantiomers of IPS-339 (7) have been prepared by this strategy.[87,88] In vitro only modest β_2-selectivity was found for IPS-339 and no chiral preference for the (S) enantiomer was observed. An explanation for this lack of chiral discrimination was based on symmetry considerations.[88] This same chiral methodology has also been applied to the synthesis of (R) and (S)-pindolol.[89]

β_1-Selective Adrenoceptor Antagonists - Atenolol and metoprolol were compared in hypertensive patients with asthma. The former was better tolerated and caused significantly fewer asthmatic attacks.[90] The β_1-selective antagonist, EMD-33512 (8), a compound structurally related to metoprolol and betaxolol, was ten-fold more effective than metoprolol in reducing heart rate.[91]

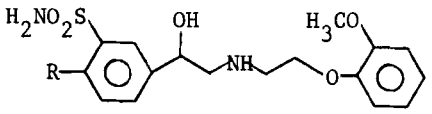
A series of 1-heteroaryl and 1-phenoxy-3-[[(substituted amido)-alkyl]amino]-2-propanols represented by 9 has provided further evidence that the aminoalkyl moiety is an important structural determinant of β_1-receptor selectivity.[92]



9

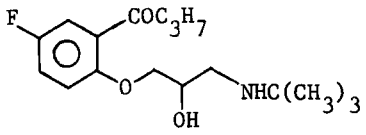
Vasodilator/β-Adrenoceptor Antagonists - Prizidilol, the structural hybrid of hydralazine and a β-adrenoceptor antagonist, lowered blood pressure acutely but raised heart rate.[93] In longer term studies,[94] blood pressure reductions were achieved without tachycardia; however, increases in plasma volume without changes in body weight were observed. In addition to the prizidilol type of vasodilator/β-blocker, studies have continued on compounds in which vasodilation is induced by α-receptor blockade. The synthesis of the four stereoisomers of labetalol by classical resolution has been reported.[95] The (R,R) isomer (Sch 19927) is a β-receptor antagonist with intrinsic sympathomimetic activity and little α-blocking ability. The (S,R) isomer possesses most of the α-adrenoceptor antagonism.[96] Structural modification of labetalol yielded

compounds having a greatly enhanced α_1-blocking component. YM-09538 (10), YM-09649 (11) and YM-09686 (12) were competitive α and β-antagonists in vitro. Using the atria and vas deferens preparations from rats, YM-09686 was 100-fold more potent in blocking the α than the β-receptor; YM-09538 was equipotent at both.[97,98]



10: R = CH_3
11: R = OCH_3
12: R = OH

In addition to β-receptor blockade, a papaverine-like action on smooth muscle has been described for butofilolol (13).[99] A vasodilating action has been demonstrated in man with BM-12434 (14); the mechanism of action is unknown but is assumed to be a direct one on the blood vessel.[100] In the dog a β-agonist component was involved in the action of sulfinolol, MK-761 and pindolol. In the rat a β-receptor agonist mediated mechanism was implicated with sulfinolol and pindolol but not MK-761 or prizidilol.[101]



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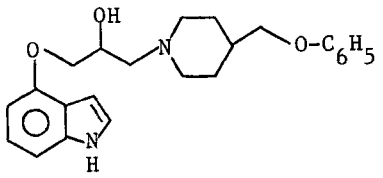
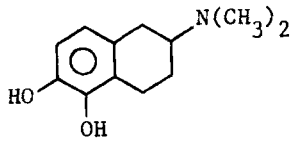


14

Miscellaneous - In man, ketanserin caused dilatation of resistance and capacitance vessels and of the renal vascular bed.[102] Studies in rats suggest that the hypotensive effect of ketanserin involves peripheral α_1 rather than 5-HT receptor antagonism.[103] An alternative mechanism of action was suggested by studies in rabbit femoral arteries; ketanserin inhibited the amplifying effect of 5-HT on the vascular responses to other vasoconstrictor substances.[104]

The peripheral dopamine receptors have been reviewed with emphasis on the potential for dopamine agonists as multitarget antihypertensive agents.[105] The dopamine agonist M-7 (15) reduced blood pressure and heart rate in rats via stimulation of presynaptic dopamine receptors on sympathetic neurons to the vasculature and presynaptic α_2-receptors on cardiac sympathetic nerve endings.[106] SKF-82526 (16), a dopamine agonist specific for the renal dopamine receptor, blunted the development of hypertension in spontaneously hypertensive rats.[107]



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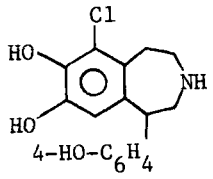

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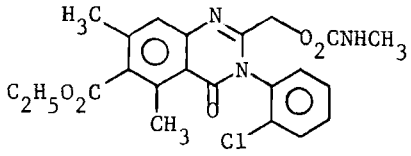
A series of quinazolinones exemplified by EG-1088 (17) reduced blood pressure in rabbits and SH rats; no single mechanism was identified.[108] Blood pressure and heart rate lowering effects were observed in rats with the oxabicyclooctanamine (18) and related examples.[109] On oral administration 15-deoxy-16-hydroxy-16-vinyl PGE_2 (DHV-PGE_2) produced a rapid and long lasting hypotensive effect in rats; topically DHV-PGE_2 was active at 30 µg/kg and a dose of 1 mg/kg had a duration greater than 24 hr. The hypotensive activity of DHV-PGE_2 methyl ester has been confirmed in man, and prolonged effects were observed using transdermal delivery.[110]



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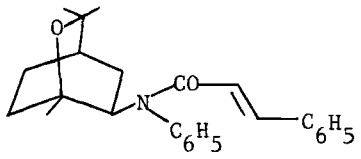

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Chapter 9. Calcium Antagonists - New Opportunities

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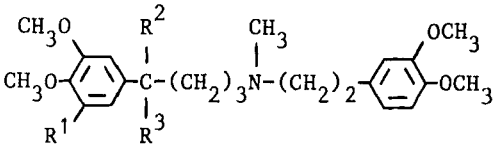
Introduction - Only three compounds from the class of calcium antagonists - verapamil, nifedipine and diltiazem - have been approved by the FDA for the treatment of angina. Results from animal experiments and recent clinical data give rise to the hope that other disorders (hypertension, asthma, migraine, etc.), which can be caused by spasms of the smooth muscle, will also respond to this new mode of action.[1]

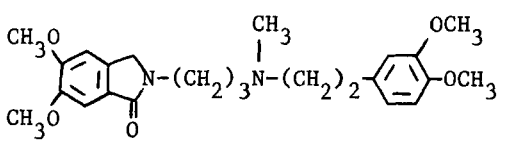
The medicinal chemistry of the calcium antagonists is characterised at present by a large number of new development compounds - comparable to the development of ß-blockers in the seventies.[2-4] However, the majority of these new products resembles in their structure the clinically established calcium antagonists verapamil, nifedipine and diltiazem. In addition, some cardiovascular drugs, which have already been introduced or are being developed, have been claimed to be calcium antagonists. Completely new structures whose pharmacological action is primarily due to inhibition of the influx of calcium into contractile cells are still rare.

Investigation of the binding sites of the basic structures having calcium-antagonistic action should give new leads for drug design.

New Drugs: Verapamil Analogues - Gallopamil (D 600, 1) is superior in potency to the parent substance verapamil, but the activity profile is largely comparable. This also applies to the enantiomers. (-)-Gallopamil has pure "calcium-antagonistic" properties (lowering the blood pressure and the dp/dt_{max} at and above 0.02 mg/kg i.v. in the rat), whereas the (+)-isomer has a significantly weaker effect in this model. However, the (+)-isomer shows an interesting antiarrhythmic effect at higher doses, possibly due to an additional stabilising effect on the membrane.[5] The greater calcium-antagonistic effect of the (-)-isomer can also be demonstrated *in vitro* on the guinea-pig ileum.[6]



1 R^1= OCH_3 R^2= iPr R^3= CN
2 R^1= H R^2R^3= $SO_2(CH_2)_3SO_2$



3

The achiral tiapamil (2) is markedly less effective than verapamil in most *in vitro* models. However, it is equally active on the potassium-depolarised coronary artery of the dog, and this leads to the conclusion that it has a certain vascular selectivity. Consistent with this, the hypotensive and negative inotropic effects are less pronounced.[7-8]

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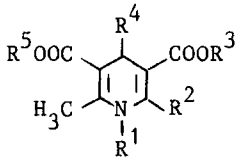
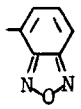
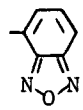
The condensed verapamil analogue AQ-A 39 (3) has been demonstrated to have specificity with respect to a bradycardic effect.[9,10] The heart rate of anaesthetised cats is decreased as a function of the dose above 0.1 mg/kg, whereas the contractility and blood pressure are only lowered at a dose 4-6 times higher. In contrast to this, the hypotensive and negative inotropic effect of verapamil occur at lower doses than the bradycardic effect.

Energies of interaction between receptor models and verapamil derivatives have been determined using the monopole-bond polarisibility method. As a result of the highly significant correlation between the calculated binding energy and the negative inotropic effect on papillary muscle of the cat, the amino acid arginine has been postulated as the possible binding site for verapamil and its derivatives.[11]

Nifedipine Analogues - Because of the possibility of wide structural variation and their superior potency, the 1,4-dihydropyridines (DHPs) of the nifedipine type are the class of calcium antagonists which has been most extensively investigated. The second generation of DHP development candidates having the greatest potency are all chiral, owing to non-identical ester functions,[12] and can exist in enantiomeric forms differing in the absolute configuration at C_4.[13] The research aim is to achieve vascular selectivity or specificity.



	R^1	R^2	R^3	R^4	R^5
4	H	CH_3	$CH_2CH_2OCH_3$	$3\text{-}NO_2\text{-}C_6H_4$	$CH(CH_3)_2$
5	H	CH_3	$(CH_2)_2N(CH_3)CH_2C_6H_5$	$3\text{-}NO_2\text{-}C_6H_4$	CH_3
6	H	CH_3	C_2H_5	$3\text{-}NO_2\text{-}C_6H_4$	CH_3
7	H	CH_3	CH_3	$2\text{-}NO_2\text{-}C_6H_4$	$CH_2CH(CH_3)_2$
8	H	CH_3	C_2H_5	$2,3\text{-}Cl_2\text{-}C_6H_3$	CH_3
9	H	CH_2OH	C_2H_5	$3\text{-}NO_2\text{-}C_6H_4$	C_2H_5
10	H	CN	CH_3	$3\text{-}NO_2\text{-}C_6H_4$	$CH(CH_3)_2$
11	$(CH_2)_2N(CH_2)_2O$	CH_3	C_2H_5	$2\text{-}CF_3\text{-}C_6H_4$	C_2H_5
12	H	CH_3	C_2H_5		C_2H_5
13	H	CH_3	CH_3		$CH(CH_3)_2$

The first step in the biotransformation[14] of nimodipine (4), which preferentially dilates cerebral vessels,[15] is probably ether cleavage of the methoxyethyl ester function. The metabolites have only weak or no calcium-antagonistic activity. (-)-Nimodipine is significantly more active on rabbit aorta strips than the (+)-antipode.[16]

Stereoselectivity is also found with the enantiomers of nicardipine (5), which has been marketed in Japan as a cerebral vasodilator since 1981. (+)-Nicardipine is about 3x more potent than the (-)-isomer in increasing the vertebral flow and lowering the blood pressure after i.v. administration to anaesthetised dogs.[17]

Nitrendipine (6) is a DHP calcium antagonist which is particularly suitable as an antihypertensive agent because of its selective vasodilatation. Blood pressure of dogs with renal hypertension is lowered by doses as low as 0.03 mg/kg p.o.[18] The IC_{50} for inhibition of potassium-induced contractions of rabbit aorta strips is 2×10^{-9}M for (-)-nitrendipine, which is 10x lower than that of the (+)-isomer.[19]

The outstanding vasodilator effectiveness of nisoldipine (7) has been demonstrated in a study of the hemodynamics in anaesthetised dogs: a dose of 5 µg/kg i.v. doubled the flow in the coronary sinus and at the same time reduced peripheral resistance.[20]

Felodipine (8) is characterised by pronounced peripheral vasodilatation, so it appears to be particularly interesting for therapy of hypertension.[21]

FR 7534 (9)[22] and FR 34235 (10) are DHP development compounds which are substituted in the 2-position (R^2) by hydroxymethyl and cyano, respectively, in place of the customary methyl group. Furthermore, FR 34235 has non-identical ester groups and has been shown to be highly active in a chronic coronary occlusion model in the dog: infusion of 0.75 µg/kg/min brought about a significant rise in the blood flow in the normal myocardium and the ischemic subepicardium.[23]

Flordipine (11) is the first DHP development compound with a substituent on the nitrogen atom. It exhibits antihypertensive activity in rats and dogs by the oral route. Vascular selectivity has been demonstrated: vasoconstriction is attenuated in veins at doses similar to that of nifedipine, whereas in arteries it is about 10x less effective (*in vitro* studies).[24,25]

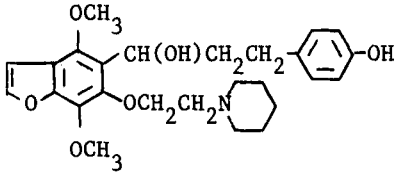
PY 108-068 (12) antagonizes the calcium-induced contractions of the rabbit aorta in concentrations comparable to nifedipine.[26] Preliminary findings in anaesthetised cats (50 µg/kg i.v.) and anaesthetised dogs (10 µg/kg i.v.) show, in contrast to nifedipine, a bradycardic effect, but no data have been published of studies in conscious animals in which reflex tachycardia might occur.[27,28]

PN 200-110 (13) resembles PY 108-068 in being characterised by benzoxadiazolyl substitution in the 4-position of the DHP nucleus, but it has non-identical ester groups. The superior effect of DHPs with non-identical ester functions is again shown by this example: PN 200-110 is 100x more potent than the achiral PY 108-068 on the coronary artery of the dog *in vitro*.[29]

An interesting correlation between the puckering of the DHP ring found by X-ray structure analysis and the calcium-antagonistic activity has been demonstrated: in the series of 4-phenyl-substituted DHPs, an ortho substituent brings about a very slight deviation from planarity of the DHP ring.[30] This is apparently an important criterion for high activity.

The chemistry of dihydropyridines has been dealt with in a recent review.[31]

New Structures - The benzofuran derivative piprofurol (14) is the most potent calcium antagonist in vitro aside from the DHPs. It has an IC_{50} of 2x 10^{-8}M in the potassium-depolarised coronary artery of the dog and thus is markedly more active than verapamil,[32] which it resembles in its profile of action in vivo (increase in coronary flow, lowering of blood pressure, bradycardia and antiarrhythmic properties). Good antihypertensive activity in the dog (at and above 1 mg/kg p.o.) is noteworthy.[33]



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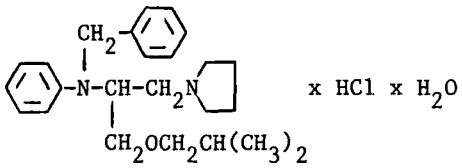
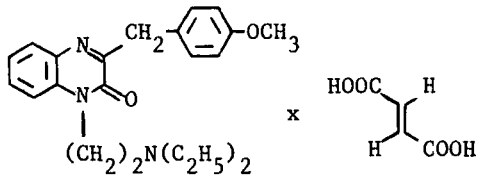


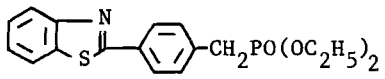
15

Another calcium antagonist having a basic structural element is bepridil (15), which is less potent than verapamil on rabbit aorta.[34] A dose of 5 mg/kg i.v. shows a marked protective effect against myocardial ischaemia after arterial occlusion (dog).[35] An antianginal effect, at 100 mg p.o. t.i.d., and good toleration have been demonstrated in a clinical double-blind study.[36]

Caroverine fumarate (16) has been commercially available as a spasmolytic for a number of years in some countries. Recent investigations have indicated that it has a calcium-antagonistic mechanism of action. Caroverine has an interesting structural relationship to diltiazem, in which a similarly substituted thiepine ring is fused to a benzene ring. Potassium-induced contractions of the rat aorta are suppressed at significantly lower concentrations (10^{-6}M) than those induced by noradrenaline. Moreover, an inhibition of $^{45}Ca^{2+}$ uptake in the potassium-depolarised aorta has been detected at a similar concentration.[37] In addition, the electrophysiological effects are comparable to those of calcium antagonists of the verapamil type, but the negative inotropic effect is markedly lower.[38]



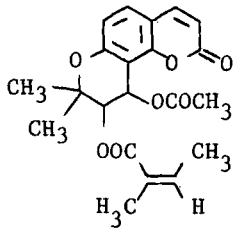
16



17

Benzothiazolylbenzylphosphonate KB-944 (17) is a completely new structure having a calcium-antagonistic action without an essential basic structural element. Potassium-induced contractions of the coronary artery of the dog are inhibited as a function of dose above 10^{-7}M.[39] Its potency and profile of action in vivo are comparable to those of diltiazem. In dogs, doses at and above 0.1 mg/kg i.v. lead to a rise in coronary sinus outflow and to a decrease in the myocardial oxygen consumption. Its

antiarrhythmic effect has been demonstrated in a variety of models. In spontaneous and renal hypertensive rats, blood pressure is lowered at and above 3 mg/kg p.o.[40]



18

Therapy with calcium antagonists is not completely new. Pyranocoumarin (18), which was isolated from extracts of roots of Peuce-danum praeruptorum Dunn, is the active principle of an old Chinese folk medicine having spasmolytic activity. Its calcium-antagonistic action has been confirmed by inhibition of potassium-induced contractions of the guinea-pig ileum and inhibition of the $^{45}Ca^{2+}$ uptake.[41]

New Indications: Acute Myocardial Ischemia, Myocardial Infarction - In isolated beating heart preparations verapamil (VER), nifedipine (NIF) and diltiazem (DIL) prevent the ischemic increase in mitochondrial Ca^{2+} content, O_2 consumption, breakdown of myocardial ATP and enzyme leakage, and improve postischemic heart contractility.[42-45] The protective action of NIF[46,47] and VER[47] correlated with the degree of negative inotropic effect during pretreatment of hearts in preischemic perfusion. In cultured neonate rat myocytes, both VER and NIF inhibited LDH release induced by hypoxic incubation.[48] In rats NIF and VER administered before ligation of the coronary artery, reduced the degree of ATP deficiency, metabolic acidosis and depression of cardiac contractility.[49] NIF and nisoldipine completely prevent ventricular fibrillation and death and reduce the total number of ectopic beats after coronary ligation in rats.[50] NIF, DIL, nisoldipine, gallopamil, bepridil[51] and FR 7534[52,53] all reduce the "area of risk" or the infarct size after coronary ligation in dogs. It is not clear whether calcium antagonists diminish the infarct size by the unloading effect of peripheral vasodilation,[54] increase of collateral circulation in the heart[55] or by specific inhibition of ischemic influx of extracellular calcium with consequent mitochondrial damage and depletion of high energy phosphate stores.[56] Proof for the therapeutic or prophylactic utility of calcium antagonists in acute myocardial infarction will have to await the completion of clinical trials in progress.[57]

Cardioplegia - The addition of NIF[58-60] or VER[61,62] to cardioplegic solutions improves reperfusion and cardiac viability after cardiopulmonary bypass in dogs. Preliminary reports seem to confirm the beneficial effect of NIF in human surgery.[57]

Cerebrovascular Disorders - Nimodipine (NIM) improves the impaired reperfusion in the brain[63] and reduces mortality[64] after cerebral ischemia in cats, and prevents stroke and death in stroke-prone spontaneously hypertensive rats.[65]

Flunarizine prevents the reduction of cerebral cortical blood flow occuring after full circulatory arrest in dogs.[66] NIM nearly doubles

cerebral blood flow in the delayed post-ischemic hypoperfusion and improves neurological recovery in dogs with complete cerebral ischemia due to temporary aortic ligation.[67]

Ongoing clinical trials have demonstrated the protective effect of NIM against cerebrovascular spasm after aneurysm surgery in humans,[68] increased cerebral blood flow in the ischemic brain of post-stroke patients,[69] and a prophylactic effect in patients with migraine.[70]

Pulmonary Hypertension - NIF, VER, DIL, gallopamil and prenylamine inhibit K^+-induced contractions and Ca^{2+} uptake in isolated guinea-pig pulmonary arteries.[71] Nisoldipine[72,73] and nitrendipine (NIT)[73] dilate the pulmonary vascular bed in cats depending on the existing tone level of pulmonary vessels. Nisoldipine specifically prevented pulmonary vasoconstriction in cats produced by an endoperoxide analogue but failed to affect vasoconstrictor response to norepinephrine or serotonin.[72]

Clinically beneficial results were obtained in numerous short-term studies in patients with primary pulmonary hypertension, congenital heart disease, pulmonary fibrosis or hypoxic pulmonary vasoconstriction.[57] NIF maintains its therapeutical effect in pulmonary hypertension patients continuously treated over 4 months.[74]

Bronchial Asthma - NIF[75] and nicardipine (but not flunarizine, cinnarizine and VER[76]) inhibited histamine or acetylcholine-induced contractions of guinea-pig tracheal preparations. In vivo, NIF attenuated histamine-induced bronchoconstriction in guinea-pigs.[76] In clinical trials, VER[77] and NIF[78] inhibited exercise-induced asthma. NIF also inhibits skin reactions to histamine, compound 48/80 and acetylcholine, and inhibits $^{45}Ca^{2+}$ uptake in mast cells stimulated by antigen IgE antibody.[79]

Atherosclerosis - VER[80,81] and DIL[81] prevent arterial calcinosis produced by overdoses of vitamin D_3 or dihydrotachysterol. VER prevents arterial calcinosis and eye cataracts in alloxan diabetic rats.[81] NIF reduces the development of atherosclerotic plaques and the increase in aortic cholesterol and Ca^{2+} content in cholesterol-fed rabbits.[82-84]

Tissue Damage - VER and NIF inhibit the development of hypertension and Ca^{2+} overload of the arterial wall in spontaneously hypertensive rats.[85] NIF prevents malignant hypertension and fibrinoid necrosis of arterial vessels[86] and induces healing of already existing vascular lesions[87] in salt-sensitive Dahl rats. NIM prevents cerebro- and renovascular necrosis and death in stroke-prone spontaneously hypertensive rats without affecting the high blood pressure.[65] Flunarizine protects rat brain against hypoxic structural damage induced by unilateral carotid occlusion and repeated exposure to a nitrogen atmosphere.[88] As calcium overload obviously plays an ultimate role in tissue necrosis,[89] its specific prevention by calcium antagonists promises fundamental progress in the pharmacotherapy of a broad variety of diseases.

Receptor Pharmacology Aspects: Alterations in transmembranal fluxes of cations, e.g., Ca^{2+}, exert intracellular reactions of enormous potency. Features of the molecular mechanism of enhancing the intracellular concentration of free (ionized) Ca^{2+} - a concomitant incidence of excitation-contraction processes - involve certain plasma membrane structures and "channels" to translocate membrane signals into cellular responses. It is generally accepted that ion currents, e.g., Na^+, Ca^{2+}, are controlled by distinct loci associated with membrane channels mediating these ionic

fluxes. Two major types have been postulated for Ca^{2+}:[90] potential-dependent Ca^{2+}-channels are activated by electrical signals, e.g., depolarization, whereas receptor-operated Ca^{2+}-channels are stimulated preferentially by membrane interaction of compounds via receptor sites.[91-94] Ca^{2+} influxes can be either enhanced by a variety of cardiostimulatory drugs, e.g., adrenaline[95] or theophylline (for further refs. see [96]) or inhibited by a structurally heterogeneous group of compounds, the "calcium antagonists",[97] "slow channel inhibitors",[98] or "Ca^{2+} entry blockers"[99] which are thought to bind to specific receptor sites and subsequently exert their pharmacologic effects. The original report on a high-affinity binding site for DHP calcium antagonists was presented in 1981, demonstrating specific binding of ^{3}H-nitrendipine (^{3}H-NIT) to cardiac membranes;[100] these results were subsequently confirmed for smooth muscle[101] and brain preparations.[102] The biochemical properties of the radioligand binding experiments are generally consistent with an interaction at a site (or sites) where DHPs exert their pharmacologic action. Binding of ^{3}H-NIT or ^{3}H-nimodipine (^{3}H-NIM)[103] is reversible, saturable and of high affinity. The equilibrium dissociation constants range between K_D=0.1-0.3 nM using ^{3}H-NIT, but, in addition a low-affinity binding component for ^{3}H-NIT was also reported in heart[100] and brain membranes. Equilibrium binding data of the neuro- and psychopharmacologically active[16,104] ^{3}H-NIM (unspecific binding 8-20%) gave a K_D value of 1.1 nM in cortical membranes,[105] and the regional distribution of the DHP receptor in various brain areas showed no major differences in the dissociation constant and receptor density.[106] *Binding of ^{3}H-NIT and ^{3}H-NIM is Ca^{2+}-dependent:*[107-109] removal of Ca^{2+} by chelators elicited a reversible reduction of DHP binding, an effect that was particularly prominent in brain tissue.[107] Modulation of ^{3}H-NIM binding by mono-, bi-, and trivalent cations[107] is generally in accord with electrophysiological findings (for further refs. see [110-112]). Cs^+, Rb^+, Mn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, La^{3+}, Ce^{3+} and Eu^{3+}, which inhibit ^{3}H-NIM or ^{3}H-NIT binding also block Ca^{2+}-channel function; binding seems to be related to the ionic crystal radii,[90] while tissue specificity may correspond to effects of anions and ionic strength.[103] The high specificity of the receptor site is confirmed by inhibition constants (K_i-values) of pharmacologically potent DHP analogues in the nano- or even subnanomolar range.[105-107] In addition, DHP derivatives of weaker affinity also exhibit less potent calcium antagonistic action.[105] The stereoselectivity, an additional criterion of specificity of action, was further evidenced with the enantiomers of NIT,[113] NIM,[107] and (+) isopropyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate (BAY E 6927).[105] The (-)-optical isomers of these DHPs generally displayed greater affinity than the racemates, or the less potent (+)-stereoisomers,[105-107] substantiating that the receptor protein contains a pharmacophoric group that preferentially recognizes the (-)-isomer. The differences in potencies of the binding data (K_i-values) between the individual enantiomers agrees well with the results (IC_{50}-values) of functional pharmacology studies.[13,105,107]

The structurally dissimilar, but pharmacologically active antagonists VER and gallopamil, cinnarizine analogues, prenylamine, bencyclane, tiapamil and others, elicited rather weak displacement of ^{3}H-NIM.[105-107] They may act preferentially via voltage dependency,[114-117] or may bind to another[118] allosteric[102] site shown by only partial inhibition of ^{3}H-NIT, ^{3}H-NIM or ^{3}H-NIF binding by VER or gallopamil,[102,107,119] or DIL.[102,119] ^{3}H-VER was reported to bind to cardiac membranes, but unfortunately revealed 40-55% unspecific binding at the K_D concentrations (4.25 nM) used.[120] The selectivity of the DHP receptor is apparent by the lack of *effects of potent DHPs on other receptors in brain and heart tissue.* Interaction of NIM, NIF, and several other DHP derivatives with ten different

receptors revealed very low affinities.[105] In order to term the DHP binding site a "receptor", the biochemical attributes of radioligand binding properties must correlate with the appropriate pharmacologic responses, e.g., in the rabbit aortic strip or in intact smooth muscle preparations. Binding characteristics of the DHP receptor correlate well (r=0.954) with pharmacologic activities in vascular smooth muscle over five orders of magnitude,[105] substantiating that the DHP receptor is physiologically and pharmacologically significant and that it mediates the action of DHP calcium antagonists. Recently, interaction of slow channel blocking calcium antagonists with myocardial α-adrenoceptors[121] was reported, and certain α-adrenergic antagonists may act as Ca^{2+} channel inhibitors.[122] Close correlation was also found between calcium antagonistic action and relief of vasoconstriction *in vivo* and *in vitro*.[123]

Several authors reported to have identified Ca^{2+}-channels simply by radioligand binding studies using ^{3}H-NIT or ^{3}H-NIM.[109,124,125] However, the mere detection of a binding site is not evidence for a receptor site, and certainly not of such a complex protein structure as a plasma membrane channel. This requires coherent results from binding, transport, electrophysiological, electronmicroscopic, and autoradiographic studies; preliminary data on the latter appeared recently.[126]

Nevertheless, ongoing investigations to correlate tissue selectivity of various newly radiolabelled ^{3}H-DHPs with their pharmacologic profiles may facilitate the understanding of the interaction of calcium entry blockers with the DHP receptor.

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Chapter 10. Agents for the Treatment of Peptic Ulcer Disease

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General - Reduction in gastric acid secretion by histamine H_2-antagonists, anticholinoceptor drugs or by compounds that affect specific enzymes within the parietal cell continues to be the main approach to therapy for peptic ulcers. Some good reviews on the mechanisms of acid production have appeared.[1-3] A comprehensive book on the pharmacology of histamine receptors includes a chapter on structure activity relationships.[4] The role of peptide hormones in the control of acid secretion,[5,6] and the antisecretory and cytoprotective properties of prostaglandins,[7,8] have been reviewed.

Clinical experience with histamine H_2-antagonists and other treatments for ulcers form the basis for many symposia.[9,10] The options for treating peptic ulcers with drugs were discussed in an editorial.[11] The assessment of antisecretory drugs,[12,13] the role of endoscopy[14] in the clinic, and surgical approaches,[15] have been reviewed.

Histamine H_2-Antagonists- The discovery of cimetidine (1) and its clinical success have provided both the rationale and stimulus for the development of improved H_2-antagonists. The pharmacology and clinical use of ranitidine (AH 19065, 2) has been reviewed,[16] and it has been the subject of numerous symposia.[17,18] Ranitidine is 4 to 10 times more potent than cimetidine in inhibiting stimulated gastric secretion in volunteers, and 150mg b.i.d. is clinically effective in healing gastric and duodenal ulcers over 4 to 6 weeks. Ranitidine is more selective than cimetidine in that it does not displace ^{3}H-dihydrotestosterone from androgen binding sites,[19] nor alter hepatic metabolism of drugs since it does not bind to cytochrome P450,[20,21] and it does not augment the response of lymphocytes from patients to *in vitro* mitogenic stimulation.[22] Although cholinomimetic effects of ranitidine have been seen in some gastrointestinal tissues in certain species,[23] they are of no clinical significance as they do not occur in man.[24,25] The parietal cells of patients treated with ranitidine for 1 year appeared normal on examination by light and electron microscopy.[26] With cimetidine over 4 weeks the population of the parietal cells does not change.[27]

Further reviews on cimetidine have appeared,[28,29] including one on the effects of cimetidine on drug metabolism.[30] In a recent clinical trial in 848 patients cimetidine b.i.d. (800mg daily) was equivalent to q.i.d. (1000mg daily).[31] The possibility that cimetidine treatment might raise intragastric nitrosamine levels and predispose to gastric cancer has been explored. In studies measuring intragastric bacterial counts and concentrations of nitrite and N-nitroso compounds during cimetidine treatment, no changes were detected during 24 hour sampling from normal volunteers,[32] but there were significant increases in these parameters in samples taken from peptic ulcer patients after an overnight fast.[33] However, a survey of 9940 patients treated with cimetidine found no evidence of an increased incidence of gastric cancer.[34] N-Nitrosocimetidine (3) was not carcinogenic in rats.[35]

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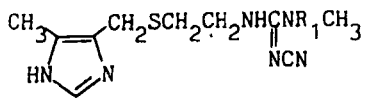
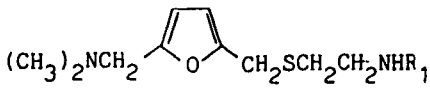
The pyrimidinone (SK&F 93479, 4) was more potent than cimetidine in the rat (x10) and dog (x16) and had a longer duration of action than either cimetidine or ranitidine.[36] In man it inhibited nocturnal gastric acid secretion at 0.6 and 0.9 mg/kg[37] and 40mg.[38] Trials were suspended following the discovery in the rat of some non-malignant changes in the mucosa at doses of 1000mg/kg/day.[39]

In man, oxmetidine (5), 400mg b.i.d., reduced mean gastric acidity over 24 hr. by 59% and it had a similar duration of action to cimetidine.[40] In a clinical trial, oxmetidine, 400mg b.i.d., was equivalent to cimetidine, 1g, in the treatment of duodenal ulcer.[41] Unlike cimetidine, oxmetidine does not penetrate the cerebrospinal fluid after a single iv dose[42] or affect the mixed function oxidase in the liver.[43] It does not raise serum prolactin levels after an i.v. bolus dose of 50 or 200mg,[44] or alter basal levels of gonadotrophins in male patients.[45] It did not affect the parietal cell population.[27]

The background to the discovery of ranitidine, the guanidinothiazole tiotidine (6), and some of SK&F's work on H_2-antagonists and histamine receptors, has been published.[46] Introduction of a benzyl group into histamine did not give new H_2-antagonists, and both H_1- and H_2-agonist activity was reduced.[47]

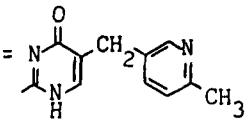
The guanidinothiazole famotidine (YM-11170, 7) lacks the usual NH in the chain. It is 40 and 50 times as potent as cimetidine in inhibiting acid secretion in the dog[48] and pylorus-ligated rat respectively.[49] Doses as low as 5-10mg reduced basal and tetragastrin-induced gastric secretion in man.[50] It was more active than cimetidine in inhibiting gastric lesions in the rat induced by aspirin or indomethacin, and it is not antiandrogenic.[49]

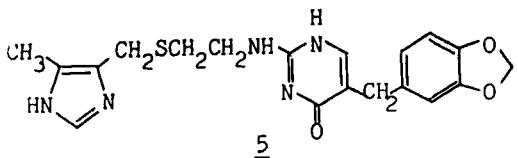




1 R_1 = H 3 R_1 = NO

2 R_1 = $-CNHCH_3$ (=$CHNO_2$) 4 R_1 =





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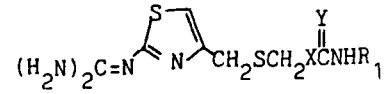


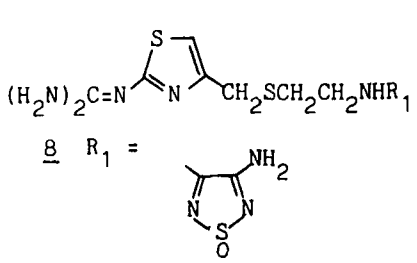
	X	Y	R_1
6	CH_2NH	NCN	CH_3
7	CH_2	NSO_2NH_2	H

One of the most significant developments in this field is the discovery of two new classes of H_2-antagonists with a long duration of action, the thiadiazoles BL-6341A (8),[51] L-643441 (9),[52] and the triazole lamtidine (AH 22216, 10).[53,54] BL-6341A is described as a competitive antagonist on guinea pig atrium, 45 times as potent as cimetidine. In the pylorus-ligated rat, it is 116 times more potent than cimetidine. In the dog 1μmol/kg p.o. is significantly longer acting than an equieffective dose of cimetidine or ranitidine in that 9 hours after dosing inhibition of acid secretion is still >50%.[51] L-643441 was 152 times as potent as cimetidine in

the rat.[52] The Merck group described L-643441 as an irreversible H_2-antagonist on guinea pig atrium.[55] In the dog, it had a similar potency to ranitidine but had a longer duration of action, in that 24 hours after an oral dose of 15mg/kg secretion was reduced by 84%.[56]

The two thiadiazoles (8,9) had significantly less affinity than cimetidine for androgen receptors and did not potentiate hexabarbital sleeping time in mice.[52]

The triazole (10) is an unsurmountable antagonist of histamine on the guinea pig atrium.[53] However in the dog, in vivo, it is a competitive antagonist, 4 to 10 times as potent as ranitidine against various secretagogues, and it has a prolonged duration of action. At 0.1mg/kg p.o., gastric secretion was still inhibited by 50% after 18 hours.[53,54]



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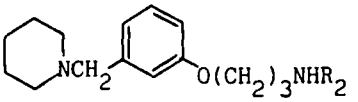
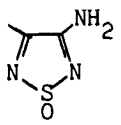


9 R_2 =



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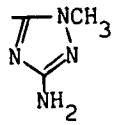


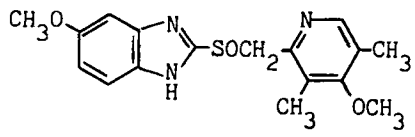
Other Antisecretory Agents

1. Inhibitors of H^+/K^+- ATPase. The benzimidazole omeprazole (H168/69,11), one of a series of compounds which act by inhibiting the parietal cell H^+/K^+-ATPase,[57] was reviewed at a symposium.[58] Omeprazole inhibits acid secretion in the dog (ED_{50} 0.3mg/kg) and at 0.6mg/kg 30-40% inhibition was present after 22-24 hours. Pentagastrin stimulated acid secretion in volunteers was inhibited by 65% at 40mg, and clinically 40mg daily for 4 weeks is effective in the treatment of duodenal ulcer. It has also been used successfully in patients with Zollinger-Ellison syndrome.[59]

2. Tricyclic Compounds. Pirenzepine (12), a selective antimuscarinic agent, has been the subject of another symposium.[60] Clinically it is about as effective as cimetidine but side effects such as dry mouth have been reported. Pirenzepine and atropine have been compared with ranitidine and cimetidine in the anaesthetised dog.[61] The anticholinoceptor agents reduced both gastric and salivary secretions, but the H_2-antagonists selectively inhibited acid secretion. In another study[62] in the rat, pirenzepine had less effect on pupil diameter than on salivary or acid secretion; ED_{50} 1.8, 0.5 and 0.7 mg/kg respectively.

A phenothiazine (LM24056, 13) inhibited gastrin, gastrin plus bethanacol but not histamine stimulated gastric secretion in the dog.[63] The long duration of action (>20 hours) in the dog at 2.5 mg/kg p.o. may in part be due to the formation of an active metabolite, desmethyl LM24056.[64] In vitro, this metabolite had a higher affinity for the muscarinic receptor than LM24056. In man at 100-300mg, LM24056 inhibited nocturnal acid secretion.[65] Dry mouth was a side effect at the highest dose.

Desmethylimipramine (14) was compared with atropine in the rat.[66] Both markedly inhibited acid secretion, but unlike atropine the former did not substantially increase pupil size, cause urinary retention, or increase heart rate. Desmethylimipramine may act at central α_2-adrenoceptors.[66]



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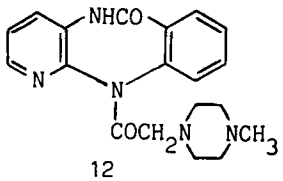

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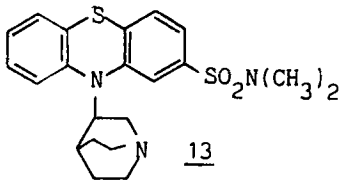

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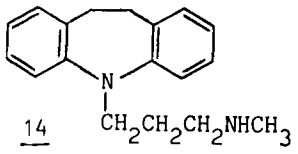

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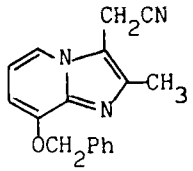
3. Gut Peptides. A number of gut peptides can inhibit gastric acid secretion, but their therapeutic use is limited by their short half life and lack of selectivity.[67] Intracranial administration of peptides such as bombesin, calcitonin, the opioid peptides and neurotensin decreased gastric acid secretion and protected against stress-induced ulcers in the rat.[5] The opiate antagonist naloxone inhibited gastric secretion elicited by intravenous met-enkephalin[68] or sham feeding[69] in man. The protective effect of somatostatin against ethanol induced gastric damage in the rat may involve an interaction with sulfhydryl groups.[70]

4. Other Structures. An imidazopyridine (SCH 28080, 15) inhibited histamine stimulated acid secretion in dogs (ED_{50} 0.09 mg/kg i.v., 4.4 mg/kg p.o).[71] It is 4 to 10 times more potent than cimetidine in the pylorus ligated rat. It prevented aspirin induced gastric lesions in the rat and protected rats from necrosis induced by ethanol (ED_{50} 4mg/kg p.o.). In man, doses of 50-200mg inhibited gastric secretion by a mechanism not related to H_2-blockade or an anticholinoceptor effect.[72]

In the anaesthetised dog, the chromone (FPL 52694, 16) inhibited pentagastrin stimulated acid secretion by 70%.[73] In conscious dogs with gastric fistulae it was more potent given intragastrically than by i.v. infusion.[74] A 32% reduction in pentagastrin stimulated acid secretion was seen in man.[75]

In man, the thioamide (RP 40749, 17), at 2mg/kg p.o., inhibited pentagastrin stimulated gastric secretion by an unknown mechanism.[76]

The amidine fenoctimine (18) inhibits acid secretion in rats and dogs.[77] In the latter, given intragastrically, it resembles cimetidine but shows prolonged activity at a dose of 6mg/kg. It is not an anticholinergic, nor an H_2-antagonist, but may act directly on parietal cells. It inhibits food and pentagastrin stimulated secretion in man and was active 8 hours after dosing.[78]



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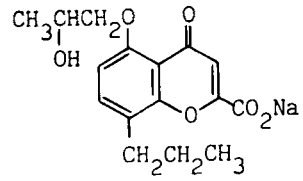

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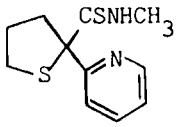

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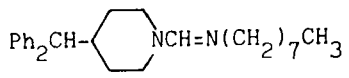

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Prostaglandins - Prostaglandins have antisecretory and cytoprotective properties, and may have a physiological role in peptic ulcer disease. Mechanisms of mucosal damage and of cytoprotection have been reviewed.[79,7] An important defence against mucosal damage may be a "mucus-bicarbonate barrier" consisting of bicarbonate ions trapped below a mucus gel layer covering the gastroduodenal mucosa.[80] A pH gradient across this layer, from a luminal pH of 2 to pH7 at the mucus/mucosa interface, has been measured in rat and human gastric mucosa.[81,82] Aspirin treatment reduced this pH gradient by lowering the intra mucus pH, an effect prevented by 16,16-dimethyl PGE_2.[81,82] 16,16-Dimethyl PGE_2 increased mucus synthesis by rat isolated mucus cells,[83] and increased the mucus gel thickness in the rat.[84] Gastric ulcer patients had gastric mucus with a weak structure.[85] Gastric bicarbonate secretion has been detected in man, and is stimulated by PGs.[86,87]

Other mechanisms involved in cytoprotection could include direct effects of PGs on the mucosal cells. 16,16-Dimethyl PGE_2 (1µg/ml) prevented ethanol induced increases in ^{51}Cr release from rat isolated gastric mucosal cells.[88] A decrease in mucosal DNA levels following ethanol was prevented by 16,16-dimethyl PGE_2 (1µg/kg s.c.) in the rat[89] and by PGE_2 (20µg/ml) in human mucosa.[90] Proliferation of gastric mucosal cells resulted from chronic administration to rats of PGE_2, (15R),15-methyl PGE_2, (arbaprostil, 19),and 16,16-dimethyl PGE_2.[91,92] Although 16,16-dimethyl PGE_2 completely prevented formation of macroscopically visible necrotic lesions in rat gastric mucosa exposed to ethanol or to indomethacin, microscopic analysis revealed that damage to the surface epithelial cells was unaffected or only reduced by the PG.[93,94,95]

Endogenous prostanoids may have a cytoprotective function. Human gastrointestinal mucosa forms PGE_2, $PGF_{2\alpha}$ and PGI_2 from arachidonic acid,[96] and PGE levels are low in gastric mucosal samples from gastric ulcer patients.[97] Arachidonic acid protected rat gastric mucosa from ethanol damage[98] and inhibited gastric acid secretion in canine parietal cells,[99] both effects being prevented by indomethacin. In the rat, paracetamol, sodium salicylate and mild irritants, such as 20% ethanol, increased mucosal PG generation and protected the mucosa from damage by 100% ethanol.[100,101] This effect was prevented by indomethacin. However in the dog, adaptive cytoprotection to bile salt did not occur,[102] and the importance of endogenous PGs to mucosal integrity in this species has been questioned.[103] In man there was no correlation between severity of mucosal damage and degree of reduction of mucosal PGE following 5 days treatment with acetyl salicylic acid.[104]

Both PGE_2 and (15R),15-methyl PGE_2 reduced gastric damage caused by non-steroidal antiinflammatory compounds, and enhanced duodenal ulcer healing in man. Faecal blood loss following indomethacin in rheumatic disease patients was inhibited by oral PGE_2, 0.33mg t.d.s., or (15R),15-methyl PGE_2, 0.05mg t.d.s.,[105] and gastric bleeding induced in volunteers by aspirin was inhibited by oral PGE_2, 0.5mg q.d.s.[106] Duodenal ulcer healing was enhanced by 4 weeks oral treatment with PGE_2, 0.5mg t.d.s. plus 1mg at night,[107] or (15R),15-methyl PGE_2 at 0.1mg q.d.s.[108] PGE_2 does not inhibit gastric acid secretion at these dose levels.[106,107]

16,16-Dimethyl PGE_2, at 0.1-1.0µg/kg p.o., inhibited gastric acid secretion in volunteers,[109] 1µg/kg t.d.s., reducing acidity over 24 hours by 60%.[110] Lower, non-antisecretory dose levels prevented the fall in gastric potential difference caused by aspirin,[111] or sodium taurocholate,[112] but not that caused by ethanol.[113]

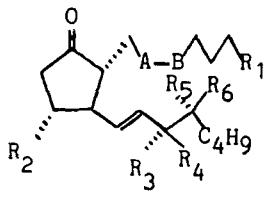
11-Methyl-16,16-dimethyl PGE_2 (RO21-6937, 20) inhibited gastric acid secretion in the dog at doses of 5µg/kg i.v. or 30µg/kg p.o.[114] and also inhibited secretion induced by a test meal in man.[115] RO21-6937 prevented chemically induced gastric or duodenal ulcers in the guinea pig and rat, but was ineffective against gastric lesions caused by ethanol in the rat. It did not produce side effects such as hypotension or diarrhea.[114]

2-Decarboxy-15-deoxy-16-hydroxy-2(hydroxyacetyl)-16-methyl PGE_1 (CL 115,574, 21) prevented experimental ulcers in the rat at doses of 3-100µg/kg p.o., inhibited gastric acid secretion in the rat (100-1000µg/kg s.c.) and dog (5-100µg/kg i.g.), and was free from behavioural effects in the dog at 500µg/kg.[116,117] In volunteers CL 115,574 was well tolerated at doses of 500-1000µg p.o., reduced basal secretion by 85% and pentagastrin-stimulated secretion by 50%.[118]

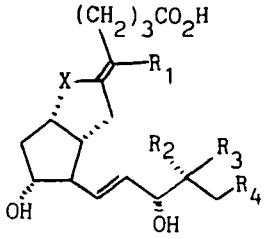
The thia PGE_1 analogue (EMD 33290, 22) was a potent, but short acting inhibitor of basal acid output in man (ED_{50} 20µg/kg p.o.). Doses of 0.7µg/kg and 36µg/kg p.o. prevented the fall in gastric potential difference caused by aspirin and sodium taurocholate, respectively.[119]

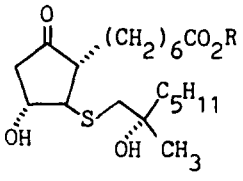
16-Methyl-16-methoxy PGE_1 methyl ester (DL646, 23) was cytoprotective in rats at doses of 0.02-0.1µg/kg p.o., inhibited gastric acid secretion at 15µg/kg i.g., and higher oral doses did not cause diarrhea in mice or rats, or hypotension in dogs.[120]

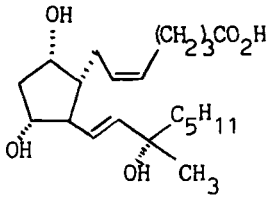
(15S),15-Methyl PGE_2 (24) infused subcutaneously at 0.015-0.03µg/kg/min, (15S),15-methyl $PGF_{2\alpha}$ (carbaprost, 25) at 1-2µg/kg/min s.c., and prostacyclin infused intravenously at 0.125-0.25µg/kg/min all inhibited gastric acid secretion in the rhesus monkey, but stimulated a non-parietal secretion to differing degrees.[121] Prostacyclin reduced aspirin-induced gastric ulceration when infused intravenously in the cat.[122] In the rat two stable prostacyclin analogues, nileprost (26) and ciloprost (27) inhibited gastric secretion, reduced indomethacin lesions[123,124] and neither induced diarrhea[123] nor inhibited it.[124] Thus it is possible to obtain PGs in which cytoprotective and antisecretory properties are separated from side effects such as diarrhea.



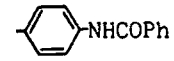
	R_1	R_2	R_3	R_4	R_5	R_6	A-B
19	CO_2H	OH	CH_3	OH	H	H	CH=CH
20	CO_2H	CH_3	OH	H	CH_3	CH_3	CH=CH
21	$COCH_2OH$	OH	H	H	CH_3/OH		CH_2CH_2
23	CO_2CH_3	OH	OH	H	OCH_3	CH_3	CH_2CH_2
24	CO_2H	OH	OH	CH_3	H	H	CH=CH







	R_1	R_2	R_3	X	R_4
26	CN	CH_3/H		O	C_3H_7
27	H	CH_3	H	CH_2	$C{\equiv}CCH_3$

22 R = 

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Non-prostaglandin Mucosal Protectants - Other drugs that increase mucosal resistance include colloidal bismuth subcitrate (De-Nol) and sucralfate, an aluminum salt of sucrose octasulphate.[11] De-Nol and sucralfate form a protective coating on the surface of peptic ulcers.[125,126] Sucralfate also inhibits pepsin and binds bile acids.[127] Both drugs are as effective as cimetidine in the treatment of peptic ulceration and have been the subject of recent symposia.[128,129] Studies have suggested a lower relapse rate after treatment with De-Nol[130] or sucralfate[131] than after cimetidine.

In Vitro Studies On Gastric Mucosa - The importance of histamine in the control of acid secretion was demonstrated in rabbit gastric gland[132] and canine parietal cell preparations,[133] where potentiating interactions occur between histamine and other secretagogues. However, pepsin secretion by isolated rabbit glands[134] or rat chief cells[135] is more responsive to the muscarinic agonist, carbachol. PGE_2 binding sites,[136] stimulation of mucus secretion[83] and cytoprotective effects of PGs[88,90] have also been studied in vitro.

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Chapter 11. Prolonged Ventricular Repolarisation - A Prevention of Severe Arrhythmias?

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Introduction - Prolonging ventricular repolarisation as a means of preventing ventricular arrhythmias has received new attention in the last year. Although it was already suggested in 1970 that an agent which prolonged cardiac repolarisation time could prevent or abolish arrhythmias,[1] it is only with the recent reports of the effects of amiodarone in life-threatening arrhythmias that interest in the mechanics of these effects and the development of new drugs has been revived. This chapter summarises some of the electrophysiological background and will review old and newer drugs which prolong ventricular repolarisation with emphasis on some recent developments.

Origin of the Cardiac Action Potential - Intracellular microelectrode techniques have permitted measurement of the electrical activity of single cardiac cells.[2] In resting fibres, the transmembrane potential, the cell interior, which is negative with respect to extracellular fluid, varies from -60 to -95 mV, depending on the cell type within the heart. This level is mainly determined by intracellular/extracellular potassium concentration ratio. At equilibrium, potassium is concentrated intracellularly and sodium and chloride predominate in extracellular fluid. This non-equilibrium distribution of potassium and sodium ions is maintained by an ongoing expenditure of energy by the cell. The cell actively pumps three sodium ions out of the cell in exchange for two potassium ions and this sodium-potassium transport process is related to membrane ATP-ase activity. Sodium ions are prevented from leaking back in again because the membrane selectively excludes them. The influx of potassium from the outside is accelerated until an equilibrium is attained, i.e., same number are passing in and out per unit time.

Following excitation, the transmembrane action potential undergoes a series of changes, resulting in inscription of the cardiac action potential (fig.). During depolarisation (phase 0 of the action potential), the initiation excitatory current causes a change in the permeability of the membrane and induces a large transit influx of sodium ions. In addition, as the potential becomes more positive, the permeability of the membrane to potassium falls, i.e., inward-going rectification, so that the total conductance during the plateau differs little from the total resting conductance during diastole. This is followed by a second inward current which is smaller and slower than the fast sodium current and which is probably carried by both sodium and calcium ions. When the slow inward current declines as a result of partial inactivation of the channels, the plateau (phases 1 and 2) drifts towards a more negative potential, and repolarisation (phase 3) is accelerated with a positive feedback effect due to deactivation of slow inward current with return of potassium conductance (inward rectification). In some tissues, a background outward potassium current may also contribute to repolarisation. Immediately after completion of phase 0-3 of the action potential, active exchange of sodium and potassium ions is required during phase 4 to restore the cell to its

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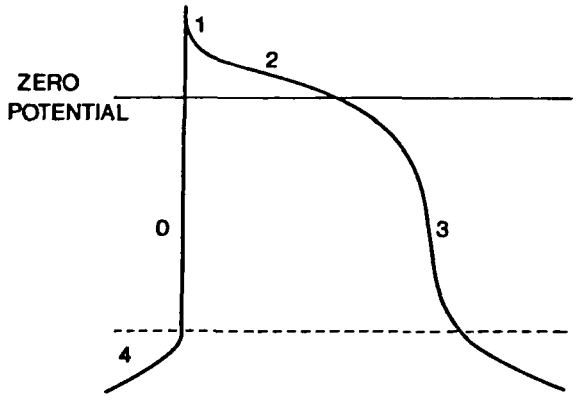
steady ionic composition.



Prolongation of the Ventricular Repolarisation Time - As voltage is the primary determinant of the time course of repolarisation, action potential duration (APD) becomes an estimate of the effective refractory period (ERP), which is the period during which a premature impulse fails to propagate. Action potential duration (APD) varies quite considerably in various regions of the heart.[3] APD in the muscle is about 150 ms shorter than in the Purkinje system, so that there is no possibility of retrograde excitation of the Purkinje fibre by the muscle. The effects of ischemia on the cellular electrophysiology of Purkinje fibres may differ depending on whether the fibres are in the major bundles of the conducting system or are peripheral and subendocardial. Whatever the effects on the individual parts of the Purkinje conducting system, dispersion of the refractory periods between ischemic and adjacent normal areas will distort the critical balance of refractory periods between adjacent areas. An association between decrease in fibrillation threshold and the dispersion of refractoriness in ventricular myocardium under diverse experimental conditions has been shown.[4] A therapeutic intervention designed to interfere should either restore this balance by accelerating or by prolonging repolarisation and refractoriness.

Indirect evidence in humans that prolonging cardiac repolarisation is antiarrhythmic stems from the observation that atrial arrhythmias are common in thyrotoxicosis but are rare in hypothyroidism. In an electrophysiological study of different thyroid states on intracellularly recorded cardiac action potentials, thyroidectomy caused a large and uniform prolongation of the APD.[5] No other electrophysiological parameters in atrial muscle were affected by variations in the thyroid state. An additional argument was the discovery that amiodarone prolonged the monophasic action potential duration (MAPD) with the consequent lengthening of the effective refractory period (ERP) in animals[6] and humans,[7] and is an extremely potent antiarrhythmic drug.[8,9]

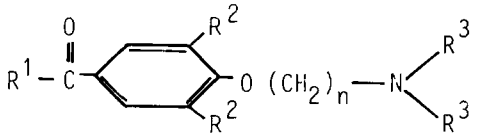
Amiodarone and Derivatives - Amiodarone (1), a benzofuran derivative, was introduced some twenty years ago as an antianginal agent, when it was shown to be a potent smooth muscle relaxant and coronary vasodilator.[8] A moderate degree of noncompetitive inhibitory effect on sympathetic excitation was also evident.[9] It was discovered that chronic amiodarone administration (20 mg/kg daily, i.p.) produced marked lengthening of the atrial and ventricular APD in the rabbit (23.7% after 3 weeks, 33.5% after 6 weeks).[1] The MAPD from the bundle of His in the dog[6] and right atrium in man[7] after 4 weeks of chronic administration was prolonged by approximately 30%.

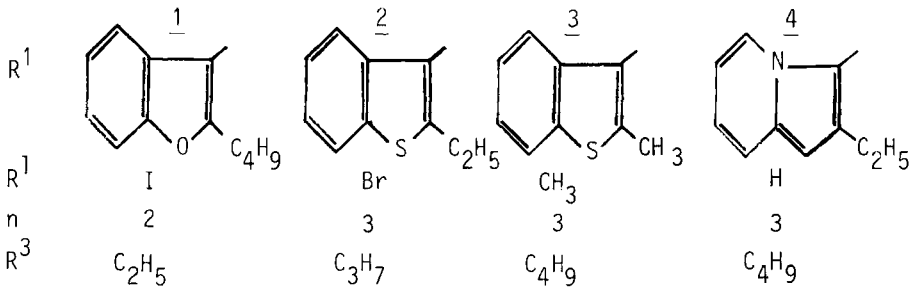
Some differences of electrophysiological effects have been observed after acute and chronic amiodarone administration in man, which may be of clinical significance. After acute intravenous administration (5 mg/kg), the ventricular ERP was unchanged while atrial ERP and anterograde conduction through the AV node was prolonged.[10-13] After chronic oral administration, changes become more prominent and ventricular effective refractory periods in contrast to the effects seen after acute administration are significantly increased.[14]

Although the first reports on its effectiveness in a variety of atrial arrhythmias including those of the Wolff-Parkinson-White syndrome go back to 1970,[15] the impressive results have been only recently reported in patients with recurrent ventricular tachycardia (VT) and fibrillation (VF).[16-25] Amiodarone (2.5-5 mg/kg, i.v.) was also successfully used in 95 patients with various arrhythmias following cardiac operations with extracorporeal circulation.[26] Another interesting observation is that the clinical efficacy of amiodarone in patients with ventricular arrhythmias may not be reliably predicted.[22,27] The reason for this disparity is unclear.

Usage of oral amiodarone in refractory, sustained life-threatening ventricular tachyarrhythmias has been difficult because of insufficient knowledge concerning its pharmacokinetics in man and because of the lack of a sensitive and reliable assay. A simple and rapid HPLC method for determining amiodarone concentration and its desethyl metabolite is now available.[27] After a single oral dose of 1400-1800 mg, oral absorption is slow and erratic.[28] Peak levels are seen after 4.9 hours and serum half-life is 7.2 hours.[29] After a mean dose of 1327 mg/day for 4.1 weeks, the concentration of its desethyl metabolite was about 50 percent that of amiodarone. Serum elimination half-life was 29 days. The recent observation that serum reverse T3 levels correlated closely with arrhythmia suppression and appearance of side effects, will enable units which have no access to HPLC methodology to monitor efficacy and certain side effects.[31]

Although amiodarone has a high therapeutic-to-toxic ratio, the major side effects are either hypo- or hyperthyroidism and pulmonary abnormalities.[32] Pulmonary abnormalities, e.g., diffuse pulmonary fibrosis and hypersensitive pneumonitis, are now recognised as the more serious potential complications of amiodarone.[22,34] Cessation of amiodarone therapy and steroid therapy is recommended. Corneal microdeposits are almost invariably seen in patients treated with amiodarone, but are reversible upon





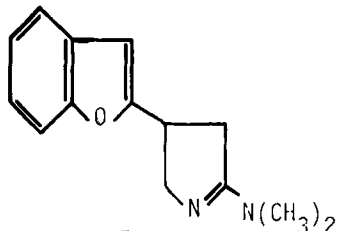
withdrawal of the drug. Cutaneous photosensitivity may appear in 3-10% of patients taking the drug. The frequency is generally dependent on the degree of exposure to the sun, although this is not always so.[34] Amiodarone interacts with several other cardiovascular agents. It increases plasma digoxin levels, probably by a displacement of digoxin from various binding sites, which may result in symptoms of digitalis toxicity.[35] Amiodarone may potentiate the anticoagulant effects of warfarin, and close monitoring of the prothrombin times is required when amiodarone is introduced and the dose of anticoagulant requires alteration.[36] Amiodarone may potentiate the bradycardia in patients on β-adrenoceptor blocker therapy or on calcium antagonists. Two cases have also been reported of torsade de pointe[37] after combination of quinidine and amiodarone. Torsades de pointe have also recently been reported on amiodarone monotherapy.[38,39]

Because of the early finding of hypo- and hyperthyroidism, and the possible relationship to the iodine atoms on the molecule, attempts have been made to synthesise derivatives not containing iodine. A bromo-analog, L-8040 (2) was administered to rabbits for 6 weeks as daily i.p. injections of 20 mg/kg.[40] APD to 90% repolarisation (APD_{90}) increased by a mean of 17% in atrial and 16% in ventricular tissue. Under identical conditions, amiodarone prolonged APD_{90} by 34% and 31%, respectively.[1] L-9146 (3) was found to have electrophysiological effects similar to those of amiodarone.[41] It is an antagonist of both α- and β-adrenoceptor-mediated cardiovascular effects of a noncompetitive type. Chronic administration to rabbits led to a mean prolongation of atrial APD_{90} by 22% (10 mg/kg/day). In ventricular tissues, APD_{90} was markedly prolonged, e.g., by 75% in ventricular wall muscle. The degree of APD_{90} prolongation was dependent on the particular tissue. As a result, APD_{90} became uniform throughout the ventricle. Butoprozine (L-9394, 4) is more active than amiodarone against certain experimental arrhythmias.[43] It was found to decrease the fast sodium and slow calcium inward currents and the delayed outward current in a dose-dependent manner in voltage clamp experiments on frog atrial and ferret ventricular fibres, and thus combines actions common to amiodarone and verapamil.[42] Intravenous injection of doses between 0.5 and 2 mg/kg of 4 led to substantial increases of the effective refractory period (ERP) in the AV node and the retrograde ventriculo-auricular pathway in 32 patients. The drug had no effects on conduction in the atria, His-Purkinje or the ventricle.[43] In patients with supraventricular tachycardias, injection of 4 resulted in termination of intranodal tachycardia (3 of 3 cases) or tachycardia with WPW-syndrome (2 of 3 cases).[44] Prifuroline (14843JL, 5) increased atrial, AV nodal and, to a lesser degree, ventricular ERP in dogs after intravenous administration.[45] In contrast to the effects of amiodarone, 5 also increased His-Purkinje conduction time, thus combining quinidine-like and amiodarone properties.

Bretylium and Other Quaternary Ammonium Compounds - Bretylium tosylate (6) was initally introduced as an antihypertensive agent because it interferes with postganglionic sympathetic nerve transmission.[46] The first prominent effect of bretylium after intravenous administration is its sympathomimetic effect.[47] Release of adrenaline from peripheral adrenergic nerve terminals has been demonstrated in the heart.[48] Bretylium also blocks the uptake of noradrenaline and adrenaline into adrenergic nerve endings.[49] The importance of these adrenergic effects of bretylium for its antiarrhythmic efficacy is not yet established. The direct cardiac electrophysiological effects of bretylium were only demonstrated in the early 70's. Bretylium produces a marked increase in APD and ERP in ventricular muscle and Purkinje fibres[50] even after surgical denervation[51] and this effect is independent of cardiac sympathetic nerve uptake.[52] At higher concentrations, rate of depolarisation, conduction velocity and automaticity were also

affected, demonstrating that electrophysiological effects are similar to amiodarone.[53] Bretylium produced greater increases in APD and ERP in the normal cells bordering the infarct zone than in the infarcted region where APD was prolonged already by ischemia and so reduces disparity between normal and infarcted regions.[54] Bretylium also increases prostaglandin synthesis which in turn increases the fibrillation threshold.[55]

Bretylium has been shown to be effective in a variety of animal models of re-entrant ventricular arrhythmias[56,57] and ventricular fibrillation.[58,59] In the presence of ischemia, bretylium raises the ventricular fibrillation threshold[60] and reduces the occurrence of either spontaneous or induced ventricular fibrillation.[61,62] As absorption in humans is somewhat poor,[63] bretylium is used parenterally in the prevention of recurrences of ventricular fibrillation resistant to most standard antiarrhythmic drugs[64-66] and for patients in cardiopulmonary arrest.[67-69] The most significant adverse effect of parenteral bretylium is hypotension due to adrenergic neuronal blockade, which might respond to protriptyline therapy.[66]



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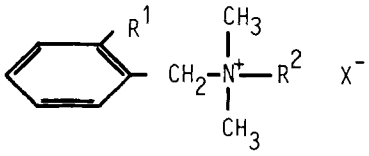
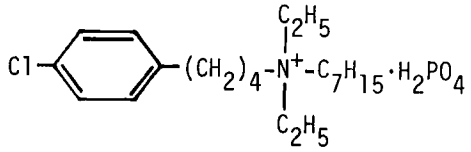


	R^1	R^2	X
6	Br	C_2H_5	$C_7H_7SO_3^-$
7	I	CH_3	Cl^-

As with amiodarone, derivatives or structurally similar compounds, lacking the ganglion blocking effects of bretylium, have been synthesized. UM-360 (7) was shown to lack the catecholamine releasing and adrenergic blocking action of bretylium but still increased the fibrillation threshold.[61] Clofilium phosphate (8) increased APD_{95} maximally by 35%, and ERP by 39%, in isolated canine Purkinje fibres.[70] At the same concentration (3×10^{-7}M), 8 had no effect on rate of rise of diastolic depolarisation, resting potential or action potential amplitude. APD_{95} in atrial muscle was not affected. The ventricular fibrillation threshold (VFT) in dogs was significantly increased after infusion of 0.5 or 1 μM/kg.[71] In 22% of the ventricular fibrillations induced during VFT measurements, spontaneous reversion to sinus rhythm occurred. A comparison of canine Purkinje fibres from infarcted and non-infarcted zones showed clofilium to be more potent in normal tissue with regard to prolongation of APD_{95}, thus leading to nearly uniform APD_{95} in both zones. Radioactive clofilium was concentrated in rat and dog heart tissue.[72] Clofilium reduced the incidence of complex ventricular arrhythmias[73] and was antifibrillatory[74] in a chronically infarcted canine model. In man, i.v. clofilium (60-300 μg/kg) increased ERP by 19 and 13% in atrium and ventricle, respectively, without slowing conduction.[75] Inducible ventricular tachycardia (VT) improved in 5 of 6 patients. In another group of 16 patients with inducible VT, clofilium abolished inducibility by electrical stimulation in five cases.[76]

Sotalol - Apart from having β-adrenoceptor blocking properties,[77] sotalol (9) prolongs APD and ERP in animals[78-82] and man.[83,84] This prolongation is dose-dependent over the range of 2×10^{-6} and 6×10^{-4}M. The effects are more marked in Purkinje fibres than in ventricular muscle. At larger

concentrations of 9 (10^{-3}M), APD and rate of rise of diastolic depolarisation were reduced. Changes in APD and ERP were magnified at slower rates of stimulation.



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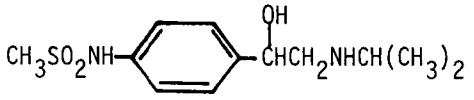
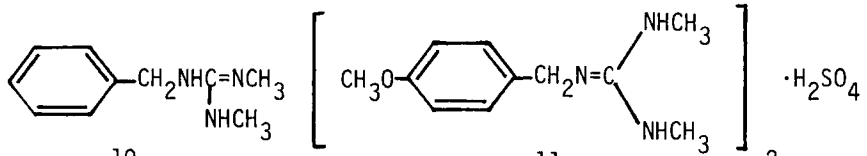


9

In 8 patients with atrial fibrillation, 40-100 mg of 9 (i.v.) saw a consistent increase (13-17%) in the ventricular repolarisation time as measured from ERP and MAPD.[82] In a double-blind study in 17 patients, a significant increase in QT-interval (from 281 to 419 ms), right atrial effective refractory period (from 235 to 289 ms), right ventricular effective refractory period (from 252 to 277 ms), right atrial monophasic action potential duration (from 263 to 307 ms) and right ventricle monophasic action potential duration (from 313 to 344 ms) was observed after 0.3 or 0.6 mg/kg i.v. of 9.[83] These variables were unchanged after 0.15 or 0.2 mg/kg i.v. or propranolol. A dose of 0.4 mg/kg i.v. or 9 prolonged atrial, ventricular and AV nodal effective refractory period and terminated AV re-entrant tachycardia in 5 out of 12 cases.[84] After a dose of 0.6 mg/kg i.v., increases were observed in AV nodal conduction, AV nodal ERP (26%) and right ventricular ERP (6%) in 15 patients.[85] A dose of 1.5 mg/kg produced significant lengthening of AH interval and ERP of right atrium, left atrium and right ventricle, in 9 patients. Retrograde ERP of the accessory pathway was also prolonged in the 3 patients studied.[86] A dose of 1.5 mg/kg i.v. increased atrial ERP from 224 to 264 milliseconds, and ventricular ERP from 209 to 245 milliseconds in 15 patients with accessory atrioventricular pathways.[87] ERP of the accessory pathway was prolonged in anteretrograde (3 out of 3) and retrograde (10 out of 10) direction. Although sotalol has undergone extensive electrophysiological investigations, there have been few recently published reports of the use of both intravenous and oral sotalol in arrhythmias.[88] From the studies which are available, it seems rather difficult to differentiate between those antiarrhythmic effects of sotalol mediated via its β-adrenoceptor blocking activities and its effect on ventricular repolarisation. However, prolongation of the QT_c interval, a measure of APD has uniformly been observed after therapeutic doses.[89,90] Adverse effects are those usually seen after β-adrenoceptor blockade. There have also been reports of torsade de pointe after large doses of sotalol (2.4-8 gm).[91]

Bethanidine and Analogs - The neuronal blocker bethanidine (10) increases VFT in the normal dog heart[92] and converts induced-VF to nonsustained VF with spontaneous conversion to sinus rhythm. Of 23 patients with recurrent multiple drug refractory VT and VF, 18 had complete suppression of spontaneous or electrophysiologically induced tachyarrhythmias; 3 were improved and 2 had no benefit.[97] Protriptylene was necessary to prevent orthostatic hypotension.

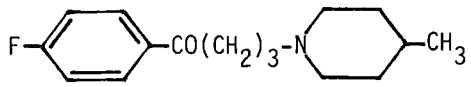


10 11

Meobentine sulphate (11), a bethanidine derivative has no neuronal blocking, sympathomimetic or anticholinergic actions.[94] Twenty mg/kg of

11, i.v., prevented re-initation of VF in 5 dogs for a range of 5-12 hrs.[95] It has no effect on diastolic excitability threshold of either normal (N) or infarct (I) tissue at the time of its antiarrhythmic activity, but it did prolong the ERP at both N (mean increase 25 ms) and I sites (mean increase 69 ms). Prolongations of ERP at I sites were both more marked and of longer duration (167 min) than at N sites (120 min) and correlated with time course of antiarrhythmic activity. 11 has not been tested yet in man.

Melperone - Melperone (FG 5111, 12), a neuroleptic butyrophenone[96] prolonged ERP in isolated guinea-pig papillary muscle without reducing contractility.[97] In anaesthetised, normal dogs, 12 led to substantial increases in atrial ERP, and to lesser increases in ventricular ERP, after i.v. infusion of 0.5-12.5 mg/kg.[98] No effects on atrial or ventricular conduction times were observed. In dogs with experimentally induced atrial fibrillation and flutter, 12 converted the arrhythmias after administration of i.v. doses of 2.5-10 mg/kg.[99] In humans, MAPD was significantly increased by 13% after 10 mg i.v. of 12.[100] In 9 patients with recent myocardial infarction and ventricular ectopic beats (VEB), i.v. administration of 50 mg significantly reduced VEBs and this treatment was superior to placebo.[101] However, only 1 patient was completely free of arrhythmias.



12

Other Drugs which Prolong Ventricular Repolarisation - Several standard anti-arrhythmic agents have been shown under certain experimental conditions to prolong ventricular repolarisation and so disopyramide will prolong APD in the presence of low potassium concentrations.[102] INPEA (nifenalol), a β-adrenoceptor blocker has been shown to prolong the APD and ERP of isolated guinea-pig atria.[80] Chronic treatment of rabbits with β-adrenoceptor blockers resulted in a prolongation of the APD of tissues taken from the hearts several days after cessation of therapy.[103] As propranolol in acute experiments had either no effect on repolarisation time or shortened it, the observations were explained by an adaptation of prolonged interruption of sympathetic control of the heart. A similar observation has been made in man where daily doses of 400 mg of metoprolol for 5 weeks resulted in a slight (6%) but significant increase in MAPD.[104]

Conclusion - Compounds with a variety of chemical structures have been shown to prolong APD and ERP of the ventricle suggesting that more than one mechanism is involved. This is not surprising given the different currents flowing during the plateau and repolarisation phase of the action potential. The compounds might also exhibit a differential effect on APD and ERP which under certain conditions might be arrhythmogenic. A greater prolongation of the APD versus ERP could result in activation of an advancing wave of depolarisation and with slower propagation of the wave front, a re-entrant arrhythmia could become sustained. Lastly, most of the agents which have been shown to be effective in life-threatening arrhythmias (amiodarone, bretylium, sotalol) have other properties, so that there is no direct evidence that the prolongation of ventricular repolarisation is the mechanism by which they act. The clinical efficacy of an agent which solely prolongs ventricular repolarisation may provide this evidence.

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Section III - Chemotherapeutic Agents

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Chapter 12. Antibacterial Agents

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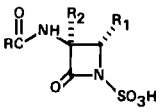
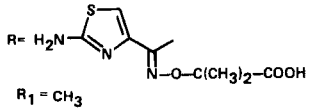
General - During 1982, β-lactam research continued to occupy a large portion of the literature dealing with discovery of new antibacterial agents. New developments in the SAR of monobactams were reported, and novel carbapenem antibiotics continued to be discovered in nature. An orally active third generation cephalosporin was disclosed, and publications on β-lactamase inhibitors appeared at a brisk rate. Aminoglycosides which are both well-tolerated and resistant to enzymatic inactivation mechanisms remained research objectives. Striking spectrum/potency improvements continued to emerge in the pyridone carboxylic acid class of synthetic antibacterial agents. Books devoted to the chemistry and biology of β-lactam antibiotics,[1-5] mode of action of antibiotics,[6] antibiotic research[7] and antibiotic screening[8] were published, as were the proceedings of the 12th International Congress of Chemotherapy[9] and of an international conference on trends in antibiotic research.[10]

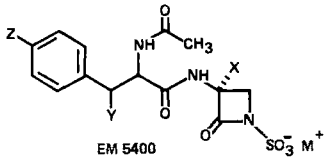
Monobactams - The structure-activity relationships leading to azthreonam (SQ 26,776), and the results of *in vitro*, *in vivo*, β-lactamase stability, mechanistic and pharmacokinetic studies of azthreonam, have been published.[11] Azthreonam has the highest affinity for penicillin-binding protein 3 (PBP-3) of Gram-negative bacteria, moderate affinity for PBP-1a, and poor affinity for PBP-1b and PBP-2, indicating a mode of action similar to that of the cephalosporins.[12] Interestingly, the $-SO_3^{(-)}$ residue of the monobactams can be replaced by other acidic activating groups, such as $-OSO_3^{(-)}$,[13,14] $-CON^{(-)}SO_2-$,[15,16] and $-P(OR)O_2^{(-)}$,[17] with retention of potent antimicrobial activity. Full papers on the fermentation, isolation, biological properties, structure determination and synthesis of SQ 26,180,[18,19] as well as five members of the EM5400 family of monobactam antibiotics[20,21] have appeared. A study comparing the behavior of azthreonam and related monobactams with third generation cephalosporins towards several β-lactamases has shown that azthreonam possesses poor affinity for β-lactamases from Gram-negative bacteria and is highly resistant to enzymatic hydrolysis.[22] Stereospecific syntheses of 3-amino-2-oxoazetidine-1-sulfonic acids, the basic nuclei of the monobactams, from L-α-amino-β-hydroxy acids, have been reported.[23,24]





$R_1 = CH_3$
$R_2 = H$
(AZTHREONAM)

$R = CH_3$
$R_1 = H$
$R_2 = OCH_3$
(SQ 26,180)



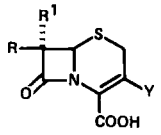
EM 5400

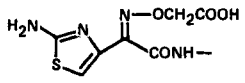
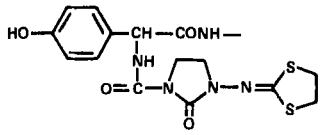
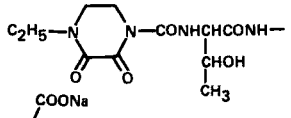
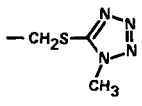
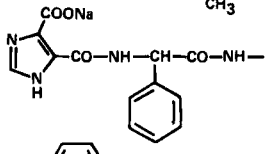
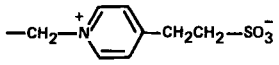
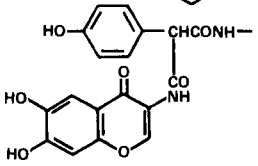
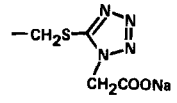
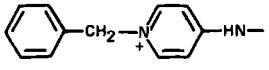
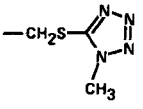
	X	Y	Z	M
SQ 26,823	OCH_3	H	H	Na
SQ 26,875	OCH_3	H	OH	K
SQ 26,700	H	H	OH	K
SQ 26,970	OCH_3	OH	$OSO_3^-Na^+$	Na
SQ 26,812	OCH_3	$OSO_3^-Na^+$	$OSO_3^-Na^+$	Na

Cephalosporins - FR 17027, a novel oral cephalosporin, has an antibacterial spectrum comparable to that of the third generation parenteral cephalosporins, with the exception of

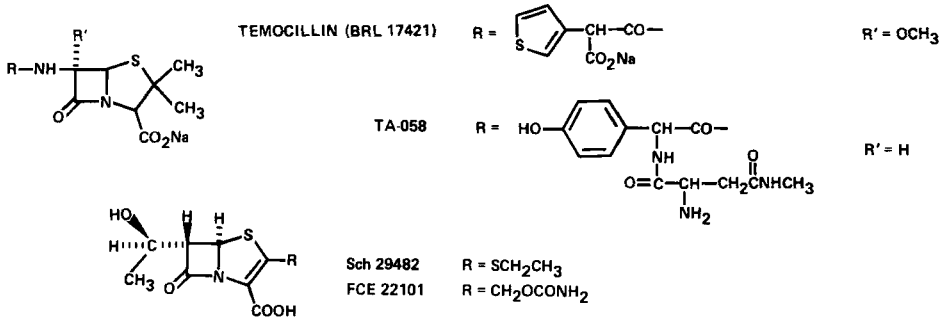
ISBN 0-12-040518-0

antipseudomonal activity.[25] It is less active than cefaclor or amoxicillin against staphylococci but is considerably more potent against Gram-negative pathogens. FR 17027 exhibited excellent stability to both penicillinases and cephalosporinases and was highly effective orally in protecting mice against lethal infections caused by a wide variety of Gram-negative bacteria. When administered orally to rats and dogs, FR 17027 was better absorbed than cephalexin and amoxicillin.[26] KIT-180 (KI-6269) is a new cephalosporin with a broad, potent antibacterial spectrum similar to that of cefoperazone.[27] T-1982, a new cephamycin antibiotic, is equal to or more active than cefmetazole and cefoperazone against various Gram-negative bacteria but is less active against staphylococci.[28] In animal infection models, T-1982 is reportedly more active than cephalosporins having comparable *in vitro* potency because of the potentiation of its bactericidal activity by complement and phagocytes.[29] The greater activity of AC 1370 (U-63,196E) *in vivo* than would be expected from its *in vitro* potency is attributed to enhancement of its bactericidal activity by serum.[30] E-0702 is a cephalosporin with a broad antibacterial spectrum which is notable for its highly potent antipseudomonal activity.[31,32] L-640,876, a structurally novel quaternary heterocyclylamino-cephem, possesses good activity against Gram-negative and Gram-positive bacteria with the exception of *Pseudomonas aeruginosa*, *Serratia marcescens* and *Streptococcus faecalis*.[33,34] In mouse protection studies, L-640,876 was highly effective against a variety of bacterial pathogens.[35] Papers on the laboratory and clinical evaluation of ceftazidime,[36] cefotaxime,[37] ceftizoxime,[38] moxalactam[39] and cefadroxil,[40] which were presented at their respective symposia, have been published. Structure-activity relationships related to ceftizoxime,[41] the chemistry and structure-activity relationships of 1-oxacephems[42] and the pharmacokinetics of the new cephalosporins,[43] have been reviewed.



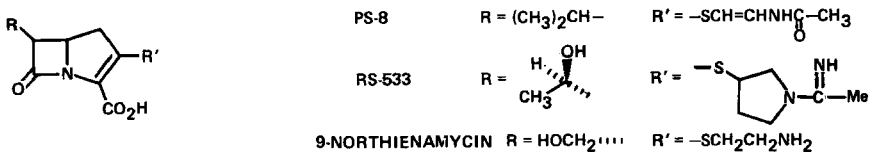
	R	Y	R'
FR 17027			H
KIT-180 (KI-6269)			H
T-1982			CH_3O
AC-1370 (U-63,196E)			H
E-0702			H
L-640,876			H

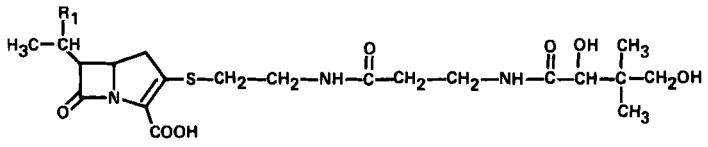
Penicillins - The laboratory evaluation of temocillin (BRL 17421), a parenteral semi-synthetic penicillin, has been the subject of numerous publications.[44-46] Temocillin is stable to a wide variety of β-lactamases and possesses good activity against most *Enterobacteriaceae*, *Haemophilus* and *Neisseria* strains but is not active against *Pseudomonas*, *Bacteroides* and Gram-positive organisms. TA-058 is reported to be equal to or better than piperacillin in antimicrobial spectrum and potency, to exhibit good pharmacokinetics in man and to be efficacious in the treatment of lower respiratory tract infections.[47,48] The results of laboratory, clinical and pharmacological studies of mezlocillin[49,50] and azlocillin[50] have been reported.



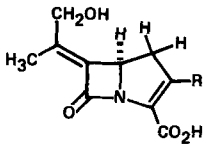
Penems - Sch 29482 is an orally active penem derivative which has broad, potent antibacterial activity, except against *Pseudomonas*, and is highly stable to a wide variety of β-lactamases. Studies of the comparative *in vitro* activity, β-lactamase stability, *in vivo* activity, and pharmacokinetics of Sch 29482, have been published.[51] Like the carbapenem antibiotics, Sch 29482 is susceptible to hydrolysis by renal dipeptidase, accounting for its low urinary recoveries.[52] FCE 22101 is a new penem which exhibited broad, potent antibacterial activity *in vitro* and *in vivo*, except against *Pseudomonas*.[53] The β-lactamase stability and mode of action of penems with various C-6 substituents have been studied.[54] Penems have the highest affinity for PBP-2 and high affinity for PBP-1A and PBP-1Bs, but reduced affinity for PBP-3. A novel, practical synthesis of penems via an oxalimide cyclization reaction has been published,[55] and a versatile method for replacing the 2-alkylthio side chains of penems with other thio side chains has been reported.[56]

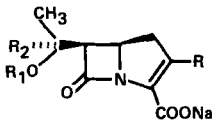
Carbapenems - Full papers on the fermentation, isolation, structure determination, antibacterial activity, and β-lactamase inhibitory activity of asparenomycin (ASM) A, B and C, have been published.[57-61] SQ 27,860 is the first bicyclic β-lactam antibiotic isolated from bacteria and is structurally the simplest carbapenem possessing broad antibacterial activity, albeit modest potency.[62] Other new carbapenem antibiotics that have been isolated from *Streptomyces* include the OA-6129 group,[63-65] C-19393 E_5,[66] 6643-X,[67] PS-8,[68] MM 27696,[69] 8-epi-thienamycin[70] and 9-northienamycin.[70] Structurally, they are minor variants of known carbapenem antibiotics and are less active than thienamycin. The relationship between carbapenem structures and their susceptibility to hydrolysis by renal dipeptidase has been studied.[52,71] A significant species variation in the degree of metabolism has been observed for thienamycin.[71] Carbapenems bearing a basic substituent in the C-2 side chain are less susceptible to hydrolysis by the dipeptidase.[71] RS-533 is a new semi-synthetic carbapenem exhibiting broad, potent activity *in vitro* and *in vivo*.[72] The synthesis of carbapenem antibiotics has been reviewed,[73] and a versatile method for replacing the 2-alkylthio side chains of carbapenem antibiotics by other thio side chains has been reported.[74]





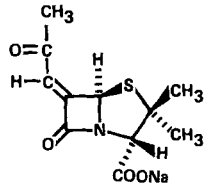
OA-6129A R_1 = –H
OA-6129B_1 R_1 = –OH
OA-6129B_2 R_1 = –OH
OA-6129C R_1 = –OSO_3H



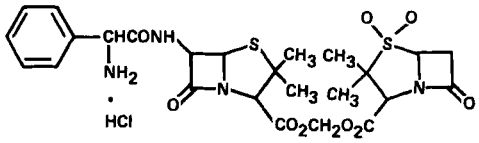


R	
ASM A	–S–C(=O)(H)–C(H)=C(H)–NHCOCH$_3$
ASM B	–S(=O)(R)–CH_2–CH_2–NHCOCH$_3$
ASM C	–S–C(H)=C(H)–NHCOCH$_3$
6643-X	–S–CH_2–CH_2–NHCOCH$_3$

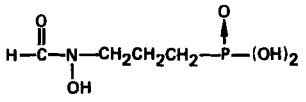
Compound	R	R_1	R_2
C-19393 S_2	–S–C(H)=C(H)–NHCOCH$_3$	SO_3Na	CH_3
MM 4550	–S–C(H)=C(H)–NHCOCH$_3$	SO_3Na	H
MM 13902	–S–C(H)=C(H)–NHCOCH$_3$	SO_3Na	H
C-19393 E_5	–S–C(H)=C(H)–NHCOCH$_3$	H	H
MM 27696	–S–C(H)=C(H)–NHCOCH$_2$CH$_3$	SO_3Na	H
PLM A SF-2103 A	–SO_3Na	SO_3Na	H
PLM B	–S(=O)–CH_2COONa	SO_3Na	H
PLM C	–S(=O)–CH(OH)–OH –S(=O)–CHO	SO_3Na	H



Ro 15-1903



SULTAMICILLIN



FOSMIDOMYCIN

β-lactamase Inhibitors - A comprehensive review of β-lactamase inhibitors has been published.[75] Augmentin, a physical combination of the β-lactamase inhibitor clavulanic acid and amoxicillin, continues to be the subject of extensive laboratory and clinical studies.[76-81] Sultamicillin, a mutual prodrug of ampicillin and sulbactam which delivers good blood levels of ampicillin and the β-lactamase inhibitor upon oral administration, has been extensively evaluated.[82-85] Ro 15-1903 is a more potent inhibitor of a variety of β-lactamases than clavulanic acid or sulbactam when preincubated with the enzymes.[87] However, *in vitro* and *in vivo* Ro 15-1903 did not demonstrate greater synergy than clavulanic acid or sulbactam against various β-lactamase-producing bacteria when combined with ampicillin, piperacillin or ceftriaxone.[88] The structurally simple penam derivative, 6β-(trifluoromethanesulfonyl)-amidopenicillanic acid sulfone, is a potent inhibitor of *Bacillus cereus* 569/H β-lactamase I and *E. coli* RTEM β-lactamase when preincubated with the enzymes.[89] Novel carbapenem SF-2103 A possesses only modest antibacterial activity but is reported to be a potent inhibitor of both

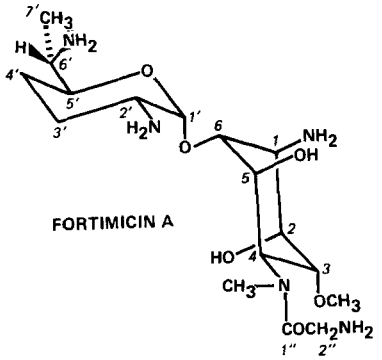
penicillinases and cephalosporinases.[90] Combinations of cephalosporins and SF-2103 A exhibited synergy both *in vitro* and *in vivo* against a variety of β-lactamase-producing organisms.[91,92] Pluracidomycin (PLM) A, B and C are new carbapenem antibiotics possessing potent β-lactamase inhibitory activity.[93] Pluracidomycin A is probably identical to SF-2103 A.[93] The β-lactamase inhibitory activities and synergistic effects of twelve 5,6-cis-carbapenem antibiotics have been compared.[94] Although MM 4550 and MM 13902 were the most active in terms of breadth of β-lactamase inhibitory spectrum and potency, C-19393 S_2 was the most active in potentiating the activity of ampicillin against penicillinase-producing bacteria.

Cell Wall Inhibitors - Structure activity relationships in the 3-(N-acyl-N-hydroxyamino)-propyl phosphonic acid series have been published and suggest superior activity for the N-formyl derivative fosmidomycin (FR-31,564).[95] Synergy was found between fosmidomycin and beta-lactam antibiotics, trimethoprim and gentamicin.[96] A reduction in antibacterial activity of phosphonic acid antibiotics in urine versus Mueller-Hinton or nutrient broth was reported.[97] Phosphonopeptides based on glycine rather than alanine (alafosfalin), are effective antibacterials and inhibit enzymes that operate on both D- and L-amino acid substrates.[98]

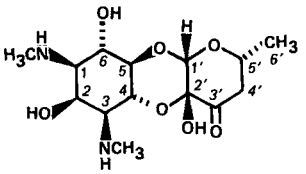
Teichomycin, a glycopeptide with good Gram-positive activity, continues to generate interest.[99-101] Configurational studies of ristocetin A have led to a proposed structural assignment which may help rationalize its mechanism of action at the molecular level.[102] A structure revision for vancomycin was also reported.[103]

Aminoglycosides - Breadth of spectrum and good potency of the aminoglycosides (AGs) continued to stimulate efforts to identify products with improved toleration and decreased susceptibility to enzymatic resistance pathways. A novel, synergistic approach to the resistance problem was disclosed with the report that 7-hydroxytropolone is a competitive inhibitor of 2"-O-adenylyltransferase and produces synergy with AGs inactivated solely by that enzyme.[104,105] Evaluation of O-demethylfortimicin A, *in vitro*[106] and *in vivo*,[107] indicates efficacy equivalent to currently used AGs with diminished ototoxic effects[108] and improved resistance to enzymatic inactivation. Further chemical modifications of fortimicin A through manipulation of substituents in the fortamine ring[109] produced improved antibacterial activity in 2-deoxy-3-demethoxy fortimicin A.[110] Decreased potency observed after 4-N-amino acylation of lysinomicin did not correlate with similar fortimicin modifications.[111] Chemical conversion of fortimicin B to dactimicin was reported.[112]

An improved synthesis of dibekacin was described,[113] and further kanamycin transformations and SAR were examined.[114-116] Novel tobramycin[116-118] and gentamicin[119] modifications were reported, as well as deoxy amikacin[120] and butirosin derivatives.[121] Variations on the sporaricins provided compounds with improved toxicity and resistance characteristics,[122-124] and work with istamycins[125-127] and sagamicins[128-132] was also described. The synthesis[133] and SAR of dihydrostreptomicin epimers[134] were reported as were transformations of paromomycin,[135] lividomycin B[136] and syntheses of paromamine[137] and glycocinnamoylspermidines.[138,139]



FORTIMICIN A



SPECTINOMYCIN

Chemical modifications of spectinomycin produced dramatic changes in antibacterial spectrum and potency. Methods for N-demethylation were found,[140] and procedures for modification of the sugar moiety were described,[141-142] which gave improved activity over spectinomycin.[143] Improved activity for lipophilic 4-dihydro-4-deoxy-4(R) amino analogs was reported.[144]

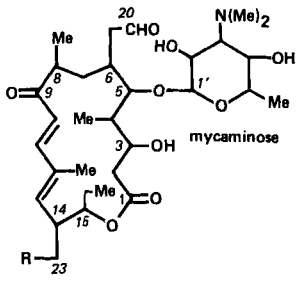
The structure of streptothricin has been fully elucidated,[145] and its synthesis reported.[146] Applications of mass spectral techniques for improved AG structural studies have been developed.[147,148]

Macrolides and Lincosamides - Comparisons among 16-membered ring macrolides related to tylosin (T) provided new SAR helping to define the relative importance of T substituents for antibacterial activity.[149,150] The mycarose and mycinose sugars were unnecessary for good activity, and antibiotics lacking these sugars have been isolated from mutants of *Streptomyces fradiae*.[151] Transformations of seco acid derivatives of mycaminosyl tylonolide (MT) diethyl acetal to macro lactones of the primary alcohol were studied, and the products showed some antibacterial activity.[152] Syntheses of 23-dialkylamino derivatives in the MT and 4'-deoxy MT series gave compounds with enhanced *in vitro* activity, particularly against Gram-negative bacteria.[153] Derivatives of T arylated or sulfonylated at the 4"-OH position were studied and SAR developed.[154] The improved activity of some of the most potent analogs was attributed to strong ribosomal binding. Competitive binding studies of 3-O-acetyl-4"-O-isovaleryl T to ribosomes from resistant *Staphylococcus aureus* further indicate that 4"-O acylation markedly enhances ribosomal binding.[155] Application of hybrid biosynthesis to 16-membered ring macrolides was reported, providing information both with regard to SAR and to the properties of the enzymes involved in the biosynthesis.[156] Mutasynthesis in the mycinamicin series provided biosynthetic intermediates to rosaramicin[157] and novel antibacterial macrolides as well.[158,159] The structure of the AR-5 antibiotics (mycinamicins) was studied by chemical degradation and X-ray analysis.[160] Chemical modifications of oleandomycin neutral sugars were examined,[161,162] and an improved synthesis of 10,11-anhydro erythromycin was reported.[163]

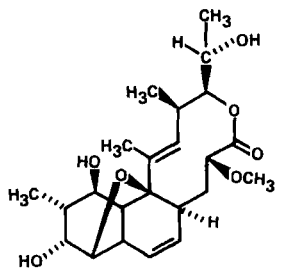
Alanine peptides of erythromycin A and of 9-(S)-erythromyclamine were prepared in an unsuccessful attempt to facilitate macrolide transport into Gram-negative cells.[164] Isolation of erythromycin F, a new possible biosynthetic precursor to erythromycin E, was reported.[165]

Chemical conversion of ten-membered ring macrolide nodusmicin to nargenicin and other 9-O esters allowed development of SAR in this class of *Staph. aureus* active antibiotics.[166] Erythromycin showed superior *in vitro* activity to midecamycin in a direct comparison,[167] and synergistic effects of midecamycin and other antibiotics such as carbenicillin, dibekacin and fosfomycin were demonstrated *in vitro* and *in vivo*.[168-170]

Pirlimycin (U5793OE), a lincosamide antibiotic, exhibited anaerobe activity *in vitro* superior to clindamycin.[171] It was also effective against aerobic Gram-positive cocci,[172] particularly in mastitis.[173]



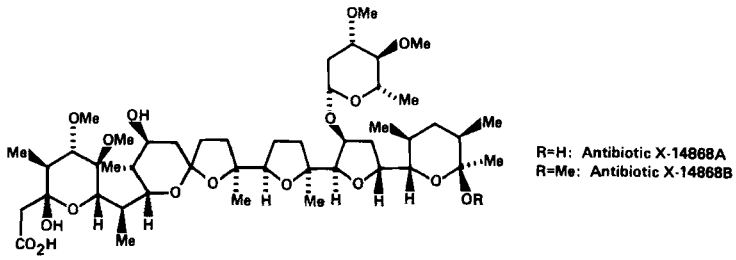
MYCAMINOSYL TYLONOLIDES



NODUSMICIN

Ansamycins - Two new 16,17-dihydrorifamycin S derivatives were identified as products of a recombinant strain of *Nocardia mediterranei*.[174] Surprisingly, the absence of the 16,17 double bond dramatically decreased antibacterial potency against Gram-positive bacteria. An interesting rearrangement of 3-hydrazino-rifamycins was discovered.[175] Enlargement of the ansa bridge with terminal carbonyl transfer from the 2-amino to 3-hydrazino moiety produced loss of antibacterial activity. Conformations of rifamycins were studied by ^{1}H-NMR and X-ray, in solution and the solid state,[176] and a new efficient diastereoselective aldol approach to synthesis of the ansa chain of rifamycin S was employed.[177] The in vitro activity of semi-synthetic rifamycin DL-473 was compared to rifamicin and found to be about two-fold less active.[178] A complete report on the structure of rubradirin was published.[179]

Polyether Ionophores - New polyether antibiotics continued to be discovered. Both X-14868 A and B exhibit potent anticoccidial activity and transport metal ions across membranes but with different specificities.[180] Ferensimycins A and B are congeners of lysocellin and are also effective against coccidiosis in fowl.[181] Cezomycin, a demethyl amino analog of A23187, was produced by controlled biosynthesis.[182] Tetronomycin, a novel polyether containing a tetronic acid instead of a carboxylic acid moiety, has been discovered and found to possess potent Gram-positive activity, as well as the toxicity characteristic of carboxylate ionophores.[183] Relatively low toxicity was found for cationomycin and the crystal structure of its thallium salt was determined.[184] Selective acylation of laidlomycin gave a series of esters, some of which displayed enhanced anticoccidial activity over the parent.[185]



Synthetic Agents - Pyridone Carboxylic Acids - Analogs of nalidixic acid continue to be of interest, as impressive improvements in spectrum and potency have been obtained with compounds like pefloxacin,[186] AT 2266,[187] DL-8280[188] and norfloxacin (AM-715, MK-0366).[189-200] SAR development for this class of orally effective antibacterials has continued.[201-204]

New Antibiotics - Some novel compounds recently discovered in antibacterial screening are tabulated below, along with their source, producing organism, and general activity.

Table I

Antibiotic	Producing Organism	Activity	Reference
Factumycin (A40A)	*Streptomyces lavendulae*	G +, G-	205
AB-315	*Gluconobacter*	G +, G-	206
Bulgecin	*Pseudomonas acidophila*	β-lactam synergist	207
AT-265	*Streptomyces rishiriensis*	G +, G-	208
Thiolactomycin (2-200)	*Nocardia*	G +, G-, AA, AF	209-212
Myxovirescins	*Myxococcus virescens*	G +, G-	213
Thiosporamicin CP-46,192	*Streptosporangium roseum*	G +	214
L-681,217	*Streptomyces*	G +, G-	215

G + (Gram-positive), G- (Gram-negative), AA (anaerobic), AF (acid fast)

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Chapter 13. Bacterial Penicillin-Binding Proteins

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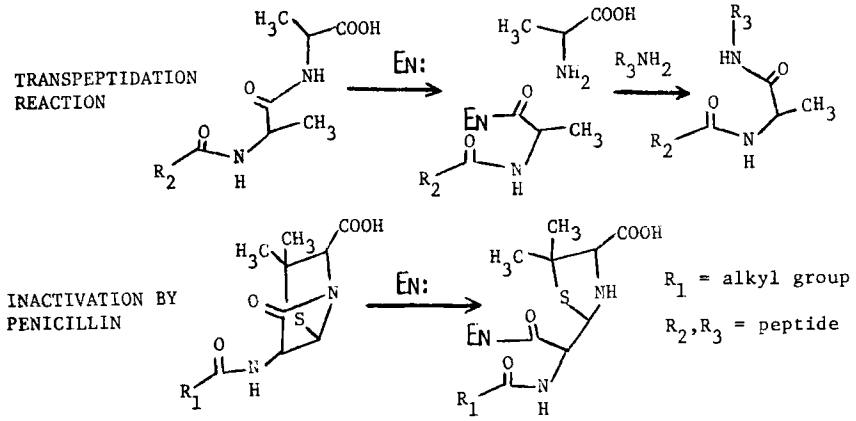
Introduction - Penicillin-binding protein (PBP) studies have significantly improved our understanding of the mechanism of action of β-lactam antibiotics in recent years. At the cellular level, they have helped elucidate some of the processes involved in bacterial cell-wall biosynthesis and morphogenesis. At the molecular level, they have contributed to our understanding of intrinsic antibiotic activity and have provided the framework for structure-activity relationships. Penicillin-sensitive enzyme (PSE) studies have focused on DD-carboxypeptidases, non-essential PBPs which are plausible models for the interaction of penicillin with its targets. Together, PBP/PSE studies represent an approach to the rational design of inhibitors of bacterial cell-wall biosynthesis. The present review focuses on properties of PBPs in some of the better-studied bacteria of clinical importance. The role of individual PBPs in bacterial susceptibility/resistance is also discussed. Additional information is available in several recent reviews.[1-5] An excellent early account of the topic has been published,[6] while a related report on β-lactamases has appeared in this series.[7]

PBPs: General - Penicillin and other β-lactam antibiotics inhibit bacterial growth by binding to specific transpeptidases which crosslink peptidoglycan,[8] the rigid, bag-like macromolecule responsible for the mechanical strength of the bacterial envelope.[9,10] Penicillin was suggested to act at the cell- wall level a few years after discovery of this remarkable antibiotic,[11,12] but the exact site and the molecular mechanism of penicillin action were only proposed in 1965.[8] Known as the Tipper-Strominger hypothesis, it suggests that penicillin acts as a steric analogue of the pentapeptide chain in growing peptidoglycan, forming a stable penicilloyl-enzyme complex with peptidoglycan transpeptidase. This model is now generally accepted and the alternative allosteric model[13] has been abandoned. In variations of the steric analogue model, penicillin acts as a transition-state analogue[14,15] or a k_{cat} inhibitor.[16]



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The steric analogue model is believed to apply to all peptidoglycan enzymes which cleave the peptide terminal bond in R-D-Ala-D-Ala. These are the DD-carboxypeptidases and the peptidoglycan transpeptidases. Both types of enzymes may proceed by similar mechanisms involving the formation of an acyl intermediate with the peptide substrate.[17-19] Both are sensitive to penicillin hence the term penicillin-sensitive enzymes and both bind penicillin covalently hence the term penicillin-binding proteins. PBPs are presumed to be PSEs, although so far this has been shown only for E. coli PBPs.[20-25] Essential PBPs are those PBPs whose deletion by mutation, or binding to β-lactam antibiotics, is associated with cell death. Therefore, intrinsic antibiotic activity, i.e., activity in the absence of β-lactamases and permeability factors,[26] is a direct consequence of binding to essential PBPs. PBP patterns, essential PBPs, and β-lactam-binding profiles are different in different bacteria.[27] Nevertheless, there are mechanistic similarities in the interaction of PBPs with penicillin and PSEs with the substrate in different bacteria.[18,19,28] With DD-carboxypeptidases, the similarity includes homologies in the amino acids lining the active site and this also extends to β-lactamases.[29,30] Alterations in PBP patterns, often observed in organisms lacking an essential PBP,[31-34] can be taken to suggest some overlap of enzymatic activities and physiological functions of PBPs within an organism.

PBPs have been found in all bacteria so far examined.[27] Typically, an organism will contain four to eight PBPs with molecular weights between 35,000 and 120,000 daltons. They are numbered by convention in the order of decreasing molecular weight. Particular PBPs vary greatly in their relative abundance ranging from a few molecules to a couple of thousand molecules per cell. Essential PBPs are usually minor proteins, of molecular weights 60,000 to 120,000 daltons. Although essential PBPs have been identified in several bacteria, physiological functions have been assigned so far only for those of E. coli and related bacteria.[35-38] Decreased binding of β-lactam antibiotics to essential PBPs has been inplicated in resistance in several organism.[39-43] The spatial and temporal distribution of PBPs in the cell is largely unknown. Minor PBPs associated with localized functions, such as septum formation, are probably concentrated at specific regions in the cytoplasmic membrane.[44] Studies with synchronously growing E. coli have uncovered transpeptidase activity associated with septation but not cell elongation.[45] In Bacillus megaterium, changes in the PBP pattern associated with sporulation have been observed.[46]

The most extensively studied PBPs are the DD-carboxypeptidases, proteins of molecular weight typically between 40,000 and 50,000 daltons. They are relatively ubiquitous in bacteria, although they are seldom essential.[47-49] Their wide distribution parallels that of β-lactamases, to which they appear to be evolutionarily related.[50] Accordingly, DD-carboxypeptidases usually possess weak β-lactamase activity.[51] Endopeptidase and transpeptidase activities (Fig. 1) are also associated with DD-carboxypeptidase, the latter being probably of no physiological importance.[35,48,52,53] DD-carboxypeptidases from Streptomyces R61 and R39, Bacillus subtilis, Bacillus stearothermophilus, Staphylococcus aureus, and E. coli have been studied as models for the interaction of penicillin with its targets.[4,53,54]

PBPs are detected by the binding of radiolabeled benzylpenicillin. Typically, whole cells or bacterial membranes are incubated for ten minutes with [^{14}C] benzylpenicillin. Proteins are then solubilized with

detergent, fractionated on sodium dodecylsulfate (SDS)-polyacrylamide gels, and fluorographed.[55] Differences in binding between whole cells and membranes have been reported recently.[56] Incubation with clavulanic acid prior to penicillin permits PBP detection in the presence of penicillinases, provided PBPs are not sensitive to clavulanic acid at the concentrations used.[27] Binding of a given β-lactam to PBPs is usually measured indirectly, as decreased binding of [^{14}C] benzylpenicillin. Binding can also be measured directly, with radiolabelled β-lactam of specific activity comparable to that of penicillin.[57] Binding results have been correlated with minimal inhibitory concentrations (MICs) and cell morphology.[58] From such studies, essential PBPs and physiological functions of individual PBPs have emerged.[58] Complications arise when: 1) binding occurs to more than one PBP at near-MIC concentration;[47,59] 2) PBP binding is not associated with discrete morphological changes;[60] and 3) more than one PBP is responsible for a given function.[48]

Purification of PBPs is usually accomplished by affinity chromatography on Sepharose, the β-lactam ligand being selected on the basis of PBP affinity. PBPs are subsequently eluted with neutral hydroxylamine.[35,61,62] Most of the purified PBPs are devoid of enzymatic activity, a fact variously attributed to inactivation during purification or inappropriate assay conditions, especially substrate.[62] Penicillin binding is obviously a necessary but not sufficient condition for full enzymatic activity.

PBPs in Gram-Negative Bacteria - In E. coli cytoplasmic membrane, seven PBPs have been consistently found (Table 1). They are coded by separate genes which are dispersed in the E. coli chromosome.[48,63] PBPs 1, 2, and 3 are essential and are involved, respectively, in elongation, shape, and septation. β-Lactam antibiotics which bind to PBP 1 cause cell lysis, while those binding to PBP 2 produce giant spherical-shaped cells, and

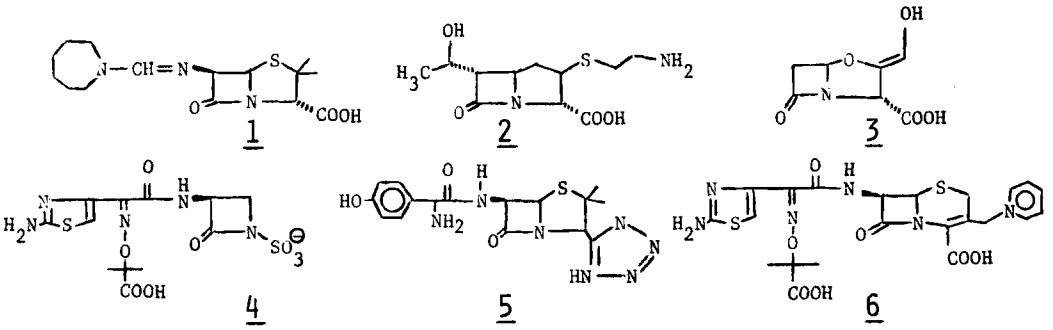
Table I

Properties of E. coli PBPs

PBP	MW	Copies/Cell	Activity	Function	Ref
1a 1b	91,000	230	transpeptidase/ transglycosylase	cell elongation	20 21
2	66,000	20	transpeptidase	cell shape	22
3	60,000	50	transpeptidase/ transglycosylase	septation	23
4	49,000	110	DD-carboxypeptidase/ endopeptidase	autolysin?	24,70
5 6	40,000	800 570	DD-carboxypeptidase/ penicillinase	regulation of crosslinking?	25,72

those binding to PBP 3 result in filamentation.[58] PBP 1 has been resolved into two genetically distinct components, a and b, with similar physiological functions although different affinities toward β-lactam antibiotics. PBP 1b has been further resolved into three genetically indistinguishable proteins.[21] PBP 1a and each of the three components of

PBP 1b are bifunctional enzymes with transpeptidase and transglycosylase activities.[20,21] Mutants lacking PBP 1a have no phenotypic defect, while those lacking PBP 1b are hypersensitive to β-lactam antibiotics, reflecting the high sensitivity of PBP 1a.[48] PBP 1b mutants have been successfully used in the discovery of naturally-occuring β-lactam antibiotics.[66] PBP 2 is highly sensitive to mecillinam (1), to some penicillins, and to a lesser extent thienamycin (2) and clavulanic acid (3). It is generally resistant to cephalosporins, and to the recently discovered monobactams (4).[57] PBP 2 is also a peptidoglycan transpeptidase.[22] PBP 3 is sensitive to penicillins, cephalosporins, and monobactams, and is, in general, less affected by structural changes on the β-lactam nucleus than are PBP 1 and 2. It is usually the target of β-lactamase-stable antibiotics, such as tetrazolyl amoxicillin (CP 35,587, 5),[67] ceftazidime (6),[68] and aztreonam (4).[69] The low molecular weight PBPs 4 and 5/6 are not essential.[70-72] Accordingly, binding of β-lactam antibiotics to these PBPs causes no growth defects.[58] PBP 4 is generally sensitive to β-lactam antibiotics and has DD-carboxypeptidase, endopeptidase, and model transpeptidase activity, all three activities occuring at the same active site.[73-75] Mutants with defective PBP 4 are viable, but have reduced peptidoglycan crosslinking.[76] PBP 5/6 is moderately sensitive to β-lactam antibiotics and has DD-carboxypeptidase and weak penicillinase activity.[51] The major activity is associated with PBP 5.[77] Predictably, mutants with defective both PBP 5 and PBP 4 have increased pentapeptide content.[76] Mutants with increased PBP 5 are spherical,[78] possibly reflecting decreased availability of pentapeptide substrate for cell elongation.[79] Complete loss of PBP 5/6 has been associated with hypersensitivity to β-lactam antibiotics.[80]



Among other gram-negative bacteria, Enterobacter, Klebsiella, and Salmonella have PBPs very similar to those of E. coli.[27,35,38] In Proteus species, PBPs are almost identical from species to species and somewhat different from, though still correlatable to, E. coli PBPs.[36] Serratia PBPs, on the other hand, vary from strain to strain, ranging from almost identical to E. coli to substantially different.[81] Like E. coli, both Proteus and Serratia possess a 36,000-dalton PBP with DD-carboxypeptidase and weak penicillinase activity.[36] The PBP pattern of Pseudomonas aeruginosa is correlatable to that of E. coli and binding of β-lactam antibiotics results in morphological changes similar to those observed in E. coli.[37] However, the concentration range over which these occur may differ as a result of differences in permeability,[82] β-lactamases,[83] and PBP affinity.[84] α-Sulfocephalosporins constitute an important exception in that PBP 3 of P. aeruginosa is very sensitive to them, while PBP 3 of E. coli is resistant. Consequently, these compounds induce filamentation and lysis in P. aeruginosa but only lysis in E. coli.[85]

In Neisseria gonorrhoeae three PBPs have been detected of molecular weights 87,000 (PBP 1), 59,000 (PBP 2) and 44,000 (PBP 3).[86] PBP 1 is the least sensitive to β-lactam antibiotics and is possibly the major peptidoglycan transpeptidase; binding of cephaloridine and penicillin results in spheroplasts.[87] PBP 2 is normally the killing site but is not a major peptidoglycan transpeptidase, since binding is not associated with inhibition of peptidoglycan crosslinking.[88] Binding to PBP 2 causes an increase in cell size and thickening of the septum.[86] PBP 3 may be a DD-carboxypeptidase similar to PBP 4 of the related Branhamella catarrhalis. Specific resistance to β-lactam antibiotics is associated with decreased binding to PBP 1 and 2,[89] while nonspecific resistance appears to correlate more closely with decreased outer membrane permeability.[90] Hemophilus influenza has eight PBPs of molecular weights 27,000 to 90,000 daltons.[91] PBP 2 (84,000) and 4 (68,000) correspond, on the basis of sensitivity to β-lactam antibiotics, to PBPs 1a and 2 of E. coli. The morphological response of this organism to β-lactam antibiotics is also similar to E. coli. Bacteroides fragilis has four PBPs of molecular weights 32,000 to 100,000 daltons.[43] PBP 2 (86,000) is the target for most β-lactam antibiotics and may be involved in septation. PBP 4 (32,000) has weak penicillinase activity and may be a DD-carboxypeptidase.

PBPs in Gram-Positive Bacteria - In Staphylococcus aureus, four PBPs of molecular weights 42,000-87,000 have been observed.[92,93] PBP 4 (42,000) is a DD-carboxypeptidase with transpeptidase and weak penicillinase activities.[52] Although it is a non-essential protein, it is involved in the secondary cross-linking of peptidoglycan.[94] Physiological, as well as mutant studies, have suggested that PBPs 2 (78,000) and 3 (75,000) are the killing sites for β-lactam antibiotics in this organism.[40,93,95] The related Staphylococcus epidermidis has a similar PBP pattern.[27] In Streptococcus faecalis five PBPs have been observed of molecular weights 42,000-105,000 daltons.[34,59,93] PBP 5 (42,000) is a DD-carboxypeptidase with penicillinase and transpeptidase activities.[96] Pharmacological studies, using several structurally diverse β-lactam antibiotics, have suggested that the killing sites are PBP 1 (105,000) and 3 (79,000).[93,97] More recent studies, however, have indicated that the situation may be more complex due to PBP inter-compensation.[98] In Streptococcus pneumoniae five PBPs have been detected of molecular weights 52,000-100,000.[99] PBP 3 (43,000) is a DD-carboxypeptidase and has been recently purified by affinity chromatography.[100] Studies with penicillin-resistant clinical isolates suggest that PBPs 1 and 2 are the killing sites for β-lactam antibiotics.[33] In Clostridium perfringens six PBPs have been observed of molecular weights 42,000-100,000.[60] PBPs 1 and 2 are relatively insensitive to β-lactam antibiotics.[101] PBPs 3 and 4 have been suggested to be the killing sites and possibly to be involved in septation.[60]

Penicillin-Sensitive Enzymes - As already stated, DD-carboxypeptidase and peptidoglycan transpeptidase are the two main types of bacterial enzymes which are inhibited by β-lactam antibiotics. DD-carboxypeptidase (M.W. range 25,000-50,000 daltons) from a variety of species have been isolated and studied extensively.[102] In contrast, peptidoglycan transpeptidases (M.W. range 90,000-100,000) have only recently been isolated and their properties are largely unknown. Therefore, most of the discussion that follows relates to DD-carboxypeptidases with (e.g., Streptomyces R61) or without (e.g., B. subtilis) in vitro transpeptidase activity.

Almost all the DD-carboxypeptidases studied thus far are readily inhibited by penicillin, 7α-methoxy-cephalosporins[84,102] and, to a lesser degree, non-methoxylated cephalosporins and monobactams.[57,103] Some are excreted into the growth medium (Streptomyces R61)[104] or are found in the soluble fraction upon rupture of the cells (E. coli DD-carboxypeptidase 1B).[51] The majority, however, are membrane bound and can be solubilized only by treatment with detergents. The enzymes are probably processed according to the signal hypothesis,[105] anchorage to the cytoplasmic membrane being achieved through a hydrophobic COOH terminus (pseudo-periplasmic enzymes).[106] DD-carboxypeptidases act on the peptide bond between the two terminal D-alanine residues of UDP-MurNAc-L-Ala-D-Glu-meso-Dap-D-Ala-D-Ala and the related synthetic substrate Diac-L-Lys-D-Ala-D-Ala.[107] Both substrates bind to the enzyme poorly, with typical K_m values of 10^{-2} M. Formation of the acyl-enzyme intermediate is rate-determining and therefore K_m includes the acylation constant. They are turned over at rates ranging from 10 (min^{-1}) for the B. subtilis enzyme[17] to 3,300 (min^{-1}) for the Streptomyces R61 enzyme.[102]

Like most peptidases, DD-carboxypeptidases also possess esterase activity, hydrolysing the depsipeptide analog of the synthetic substrate, Diac-L-Lys-D-Ala-D-Lactate.[17] The K_m for the depsipeptide is substantially lower than that of the corresponding peptide, reflecting a higher acylation rate.[17] The low K_i values of penicillin (10^{-9} M in the case of Streptomyces R61 DD-carboxypeptidase) have been attributed to a fast acylation step, the initial reversible binding having an equilibrium constant comparable to the K_m for the substrate.[108] In addition to exopeptidase and esterase activities, DD-carboxypeptidases possess endopeptidase, transpeptidase, and penicillinase activities, all five activities most likely occurring at the same active site (Fig. 1).[102,109]

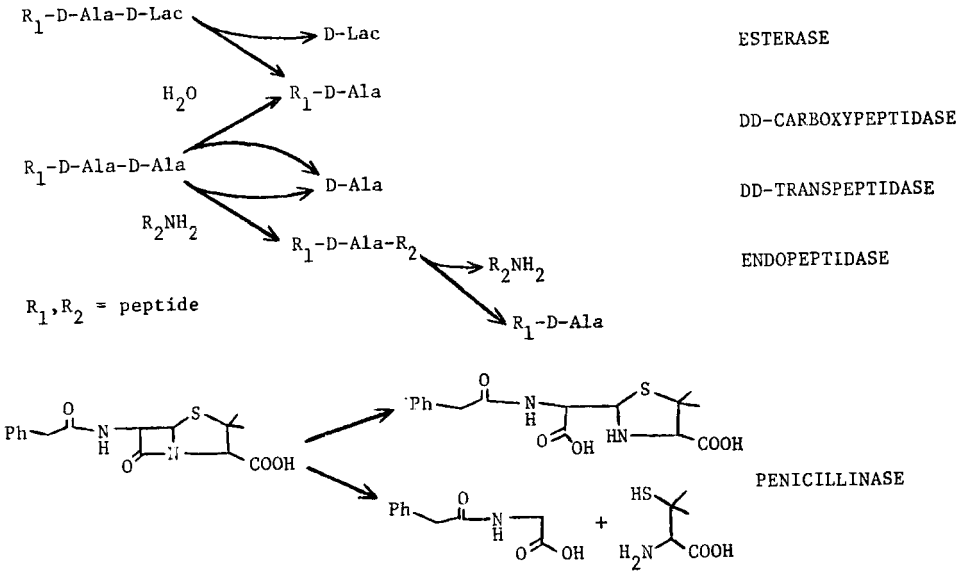


Figure 1: Enzymatic activities of DD-carboxypeptidases.

The similarity of the endopeptidase and DD-carboxypeptidase reactions is evident since α to the susceptible peptide bond is a free carboxyl group on the D-asymmetric center of diaminopimelic acid in the former and the carboxyl group of D-Ala in the latter.

Depending on the nature of the nucleophilic acceptor, DD-carboxypeptidase-associated transpeptidase activity can be divided into two types: (i) acceptor is hydroxylamine, glycine, or other amino acid and probably binds at the donor site; i.e., transpeptidation is reversed hydrolysis; (ii) acceptor is Gly-L-Ala or other peptide and binds at a site topologically distinct from the donor site, (natural-model transpeptidation).[110] Until recently, the natural-model transpeptidation was the only model for the interaction of β-lactam antibiotics with their enzyme targets.

DD-carboxypeptidase-associated penicillinase activity can be divided into two categories depending on the type of penicillin cleavage: (i) exclusive cleavage at the amide bond of the β-lactam producing penicilloic acid; (ii) additional cleavage at the C_5-C_6 of the β-lactam producing phenylacetylglycine and penicillamine.[5] The first type is found in PBP 4 of S. aureus, PBP 5/6 of Enterobacteria and probably PBP 3 of N. gonorrhoeae. It is associated with fast release of hydrolysis products (typical $t_{\frac{1}{2}}$ = 5 min). The second type is found in PBP 5 of B. stearothermophilus[111] and Streptomyces R61.[112] It is associated with slow release of hydrolysis products (typical $t_{\frac{1}{2}}$ = 2 hr). Interestingly, a similar fragmentation of penicillin sulfoxides by the related β-lactamases has been reported,[113] indicating similarity of the active-site environments in DD-carboxypeptidases and β-lactamases. In either cleavage, a covalent penicilloyl-enzyme complex is formed involving a serine residue on the enzyme.[114,115] This serine residue appears to be conserved in the amino acid sequence of several DD-carboxypeptidases.[30] The half-time of release is not substantially affected by the nature of the penicillin side chain during secondary fragmentation.[102,103] However, complete lack of the side chain increases the rate of release, possibly by altering the reaction pathway.[103] DD-carboxypeptidases do not have significant cephalosporinase activity. With the R61 enzyme, half-times of release ranged from 3 days to about one month, depending on the side chain.[108,116] Cephalosporin release probably does not involve secondary fragmentation.[117] A similar situation exists with monobactams.[103]

Until recently, peptidoglycan transpeptidase was studied, in cell wall-membrane preparations[118,119] or bacterial cells made permeable to exogeneous nucleotide sugar peptidoglycan precursors by treatment with toluene[120] or ether.[79,121] In these studies, multiple transpeptidases could not be differentiated. In addition, assays were often affected by the presence of DD-carboxypeptidases.[122] Nevertheless, peptidoglycan transpeptidase activity has been correlated with specific PBPs in several organisms with varying degrees of success.[20,32] Generally, P. aeruginosa peptidoglycan transpeptidase shows sensitivity to β-lactam antibiotics equal to its E. coli counterpart, indicating that the characteristic β-lactam resistance of the former organism is not due to a change in affinity of the antibiotic target.[123,124] Peptidoglycan transpeptidase has been recently purified from E. coli membranes and identified to be PBP 1a,[20] 1b,[21] 2,[22] and 3.[23] With the exception of PBP 2, these PBPS are bifunctional, acting both as transpeptidases and transglycosylases. The former activity is sensitive to β-lactam antibiotics, while the latter activity is sensitive to the phosphoglycolipid moenomycin.[125]

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Chapter 14. Antineoplastic Agents

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Introduction - The goal of synthesizing more selective antitumor agents through the rational design of target-specific compounds; the ability to overcome drug resistance by the judicious use of combination chemotherapy, improved delivery systems, and prodrugs; and ultimately, the possibility of achieving prophylaxis, tumor regression, and inhibition of metastases by chemical means, continued to be important objectives during 1982. Parallel to these efforts, a considerable amount of work continued on the isolation and characterization of new antitumor agents from natural sources. The mechanism and development of chloroethylnitrosoureas,[1] the development of radiosensitizers,[2] the biological activity of purine and pyrimidine analogs,[3] and the activity of nucleoside and nucleotide analogs as inhibitors of the critical enzyme inosine monophosphate dehydrogenase,[4] have been reviewed. Results of the first conference on prostaglandins and cancer have been published and the role of retinoids as chemopreventive and anticancer agents has been reviewed.[5,6] Other studies dealing with the mechanism of action of antitumor antibiotics have appeared[7,8] and the mechanism of action of interferon and its role in cancer was reviewed.[9,10] Clinically, an important advance was represented by the treatment of relapsed leukemic AML patients with ara-C, VP-16-213, vincristine and vinblastine with a 75% success rate.[11]

Alkylating Agents - A novel water soluble nitrosourea, 1-(2-chloroethyl)-3-isobutyl-3-(β-maltosyl)-1-nitrosourea, produced > 60 day survivors in all L1210 tumor-bearing mice when employed at a dose of 100 mg/kg (qd 1-5), either ip or po.[12] The drug also had a life-prolonging effect on early and advanced forms of LL carcinoma and produced complete regression against the MX human mammary carcinoma in athymic mice.[13] The maltosyl group proved to be essential for good activity.[14] Other very active 3,3-disubstituted nitrosoureas in which the nitrosoureido group was attached to sugar moieties, such as methyl glucose or arabinose, produced ILS values ~ 700% at doses ranging from 25 to 50 mg/kg.[15,16] The 1-(2-chloroethyl)nitrosourea congener of the L-aminoacid amide sarcosinamide was very active amongst a series of amino acid analogs synthesized as carriers of the active moiety. This material, at doses between 96-225 mg/kg, cured all mice by day 60 (ILS > 700%).[17] Pure N,N'-bis[(2-chloroethyl)nitrosocarbamoyl]cystamine produced > 50% cures in L1210 leukemic mice at 10 mg/kg. This compound was regiospecifically synthesized by a novel procedure that allowed the selective introduction of the NO group at the nitrogen atom bearing the alkyl group.[18] PCNU, a piperidone-2,6-dione chloroethylnitrosourea, commenced Phase II clinical trials. This compound has shown consistent activity against brain tumors.[19] L1210 cells resistant to the cytotoxic agent L-phenylalanine mustard (L-PAM) were completely sensitized to the drug by reducing the intracellular concentration of glutathione.[20]

Folic Acid Antagonists - Synthetic efforts in the area of 1-deaza-7,8-dihydropteridines continued in light of the confirmed activity of the

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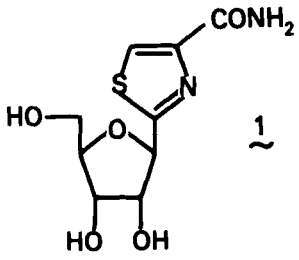
parent ethyl 5-amino-1,2-dihydro-3-[(N-methylanilino)methyl]pyrido[3,4-b]-pyrazin-7-yl-carbamate against P388 cells resistant to both methotrexate (MTX) and vincristine. The N-methyl-4-methoxyanilinomethyl analog produced an ILS of 150% after a single dose of 25 mg/kg against the P388 MTX-resistant line. This group of compounds exert their antitumor activity by causing mitotic arrest and not by inhibiting dihydrofolate reductase (DHFR).[21] 5,8-Dideazaisofolic acid was found active in vitro (ED_{50} ~ $5x10^{-6}$-10^{-7}M) against seven MTX-nonresponsive human tumor cell lines. It inhibited the development of human osteosarcoma in 70% of the hamsters treated with 80 mg/kg when given at the time of tumor implantation and 10 days later.[22] Alteration of the lipophilic character of the C_9-N_{10} bridge of MTX by 10-alkyl-10-deaza analogs produced compounds with improved transport properties. The 10-methyl-10-deaza analog gave an ILS value of 235% against L1210 in mice at 18 mg/kg.[23] An interesting synergism between MTX and epipodophyllotoxins (VM-26 and VP-16-213) was observed in relation to increase uptake and polyglutamation of MTX in Erlich ascites in vitro.[24] MTX-resistant hepatoma cells lacking an efficient transport of the drug, accumulated, hydrolyzed, and glutamylated MTX as much as the wild-type cells when MTX was given as a poly(L-lysine) complex.[25] Similarly, a clone of human CCRF-CEM cells resistant to MTX was sensitive to MTX encapsulated in cationic unilaminar vesicles.[26] Metoprine, by virtue of its enhanced lipophilic character and increased penetration by passive diffusion, showed activity against transport-deficient L1210 cells resistant to MTX. ILS values of 138 and 129% were obtained at 28 mg/kg qd 1-3.[27] In order to circumvent some of the clinical limitations of metoprine in clinical trials due to interference with the metabolism of histamine, other lipophilic DHFR inhibitors were investigated. 2,4-Diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-d]pyrimidine showed potent DHFR inhibition and no interference with histamine N-methyl transferase. The compound showed very fast entry into the cells and proved to be active against P388 (ILS 64%, 100 mg/kg), S180 (ILS 209%, 200 mg/kg) and Erlich ascites (ILS 263%, 100 mg/kg).[28]

Purine and Pyrimidine Antagonists - 2-β-D-Ribofuranosylthiazole-4-carboxamide (1) is being pursued as a high priority clinical candidate for the treatment of lung tumors and metastasis. Remarkable activity against LL carcinoma in mice was obtained at 25-50 mg/kg (qd 1-9) where it produced 100% tumor-free 60-day survivors.[29] This drug appears to be metabolized to an NAD-like structure where the nicotinamide portion is replaced by thiazole-4-carboxamide.[30] The important compound 5'-deoxy-5-fluorouridine (5'-dFUrd), which functions as a 5-FU prodrug, continues to generate a great deal of interest. A relative higher activity of pyrimidine nucleoside phosphorylase found in tumor tissue and capable of cleaving the drug to 5-FU may explain the selectivity of this drug in vivo.[31] Other analogs which included Cl, F, or OMs substituents at the 5'-position gave superior ILS values (58-64%) against murine L1210 leukemia at 250 mg/kg (qd 1-5) when compared to the parent 5'-dFUrd.[32] Other 5-FU derivatives included the N_1- and N_3-phthalidyl analogs which showed good activity against two solid tumors (MH134 and Meth A). The N_3-analog caused reduction in tumor weight of 81-83% at 200 mg/kg po given twice for 20 days.[33] The N_1-undecanoyloxymethyl and N_1-dodecanoyloxymethyl derivatives of 5-FU were active orally or ip against L1210 leukemia in mice. The dodecanoyl derivative at 100



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mg/kg ip produced an ILS of 106% with a therapeutic ratio of 23.[34] The 3',4'-dehydro analog of ftorafur showed superior activity than the parent (ILS 56% vs 8%) against murine L1210 leukemia at an equal dose of 50 mg/kg.[35] A curative effect with minimal toxicity was reported for 3'-amino-3'-deoxythymidine against murine L1210 leukemia (ILS 144%, at 160 mg/kg qdx2 1-3).[36] The antitumor activity of pseudouridinedicarboxaldehyde, the periodate oxidation product of the C-nucleoside pseudouridine, appears to be related to its ability to produce cell arrest in the G_2+M phase. An ILS value of 90% was obtained at a dose of 100 mg/kg (qd 1,4,7) against L1210 leukemia in mice.[37] 2'-Fluoro-5-methyl-1-β-D-arabinofuranosyluracil (FMAU) was very active against ara-C resistant L1210 and P815 lines *in vitro* and *in vivo*. ILS values of 86 and 250% were observed, respectively, for each tumor at 800 mg/kg qd 1-9.[38] Several phospholipid derivatives of ara-C in which the fatty acid side chains have been varied (ara-CDP-L-dipalmitin, L-diesterarin, L-dimyristin, and L-diolin) exhibited ILS values of 188, 167, 153, and 153%, respectively, at 40 mg/kg/day for 5 days. These diphosphate prodrugs appear to be more active than the monophosphates.[39] Liposome encapsulated ara-CTP helped overcome ara-C resistance in a mouse lymphoma cell line.[40] 3-Deazaguanine was found active ip against L1210 sensitive (ILS 63%) and ara-C resistant (ILS 50%) tumors in mice at doses ranging from 40-80 mg/kg.[41] A 6-mercaptopurine glucuronate behaved as a latent selective agent against L1210 cells in culture while showing no inhibition of growth against non-tumor Chinese hamster lung fibroblasts. This selectivity is attributed to the higher levels of β-glucuronidase that exist in cancer tissues.[42] Acylated derivatives (n-butyryl and n-hexanoyl) of P^1,P^2-bis(6-mercaptopurine-9-β-D-ribofuranoside-5') pyrophosphate partially circumvented the resistance to mercaptopurine riboside in L1210/MPR cells by an increase in uptake of the intact molecule.[43] This compound may have a totally different mechanism of action according to another study.[44] A discussion dealing with the role of the conformation of nucleosides and nucleotides in enzyme reactions and its relevance to the design of antitumor and antiviral agents has been published.[45]

Anthracyclines - The trend observed in this important class of drugs is towards the development of more active compounds through chemical manipulation of the sugar moiety. Biological studies with the newer analogs suggest that these antibiotics have other sites of action besides nuclear DNA. Replacement of the aminosugar daunosamine in adriamycin (ADM) and daunomycin (DNM) analogs by 2-amino-pyranosyl or 3-amino-3,5-dideoxy-D-ribofuranosyl moieties, resulted in compounds equivalent to ADM itself when tested *in vivo*, despite a lower *in vitro* cytotoxicity and a lower order of interaction with DNA.[46] When ADM was coupled to an insoluble agarose support to block its entry into the cell, the material still showed potent cytotoxicity against L1210 cells in culture. This effect could only be explained by the drug's interaction with cell surface components.[47] Among more than 500 analogs tested by the NCI, a 3'-deamino-3'-morpholino derivative of daunorubicin (DNR) is being described as the most potent anthracycline antibiotic, capable of producing comparable increases in life-span to those achieved with ADM in mice but at a dose forty times lower (0.2 mg/kg).[48] 4-O'-tetrahydropyranyl-ADM was found superior to ADM against *in vivo* P388, B16, LL, and colon adenocarcinoma 38. This fact combined with its reported lower cardiotoxicity is considered to be significant.[49] Rat peritoneal macrophages collected 24 h after ip administration of ADM (10 mg/kg) were rendered cytotoxic to syngeneic cancer cells in culture. Such *in vivo* labelled macrophages accumulated ADM in cytoplasmic vacuoles which was then transferred into the nuclei of cancer cells.[50] Another area of active research was the

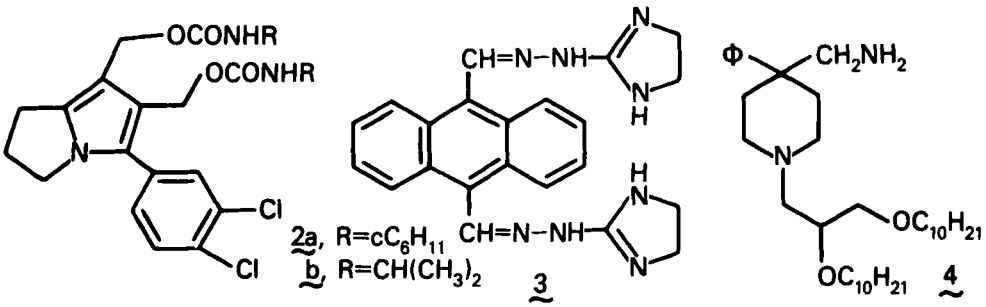
coupling of anthracyclines to polymers and macromolecules,[51,52] and encapsulation of the drugs into liposomes to alter transport properties,[53,54] reduce toxicity, and increase activity.

Aminoacids and Peptides - Four small peptides (MW ~ 3000-5000) isolated from Palythoa species showed in vitro activity against P388 cells (ED_{50} 0.02-0.0023 μg/ml). The A and B isomers produced ILS values of 22 and 32% at 150-300 μg/kg against P388 leukemia in mice.[55] Protamine, an arginine-rich protein, inhibited the growth of new capillary vessels necessary for tumor growth. In mice bearing LL or B16 tumors a dose of 60 mg/kg sc every 12 h inhibited lung metastases (77-92%), despite the lack of cytotoxicity towards these cells in vitro.[56] A histidine analog, histidinol, appears to be effective in combination with phase-specific anticancer agents by being capable of maintaining normal cells (CHO cells, LR73 line) in a G_0-like stage while transformed cells continue their cyclic transit and vulnerability to cytotoxic agents. L-histidinol (1mM) protected normal cells from 5-20 mg/ml of ara-C.[57]

Steroids, Prostaglandins and Analogs - A dichloro cyclopropyl derivative of stilbene which behaved as a "pure" antiestrogen appeared to be superior to tamoxifen in reducing the growth and occurrence of a DMBA-induced rat mammary carcinoma.[58] The transformation of stilbenes into more rigid indene-type structures does not appear to reduce receptor affinity.[59] 3,3'-Diacetoxy-α,β-diethylstilbene and the corresponding epoxide exhibited strong antitumor activity against a DMBA-induced hormone-dependent mammary carcinoma of the rat. The epoxide produced complete remission in 76% of the treated animals at 1 mg/kg/day for 28 days.[60] A SAR study in a series of 1,1,2-triphenylbut-1-enes in relation to estradiol receptor affinity, indicated that p-substitution in the E-isomer series was associated with higher receptor affinity and better antitumor activity.[61] Among several aromatase inhibitors, 10-propargylestr-4-ene-3,17-dione, behaved as the most potent compound (8000 times more potent than aminoglutethimide) in MCF-7 human breast cancer cells.[62] The proceedings of a conference dealing with the subject of aromatase inhibitors and new perspectives in the chemotherapy of breast cancer were published.[63] Prostaglandin PGD_2 demonstrated potent cytotoxicity against L1210 cells in culture. Almost complete inhibition of growth was achieved at 5 μg/ml.[64] On the other hand, prostaglandin synthesis inhibitors such as, flurbiprofen and indomethacin, increased the survival of mice after surgical excision of a transplantable adenocarcinoma when treated in conjunction with melphalan or MTX.[65]

Miscellaneous Synthetic Agents - Several selected bis(acyloxymethyl) derivatives of pyrroles and pyrrolizidines with significant and reproducible activity against P388 leukemia in mice were evaluated in a panel of experimental tumors. Compounds 2a and 2b showed good activity against B16 melanoma in mice, achieving 3 out of 10 cures for each compound at doses of 50 and 25 mg/kg, respectively.[66] Some cyanine dyes used mainly as photographic sensitizers showed marked prolongation of survival in mice bearing P388 and B16 tumors. ILS values of 82% at 10 mg/kg (P388) and 137% at 2.4 mg/kg (B16) were obtained for the most active congener.[67] A new benzothiazolo[3,2-a]quinolinium salt was shown to be very active against P388 murine leukemia producing an ILS of 118% at 200 mg/kg after a single dose. The activity correlated well with the degree of interaction with DNA.[68] Two 7-hydroxylated lucanthone analogs, predicted to have an increased association constant with DNA, demonstrated increased activity over the parent drug against P388 leukemia in mice producing ILS values of 88% (50 mg/kg) and 165% (64 mg/kg),

respectively.[69] Selected among a series of 9,10-anthracenedicarboxaldehyde bishydrazones, compound 3 showed activity against L1210 and P388 leukemia in mice with ILS values of 151 and 173% after a single dose of 125 and 150 mg/kg for each respective tumor.[70] Silylation at O or N-positions of several established antitumor agents suggests that these compounds may behave as useful prodrugs. The O-tribenzylsilyl carbamate, $(ClCH_2CH_2)_2N\text{-}COOSi(CH_2C_6H_5)_3$, produced an ILS of 114% at 50 mg/kg against P388 leukemia in mice.[71] A novel lipoidal amine 4 showed potent antimetastatic activity against B16 melanoma. After surgical removal of the primary tumor, the compound, administered at 0.15-2.5 mg/kg iv, increased the number of metastasis-free animals from 19% (controls) to 56%.[72] A high antimetastatic effect after removal of a LL primary tumor in mice was also displayed by 1-p-(3,3-dimethyl-1-triazeno)benzoic acid and 1-p-tolyl-3,3-dimethyltriazene, which produced 23% to 43% cures when administered daily for 8 days prior to tumor removal at a dose range of 25-50 mg/kg.[73] Cimetidine, a commonly used antiulcer drug in cancer patients, has no antitumor effect. However, when used in a single dose regimen at 100 mg/kg in combination with cyclophosphamide, it produced increases in survival of P388 leukemic mice comparable to those obtained after doubling the dose of cyclophosphamide but without increased toxicity.[74]



Metal Complexes and Polymers - The platinum complexes dominated the scene of recent research efforts in terms of novel structures and studies aimed at understanding their mechanism of action at the molecular level. Cis-diammine-1,1-cyclobutane dicarboxylate Pt(II), which possesses good antitumor activity, was evaluated in 60 patients. It represents a possible alternative to cis-diamminedichloro Pt(II) (cisplatin, CDDP) because it is less emetic and signficantly less nephrotoxic.[75] The selenoguanine Pt(II) complex proved to be active against a murine lymphoma (L5178Y/MP) resistant to mercaptopurine. The sulfur analog exerted its cytotoxic effect after hydrolysis to thioguanine, whereas the seleno analog was active *per se*.[76] A new complex, cis-dichloro-bis[1-(2-hydroxyethyl)-2-methyl-5-nitroimidazol-N^3] Pt(II), with low toxicity towards Chinese hamster ovary cells, was superior (10-fold) to metronidazole as a radiosensitizer toward hypoxic cells *in vitro* at 50 μM.[77] An extensive study of a series of 28 new analogs of CDDP which included Pt(II) and Pt(IV) complexes was published.[78] Using small oligodeoxynucleotides, the interaction between DNA and CDDP was studied. The products were separated and characterized by NMR.[79,80] Bioactivation of CDDP and cis-[Pt(diaminocyclohexane)Cl_2] involved intracellular hydrolysis to reactive platinum species containing aquo ligands such as cis-$[(NH_3)_2Pt(H_2O)_2]^{+2}$. The degree of intra/extracellular reactivity of Pt complexes may depend on the relative ease of hydrolysis of the non-amine ligand.[81] S180 cells treated with CDDP lose their surface charge associated with nucleic acids. These surface nucleic acids, detected by electrophoretic

techniques on all of several types of tumor cells, were absent on normal cells.[82] Pt-Ig complexes were prepared with K_2PtCl_4 and a specific Ig towards a particular tumor line (Moloney virus-induced lymphoma and a B-cell leukemia). The specific Pt-Ig complexes inhibited DNA synthesis more efficiently than nonspecific Pt-Ig complexes.[83] A new N-phosphonoacetyl-L-aspartate Pt(II) complex demonstrated good activity against murine tumors and human xenografts. Against the ADJ-Pc6 plasma cell tumor, the ILS was 351% at 25 mg/kg with 60% survivors on day 75.[84] The Cu(II) complex of an otherwise inactive ligand derivative of 2-acetylpyridine thiosemicarbazone showed the highest level of activity among a series of transition metal complexes of the ligand (ILS=64% at 6.25 mg/kg).[85] An L-asparaginase-albumin polymer more resistant to biodegradation was found 20 times more active than the free enzyme. In mice bearing 6C3HED lymphosarcoma, 50% of the treated animals survived for 27 days as opposed to 13 days for the controls.[86] A low molecular weight polyelectrolite polymer composed of amide and ammonium salt groups, separated by ethylene units, was evaluated *in vivo* against a series of tumor systems. Activity against LL was observed over a dose range of 60-2500 mg/kg with exceptionally low toxicity.[87]

Fermentation Products and Antibiotics - The first total synthesis of bleomycin was reported.[88] The physico-chemical properties of this antibiotic in relation to its mechanism of action have been reviewed.[89] The role of the bisthiazole moiety of bleomycin in DNA binding was studied using DNA fragments of defined sequences as substrates.[90] Different site specificities for DNA fragmentation by bleomycin and talisomycin were demonstrated and correlated with structural parameters.[91] Saframycins A and C, both antibiotics containing two heterocyclic quinone moieties, interact with DNA and produce single strand scissions by the generation of oxygen-mediated reactive $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$.[92] A SAR study of dextran conjugates of mitomycin C revealed a positive correlation with polymer size. The MW ~ 500,000 polymer produced an ILS of 105% at 10 mg/kg against B16 tumor-bearing mice.[93] An increase in activity and therapeutic index over actinomycin D was obtained with the 7-(2,3-epoxypropoxy) actinomycin D analog. At optimal doses of 1.2-1.8 mg/kg, it produced cures in 3 out of 7 mice treated against P388 leukemia.[94] The complete structure of the antitumor antibiotic carzinophilin A was described. This compound is considered to be the first natural intercalative bisalkylator with two aziridine moieties undergoing an acid-catalyzed interstrand crosslinking with DNA.[95] Continuing studies with the highly active DNA-binding antibiotic CC-1065 (NSC-298223) revealed this compound as one of the most potent cytotoxic agents known (400 times more cytotoxic than adriamycin), causing 90% growth inhibition of L1210 cells at 0.05 ng/ml.[96] Fredericamycin A, a novel cytotoxic antibiotic with an asymmetric spiro carbon that gives the molecule an unusual L-shape structure has been completely characterized.[97]

Natural Products - A complex phyllanthoside isolated from *Phyllanthus acuminata* was characterized and found curative at 8 mg/kg against P388 leukemia in mice. ILS values at 4-16 mg/kg were in the range of 62-72%. Curative levels of activity were also detected against B16 melanoma.[98] Microhelenin E, a new antileukemic norpseudoguaianolide from *Helenicum microcephalum* was active at 8 mg/kg against P388 murine leukemia (ILS 66%).[99] In search for anguidin congeners with superior activity over the parent compound, two 3,8-diketo analogs with the highest activities against various murine tumors were discovered. ILS values against P388 leukemia were 170 and 206%, respectively, with multiple survivors observed at 1.6 mg/kg.[100] A series of quassinoids related to the clinical

Phase II agent bruceantin, which included several active bisbrusatolyl dicarboxylic acid esters, showed a strong correlation between antileukemic activity and ability to inhibit protein synthesis in P388 leukemic cells.[101] Four toxic proteins recently isolated form the seeds of Abrus precatorius were shown to be active against S180 cells in vivo. Life span increases from 71 to more than 400% were observed at very low doses (0.04-0.12 μg/kg).[102] A SAR study for VP-16-213, VM26 and podophyllotoxin was published.[103]

Immunotherapeutics and Anticarcinogens - Retinoic acid (RA) was shown to effectively inhibit the clonal growth of the human cell lines KG-1, acute myeloblastic leukemia, HL-60, and acute promyelocytic leukemia, at very low concentrations (2-25nM). RA also inhibited clonal growth of leukemic cells from 5 of 7 patients with AML.[104] The conversion of a malignant murine embryonal carcinoma to benign teratomas was also achieved chemically with RA and dimethylacetamide in vivo either systemically or by intratumor injections.[105] It was demonstrated that N-(4-hydroxyphenyl)retinamide (4-HPR) was highly effective in inhibiting a chemically-induced hormone-independent ovarian cancer in rats without affecting the ovarian hormone action.[106] Similarly, retinyl acetate was found to be equally effective in inhibiting the induction of ovarian hormone-responsive and nonresponsive mammary carcinomas chemically induced by DMBA.[107] The activity of small molecular weight immunomodulators, capable of binding to cell surface enzymes has been studied. The activity of one of such agents, bestatin, capable of annhilating minimal residual tumor in the lungs, was discussed in detail.[108] The stearoyl derivative of N^2(γ-D-glutamyl)-meso-2(L),2'(D)diaminopimelic acid was highly effective in inhibiting tumor growth of Meth-A fibrosarcoma in mice, producing complete suppression at 100 μg/ site.[109] The toxic subunit of ricin was conjugated to a monoclonal murine antibody specific for the common acute lymphoblastic leukemia (CALLA) antigen expressed only on human lymphoblastic leukemia cells. This conjugate proved to be a potent cytotoxin for CALLA-positive cells growing in vitro, producing 50% inhibition at 2×10^{-10}M.[110] The anthracycline daunomycin after covalent attachment via a dextran bridge to specific antibodies against rat α-feto protein was more effective than daunomycin alone. Against rat ascites (AH66), the specific conjugate produced > 80% cures at 2 mg/rat.[111] BCG plus conventional chemotherapy significantly increased the survival of patients with prostate cancer when compared with conventional therapy alone.[112]

QSAR - In a series of neutral 2-nitroimidazoles the importance of electron affinity and octanol/water partition coefficients in relation to the drugs' antitumor enhancement properties of CCNU versus radiosensitization were studied. Lipophilicity appears to be more critical for the antitumor enhancement properties of CCNU.[113] Qualitative and quantitative SAR studies of the classical antifolates were conducted with 32 new MTX analogs. Minimal topological differences were used to get the best fitting with the unknown shape of the receptor cavity.[114] QSAR comparison of the inhibition of growth of MTX-sensitive and resistant leukemia cells in culture by a series of 45 triazenes was performed and compared with the degree of inhibition of DHFR. The results showed that potent triazine inhibitors against resistant tumors can be achieved by strongly hydrophobic compounds.[115] A similar trend was found for the diaminopyrimidines.[116] A QSAR study of 509 tumor-active members of the 9-anilino-acridine family was completed. The study revealed the capability of this approach in handling large amounts of biological information.[117]

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Chapter 15. Antiviral Agents

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Infectious viral diseases remain an important medical problem as attested to by the significant morbidity associated with influenza disease and the high incidence of genital herpes simplex virus (HSV) infections. Important advances in antiviral chemotherapy have been made in recent years with the FDA approval of idoxuridine, vidarabine and trifluridine for the topical treatment of herpetic keratitis and of amantadine for oral treatment of all influenza A virus infections. The past year has seen the approval of acyclovir, the first selective antiherpetic drug for treatment of primary genital herpes and mucocutaneous HSV infections. Acyclovir, with its unique mechanism of action, although one shared to some degree by a number of new antiviral pyrimidine nucleosides, may mark the beginning of a period of development of drugs with selective antiviral activity against several classes of viruses.

In this Chapter we review both new and old compounds with activity against DNA viruses and include a brief update of advances in the RNA area. Interferon inducers, which were discussed in the 1982 Annual Reports, are not included.[1] Antiviral agents with activity against DNA viruses and RNA viruses were summarized in the 1980[2] and 1981[3] Annual Reports, respectively. Several reviews which have appeared during the past two years give a comprehensive overview of the most promising clinical and experimental antiviral agents[4-14] and provide additional background on viral diseases.[15-18]

AGENTS ACTIVE PRIMARILY AGAINST DNA VIRUSES

Of the DNA viruses, the herpes group is the source of the most common viral illnesses in man. The family consists of herpes simplex virus (HSV) types 1 and 2 (cause of "cold sores", encephalitis, eye and genital infections), varicella zoster (VZV) (chickenpox and shingles), cytomegalovirus (CMV) (pneumonia, CNS diseases, and disseminated infections especially in neonates) and Epstein-Barr virus (EBV) (mononucleosis). HSV-2 has been implicated in cervical carcinoma, and EBV may be the causative agent of nasopharyngeal cancer, immunoblastic lymphoma and Burkitt's lymphoma. All of the herpesviruses share the characteristic of undergoing periods of dormancy during which they reside either in ganglionic sites or, in the case of EBV and possibly CMV, in lymphocytes. For reasons that are not well understood, these latent viruses may be reactivated to initiate recurrent or new forms of illness. Recent research has led to a better understanding of virus replication, particularly those steps specifically directed by viral enzymes. Several mechanisms are operative for the inhibition of the herpesvirus. As a preliminary step a compound may be an alternate substrate of the viral-induced thymidine kinase (TK). After processing to the triphosphate form, the agent may be an alternate

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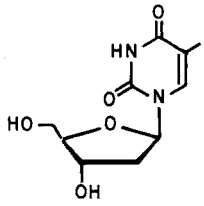
substrate for and/or a competitive inhibitor of the viral DNA polymerase. Incorporation of fraudulent nucleosides into the viral DNA may terminate or delay chain elongation and also can cause distortion of the secondary and tertiary structure of the DNA molecule.

Pyrimidine Nucleosides

Idoxuridine (5-iodo-2'-deoxyuridine, IDU) - This pyrimidine nucleoside was the first drug approved for clinical use in the treatment of herpes keratitis, a sight threatening eye infection. Because of its systemic toxicity, idoxuridine (1) is used mainly as a topical preparation and is a standard drug for herpetic eye infections. Solutions of IDU in DMSO have been successfully used in treating herpes zoster,[19] particularly trigeminal zoster, but not for thoracic involvement.[20] A 30% DMSO solution of IDU was ineffective and possibly hazardous in genital herpes therapy.[21] In controlled animal studies, 5-iodo-3',5'-diacetyl-2'-deoxyuridine, a prodrug form, significantly reduced ulcerative keratitis.[22]



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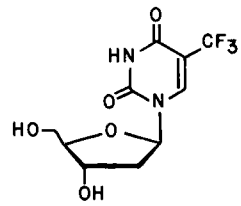
Trifluridine (5-trifluoromethyl-2'-deoxyuridine, TFT) - Trifluridine (2) has become one of the standard drugs for the treatment of herpetic keratitis, particularly in patients unresponsive or hypersensitive to other antiviral agents.[10,11] Trifluridine is converted to the 5'-monophosphate by viral and cellular kinases, and the subsequently formed triphosphate is incorporated into viral and cellular DNA but does not appear to cause chain termination.[23] Because of systemic toxicity TFT is used only topically. TFT showed in vitro activity against human CMV (HCMV) with a therapeutic ratio of 108, suggesting that it might have clinical use as an anti-HCMV agent.[24] A combination of acyclovir and TFT on clinical isolates of HCMV was synergistic against three out of four strains and may be of clinical use in the treatment of this disease.[25]



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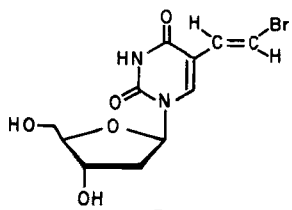
E-5-(2-Bromovinyl)-2'-deoxyuridine (BVDU) - BVDU (3) is a potent inhibitor of HSV-1, VZV and pseudorabies virus, but it has weak activity against HSV-2.[26,27] Indeed, BVDU may serve as a useful clinical probe to distinguish between the two types of HSV.[28] Its mechanism of action is similar to that of other antiherpetic pyrimidine nucleosides,[29] and host cell toxicity is very low.[26] The selectivity of BVDU for HSV-1 over HSV-2 may be due to differences in their thymidine kinase (TK).[30] Both viral enzymes appear to be bifunctional, capable of phosphorylating nucleosides and also their monophosphates. BVDU is efficiently phosphorylated by both type 1 and type 2 TK's, but the monophosphate of BVDU is a poor substrate for the type 2 enzyme.[30] The triphosphate of BVDU is equally active against both viral DNA polymerases.[29] In controlled studies, BVDU was more effective than TFT in the treatment of herpetic iritis in rabbits[31] and showed substantial healing of the more deeply penetrating eye disease, stromal keratitis, in rabbits[32] and in man.[32,33] In orofacial HSV-1 infections in hairless mice, early initiation of BVDU therapy, either systemically or topically, significantly prevented establishment of latent virus in the trigeminal ganglia but had no effect on established virus.[34] In uncontrolled trials, oral BVDU was efficacious in the treatment of six patients



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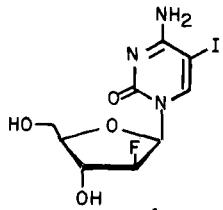
suffering from herpes zoster ophthalmicus with no toxic effects.[35] The arabinosyl analog of BVDU was ten times less active than BVDU against HSV-1 in vitro. Unlike BVDU, the arabinoside is not incorporated into replicating DNA in virus infected cells.[36]

2'-Fluoro-5-iodo-1-β-D-arabinofuranosylcytosine (FIAC) - This cytosine derivative has activity against HSV-1, HSV-2, VZV and CMV. IC_{90} values range from 0.0025-0.0116 μM and 0.0044-0.0126 μM for HSV-1 and HSV-2, respectively, with IC_{50} values for VZV and CMV of 0.01 μM and 4 μM, respectively.[37] FIAC (4) has only minimal cytotoxicity in uninfected Vero cells which is reversed by 2'-deoxycytidine. The latter compound does not compromise the antiviral effect of FIAC, so this combination may have clinical application for potential toxicity problems.[38] As with several other pyrimidine nucleosides, FIAC is first phosphorylated by the viral thymidine kinase. The subsequently formed FIAC triphosphate is more inhibitory to the HSV-1 and HSV-2 DNA polymerases than to the cellular polymerases and appears to be an alternate substrate only for the viral DNA polymerase.[39] In metabolism studies on [2-^{14}C] FIAC, the predominant metabolites were the deaminated derivatives 2'-fluoro-5-iodo-1-β-D-arabinofuranosyluracil (FIAU) (73%), 2'-fluoro-5-methyl-1-β-D-arabinofuranosyluracil (FMAU) (5.4%), and 2'-fluoro-1-β-D-arabinofuranosyluracil (FAU) (2.3%).[40] These metabolites, particularly FMAU, are also potent antiherpetic agents. FIAC is active in vivo as demonstrated by the rescue of the majority of mice inoculated with 20 MLD HSV-1 24 hours prior to drug treatment.[41] The metabolite, FMAU, was 10-100 times more protective, and both compounds are effective against HSV-2 and CMV.[41] Uncontrolled clinical trials with FIAC in patients with herpes zoster were reported to show significant benefit.[41] FMAU, which is currently in clinical trial as an antiherpetic agent, is also active against ara-C resistant leukemias.[41]



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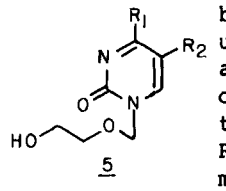
Pyrimidine Acyclic Nucleosides - The antiviral activity of acyclovir has stimulated interest in acyclic analogs of pyrimidine nucleosides. A number of 2-hydroxyethoxymethyl derivatives of cytosine, uracil, thymine and 5-halouracils (5) showed little or no antiviral activity, although some analogs were inhibitors of E. coli growth in vitro.[42,43] A study of the nucleotides of some 5-alkyl acyclouridine analogs (5; R_1 = OH, R_2 = alkyl) on HSV-1 DNA polymerase demonstrated that the mono-, di- and triphosphates of 5-propyl-1-(2-hydroxyethoxymethyl)uracil (5; R_1 = OH, R_2 = $CH_2CH_2CH_3$) were inhibitory with the IC_{50} of the triphosphate equal to 0.3 μM. However, 5 itself was not inhibitory to HSV at 100 μM concentration suggesting that lack of phosphorylation may be partly responsible for the lack of activity.[44]



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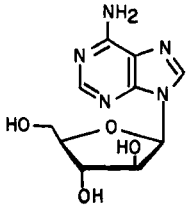
Purine Nucleosides

Vidarabine (9-(1-β-D-arabinofuranosyl)adenine, ara-A) - Ara-A (6) is a synthetic nucleoside with in vitro activity against HSV-1, HSV-2, VZV, EBV, CMV, vaccinia, variola and hepatitis B viruses.[45] The active form of the drug is the 5'-triphosphate (ara-ATP) which appears to inhibit the synthesis of viral DNA and is competitive with d-ATP. Ara-ATP is also inhibitory to cellular DNA polymerases but to a lesser extent than HSV DNA polymerase. Vidarabine ointment is used for the therapy of ocular herpes diseases.[46] The use of a combination of ara-A, acyclovir and the

adenosine deaminase inhibitor, 2'-deoxycoformycin, has been shown to significantly reduce the severity of herpes keratitis in rabbits caused by resistant strains against which neither drug alone was effective in vitro.[47] Ara-A is the only drug approved in the U.S. for the systemic treatment of HSV encephalitis.[48] A more water-soluble form, the 5'-monophosphate (ara-AMP), is under investigation.[49] Topical ara-AMP was ineffective in a controlled, double-blind trial for genital herpes.[50] In controlled, double-blind trials of immunocompromised patients with herpes zoster[51] or chicken pox,[52] ara-A accelerated cutaneous healing of lesions and when administered within 72 hours of onset, reduced or prevented spread of the infection to the viscera with minimal clinical toxicity.



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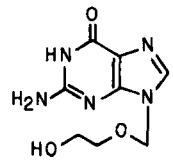
Ara-A and ara-AMP have been under intense evaluation in the therapy of chronic hepatitis B virus (HBV) infections, both alone and in combination with interferon.[53,54] In a study of five patients with chronic hepatitis B receiving doses of ara-A of 5-10 mg/kg/day for 5 days, a transient fall in the levels of HBV associated DNA polymerase was demonstrated in all patients, with the effect lasting two to three months in three cases.[55] In an uncontrolled trial, the combination of ara-A and human leukocyte interferon showed little clinical efficacy and considerable neurotoxicity in bone marrow transplant recipients with CMV pneumonia.[56] Toxicity was also encountered with this combination in chronic HBV patients, the effect being dose dependent and reversible.[57]

Acyclovir (9-(2-hydroxyethoxymethyl)guanine, ACV) - Acyclovir (7) is a purine nucleoside with activity against both human and animal herpes viruses.[58] Acyclovir, which is essentially non-toxic to uninfected cells, is phosphorylated by the viral TK to the monophosphate form in herpesvirus infected cells.[58] Further conversion by cellular kinases[59,60] produces the triphosphate which is both a substrate and a potent inhibitor of the viral DNA polymerase. The incorporation of ACV into viral DNA is self-limiting since it lacks a 3'-hydroxy group, thereby acting as a chain terminator.[13] In 1982 acyclovir was approved by the FDA for the topical and i.v. treatment of primary (initial) genital herpes and for cutaneous herpes simplex infections in immunocompromised patients. An oral formulation is under review.[61] Ongoing clinical trials with acyclovir for a wide range of herpes infections have produced an extensive literature in the past few years of which the following is a brief sample. The proceedings of an acyclovir symposium in 1981 has been published.[62] Acyclovir decreased viral shedding and shortened the duration of pain and lesion healing in the treatment of primary genital herpes.[63] Because of the rapid development and short duration of recurrent (as opposed to primary) herpes genitalis (HSG) and labialis, ACV ointment has not shown statistically significant clinical benefit for these indications in immunocompetent patients.[63,64] However, the oral form has shown efficacy for HSG.[65] Studies involving the use of the drug in prodromal stages are currently underway.[61,65] Successful therapy of herpetic keratitis,[66] zoster (shingles),[67] chicken pox,[68] and neonatal herpes[69] infections has been demonstrated. In the treatment of herpes encephalitis, an often fatal infection, studies in animal models have shown significant decrease in mortality.[70] Use of the drug in diseases caused by Epstein-Barr virus and human cytomegalovirus (HCMV) has produced both positive and negative findings.[71-73] Although neither of these viruses is known to induce a thymidine kinase, low levels of ACV triphosphate produced by viral-stimulated cellular enzymes appear to be sufficient to inhibit the viral DNA polymerase, especially the more sensi-



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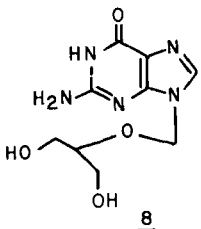
tive EBV enzyme.[74] However, CMV inhibition varies with the strain, and there is no correlation between in vitro susceptibility and clinical benefit.[72] In other studies acyclovir has been reported to transiently lower levels of HBV associated DNA polymerase in patients infected with chronic hepatitis B (another DNA virus), but no permanent clinical effects were noted.[75] Development of strains resistant to acyclovir, always a serious potential problem with anti-infective drugs, has been intensively investigated.[76,77] The lowered virulence in animals of TK-deficient mutants created in vitro has been shown.[78] Resistance in vitro is usually associated with either reduced thymidine kinase activity[79] or altered viral DNA polymerase.[80] In clinical situations there have been a few incidences of herpes strains resistant to acyclovir, all due to altered TK. Several studies with ACV have shown that generally, if therapy is commenced early in the course of the infection, establishment of latency can be prevented, but the drug has no significant effect on already latent viruses.[13] One of the most beneficial uses of ACV has been in prophylactic action against recurrent HSV infections in immunocompromised patients, whose subnormal immune defense exposes them to especially severe and often life threatening viral illnesses. This was demonstrated in a double-blind, controlled trial of bone marrow transplant recipients. Of subjects in the drug group, who were maintained on acyclovir prior and subsequent to surgery, none developed generalized herpes infections during therapy, whereas 70% of the placebo group did.[81]

9-(2-Hydroxy-1-(hydroxymethyl)ethoxymethyl)guanine (8) - In 1982 four independent groups of investigators reported the antiherpetic activity of



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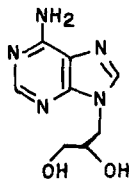
8, an analog closely related to acyclovir, differing only in the addition of a hydroxymethyl group on the acyclic chain.[82-85] Also variously termed BIOLF-62,[83] DHPG[84] and 2'NDG,[85] the compound has been reported to be superior to acyclovir in potency and bioavailability in selected in vivo studies. The general mechanism of action appears to be similar to that of acyclovir. However, it is claimed that in vitro 8 has activity against some HSV-1 strains which are resistant to acyclovir.[82] Compound 8 is equally active against HSV-1 and HSV-2 (IC_{50} = 0.2-2.0 μM),[82,83] but there are conflicting reports on its activity against the other herpesviruses. One group claims potency against EBV and CMV greater than acyclovir (IC_{50} = 1.0 and 1.0-5.4 μM, respectively),[83] while another found little or no activity with these two viruses at non-toxic doses.[82] Activity against equine herpesvirus and VZV was also reported.[82,83] The compound shows little toxicity in vitro or in vivo.[82-84] Significant reduction in mortality of mice infected with HSV-2 was reported in studies using 80 mg/kg/day of 8 orally.[83] In studies with HSV-1 thymidine kinase, 8 was claimed to be a superior substrate for the phosphorylating enzymes, with the K_m of the viral thymidine kinase for 8 comparable to thymidine (66 and 8.5 μM, respectively) and considerably lower than that of ACV (426 μM).[84]

(S)-9-(2,3-Dihydroxypropyl)adenine (DHPA) - DHPA (9) has antiviral activity against a number of DNA and RNA viruses.[3] The principal mechanism of action may be inhibition of S-adenosyl-L-homocysteine (AdoHcy) hydrolase.[86] Inhibition of this hydrolase leads to an accumulation of AdoHcy, which results in inhibition of viral mRNA methylation, an obligatory step for viral maturation.[5] DHPA has been reported to have a toxic effect on testicular germ cells in mice at a dose otherwise nontoxic to the host or to other organs.[87] This effect is reversible, and the



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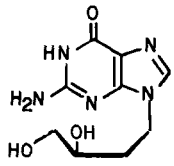
resulting offspring were seemingly normal. However, when evaluated in the developing chick embryo, DHPA manifested strong embryotoxic actions at 30- and 100-μg doses.[88] Unchanged drug appears to be responsible for the embryotoxicity which may be mediated via the inhibitory properties of DHPA towards AdoHcy hydrolase.

(R)-9-(3,4-Dihydroxybutyl)guanine (DHBG) - This new compound (10) is a selective antiherpetic agent which reduces HSV-1 and HSV-2 plaque formation by >90% at 5-10 μM, whereas the IC_{50} for uninfected cells is about 500 μM. Its activity is mediated by the HSV thymidine kinase with a K_i = 1.5 μM for the HSV-1 TK and a K_i >250 μM for the cellular enzyme. In placebo-controlled studies, a 5% ointment of the racemic drug showed good therapeutic effects on HSV-1 cutaneous lesions in guinea pigs and in herpes keratitis in rabbits. Oral DHBG reduced mortality from HSV-2 systemic infections in mice by >50% when given twice daily at 25 mg/kg.[89]



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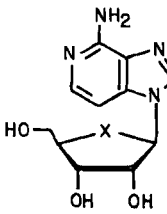
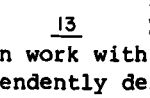
3-Deazaadenosine and 3-deazaaristeromycin - 3-Deazaadenosine (11) and its carbocyclic analog 12 comprise a new class of antiviral agents of undetermined potential. Both compounds are active against HSV-1 and HL-23 C-type virus.[90,91] The antiviral effect of 12 was correlated with accumulation of S-adenosyl-L-homocysteine (AdoHcy), presumably due to inhibition of AdoHcy hydrolase.[90] A probable mechanism for its antiviral activity may be inhibition of the methylation by AdoHcy of viral mRNA methylation at the 5'-cap. Since 12 was not phosphorylated by L1210 leukemia cells, it is probably not incorporated into RNA or DNA and may be a potential antiviral agent without some of the undesirable effects of other antiviral nucleosides.[90]



11, X = O
12, X = CH_2

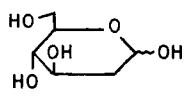
Other Agents

Foscarnet (phosphonoformic acid, PFA) - Foscarnet (13) is an inhibitor of HSV replication both in vitro and in vivo.[92] It is more potent than phosphonoacetic acid (PAA) against HSV replication in cell culture[93] and causes less severe skin irritation.[94] Clinical interest in PAA has lagged due to concern about its deposition in bone.[6] The literature on PAA has been recently reviewed.[95] PFA is a selective inhibitor of HSV-induced DNA polymerase, as has been substantiated in studies on HSV in HeLa BU cells[93] and in work with EBV virus.[96] Studies on the DNA polymerase from five independently derived PFA-resistant variants of HSV-1 suggest that resistance may result from several different types of active-site alterations.[97] Structure-activity relationships have been studied for pyrophosphate analogs on isolated CMV DNA polymerase and CMV multiplication,[98] as well as on avian myeloblastosis virus reverse transcriptase.[99] The in vitro effect of PFA in combination with acyclovir, BVDU and 8 against HSV-1 and HSV-2 has been reported.[100] The combination of PFA and 8 was strongly synergistic for both types 1 and 2. Foscarnet cream has been reported to be more effective than acyclovir in the treatment of cutaneous HSV-1 infections in guinea pigs.[101]



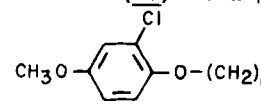
13

2-Deoxy-D-glucose (2-DG) - 2-Deoxy-D-glucose (14) is an inhibitor of protein glycosylation which has been reported to have selective antiviral activity against enveloped viruses including HSV.[102] Although an earlier report claimed that human herpes genitalis could be successfully treated by topical application of 2-DG,[103] the actual in vivo efficacy of 2-DG has remained controversial.[104] Two recent studies in experimental animal models (guinea pigs and mice) have found 2-DG to be ineffective in the treatment of herpetic cutaneous or genital infections.[105,106]

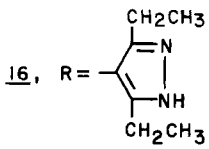


14

Arildone [4-(6-(2-chloro-4-methoxyphenoxy)hexyl-3,5-heptanedione] - Arildone (15) is a β-diketone which selectively inhibits replication of some DNA and RNA viruses by blocking virion uncoating.[3] The compound, which is in clinical trials for HSV infections,[107] has recently been reported to be orally active in protecting mice against poliovirus-induced paralysis and death.[108] This work further demonstrates the usefulness of agents blocking virion uncoating in the systemic treatment of viral infections. Studies on the metabolism of arildone[109] and techniques for its assay in physiological samples have been reported.[110] A water-soluble pyrazole derivative of arildone, Win 41258-3 (16), has antiviral activity against HSV-1 and HSV-2 in tissue culture.[111] In testing in two different animal models, 16 had a significant therapeutic effect in the topical treatment of HSV.[111]

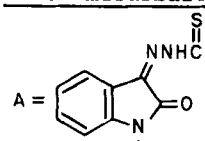


15, R = $-CH(CCH_2CH_3)_2$

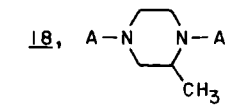


16, R =

Thiosemicarbazones - The thiosemicarbazones represent a milestone in antiviral chemotherapy due to the early use of methisazone (17) in clinical medicine. An extensive review on the antiviral activities of thiosemicarbazones has been published.[112] The dimeric methisazone analog, TSKI-VI (18), is active against vaccina virus, both in vitro and in vivo. In the treatment of ectodermal lesions in mice, TSKI-VI had anti-vaccinia virus properties similar to methisazone.[113] A series of 2-acetylpyridine thiosemicarbazones has been found to selectively inhibit replication of HSV-1 and HSV-2 with mean 50% inhibition values of 1.3 μg/ml and 0.22 μg/ml, respectively.[114]



17, A-H



18,

RNA Viruses

Significant progress has been made in the clinical use and development of agents active against RNA viruses. Clinical efficacy with both amantadine and rimantadine for the treatment of influenza A infections has been further substantiated.[115] Ribovirin appears to be effective against influenza when administered by inhalation of a small-particle aerosol through a face mask.[116] A new compound, sodium 5-aminosulfonyl-2,4-dichlorobenzoate, has good in vitro activity against several strains of influenza virus and was effective in reducing mortality in mice.[117] Several other agents have been reported with potent in vitro activity against rhinovirus. Enviroxime has an excellent serotype profile in vitro, but activity has not been substantiated in clinical trials.[118] 4',6-Dichloroflavan is another new compound with potent in vitro rhinovirus activity.[119] Studies on 4',5-dihydroxy-3,3',7-trimethoxyflavone, a natural product,[120] have led to the development of 4'-ethoxy-2'-hydroxy-

4,6'-dimethoxychalcone which was reported to have activity against 46 of 53 rhinovirus serotypes.[121] Good *in vitro* activity against seventy-two of ninety rhinovirus serotypes has been found for 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile.[122]

From this brief overview, it is evident that agents with potent *in vitro* activity against some RNA viruses have emerged but, with the exception of agents active against influenza, clinical efficacy remains to be demonstrated. Thus, the development of antiviral agents against respiratory diseases remains a challenge to the medicinal chemist.

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Chapter 16. Immunotherapy of Infectious Diseases

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Introduction - Immunotherapy, defined as the use of either naturally occurring substances or drugs to modify the immune response to a particular antigen, offers a possible modality to improve our ability to prevent or treat infectious diseases. Altnough antibiotics have made a dramatic difference in the morbidity and mortality of infectious disease, improvements are still necessary. For instance, bacterial meningitis carries a 5-10 percent mortality during childhood and as many as 50 percent of the survivors may suffer some neurologic sequellae; neonatal meningitis carries an even worse prognosis. Infections with gram negative bacteria complicated by septic shock continue to carry an unacceptably high mortality and serious viral infections still pose a major problem since there are few efficacious antiviral drugs.

The ability to manipulate the immune response is becoming more of a reality as our understanding of the immune system improves. The first intentional manipulation of the immune response dates back almost 200 years to Jenner's observation that prior infection with cowpox prevented subsequent infection with smallpox. He was then able to translate his observation into the first successful active immunization with an attenuated live virus vaccine. In 1901 von Behring was awarded the first Nobel prize in Medicine for his work in developing passive as well as active immunization against bacterial toxins. In the early 1900's specific immune animal sera were successfully used in the treatment of established infections caused by N. meningitidis, S. pneumoniae and H. influenzae. The rapid development of effective antimicrobial drugs, coupled with the complications associated with the administration of animal antisera, led to a diminished interest in the immunotherapy of established infections.

The past decade has seen a renewed interest in manipulating the immune response to both prevent and treat infections. The manipulation of the immune system by drugs, natural or synthetic products, or derivatives of these products represents an attractive adjunct in the treatment of infections. Many of the experimental foundations for this approach have been laid by the expanding knowledge of endogenous regulators and mediators of lympho-myeloid cooperation and differentiation. Human interferon and specific monoclonal antibodies have been produced in vitro and both have been used or will shortly be used in clinical trials against numerous viruses and pathogenic organisms. The role of these modulatory agents may be three-fold: a) enhancement of phagocytosis of infectious agents; b) restoration of impaired immune functions; and c) treatment

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of infection(s) without exerting selective pressure on microbial populations which is an inherent problem with antibiotic therapy.

This review will serve to update the reader on the rationale for immunotherapy and some of the more recent and significant advances in the immunotherapy of infectious disease.

Immunosuppression - Aside from the various forms of congenital and acquired immunodeficiencies or specific immune defects, there are a variety of conditions in which the immune system is compromised and the defect in the system is unknown. These are associated with increased infectious complications. For instance, malnutrition can adversely affect the immune host defenses resulting in increased susceptibility to infection.[1] Cancer patients undergoing treatment by chemotherapy, x-irradiation or surgery represent a large population susceptible to infections.

The wide spectrum of neoplastic diseases and accompanying treatments make more than a brief discussion of these disease conditions impossible. Each neoplasm has a preferred method of treatment which may produce a unique defect in the immune system. In neoplastic disease, infection has been associated with more intensive and prolonged therapy.[2] In general, the immunologic defects associated with cancer therapy fall into three major types: (1) decrease in neutrophils, (2) altered immunoglobulin levels, and (3) lowered cell-mediated immunity. The major defect predisposing to infection appears to be a decreased neutrophil or granulocyte count. Impaired cell-mediated immunity is associated with increased viral and fungal infections.

Several defects in the immune system have been associated with alcoholic and cirrhotic patients including abnormal pulmonary defense mechanisms and systemic defense mechanisms associated with defective granulocyte function and decreased numbers. Other host defense alterations associated with alcoholism include decreased serum complement levels and abnormal leukocyte chemotaxis. Defects in cell mediated immunity are reflected in a decreased ability to be sensitized to the antigen keyhole limpet hemocyanin.[6]

Diabetic patients present a unique immunocompromised profile since it is controversial whether resistance to infection in the well-managed diabetic patient is considered normal. Abnormalities in the diabetic's immune system with respect to cell-mediated immunity and neutrophil function are controversial. There is a consensus, however, that infections do create special problems in diabetics since the infection can lead to difficulty in management of the disease.[4]

Infection is the most important complication and cause of death in acute renal failure. Two-thirds of patients with chronic renal failure have a high incidence of infection and one-third of these patients succumb to infection. These complications are associated with impaired leukocyte function and cell mediated immunity. Currently, renal allograft transplantation is considered an accepted method of treatment for chronic renal failure. While successful renal transplantation will reverse the defects associated with chronic uremia, immunosuppressive agents to prevent rejection predispose the transplant recipient to infections. The lifelong balance between adequate immunosuppression to allow acceptance of the graft and over-immunosuppression leading to infection is critical to these patients. Various defects in immune mechanisms in the renal

transplant patient ranging from leukocyte to T and B cell and monocyte function have been reported. Most of these defects are critical within the first three months after transplantation.[8]

Trauma, whether caused by physical injury, burn or surgery, has been associated with various kinds of suppression of the immune system. It is well known that burn patients, for example, are exquisitely sensitive and have a high incidence of Pseudomonas infections. This increased incidence in infection is probably associated with decreased neutrophil function and possibly the appearance of suppressor T cells which actively suppress a normal immune response.[6]

Finally, the proportion of people in the U.S. over 65 years of age will increase to approximately 45 million by the year 2020. Loss of immune function occurs with aging in animals and man.[7-9] The age-associated decline in the immune response represents an opportunity to restore the response or halt its decline. T lymphocyte function is most severely affected and manifested by a decline in delayed-type hypersensitivity reactions and delay in allograft rejection. The elderly have a decrease in antibody responses to foreign antigens, poor persistence of antibody responses, poor generation of memory responses, and an increased incidence of autoantibodies and benign monoclonal gammopathies. Such abnormalities of immune function may contribute to the increased incidence of infections in the elderly.[7]

Several explanations have been offered for this immunologic senescence. With age, the thymus gland involutes and has a decreased capacity to promote T lymphocyte differentiation. Further, hormones secreted by the thymus gland can not be detected in humans older than 60 years of age.[10] Alteration of T cell subpopulations, a decrease in T cell proliferation responses,[11-13] and a marked deficiency of E-inducible T lymphocyte precursors have been recently described in elderly humans[14]. These findings could reflect either an absolute deficiency of inducible precursors or an impaired response of precursors to inductive signals. It has been recently shown that T cells of elderly adults and animals both produce less T-cell growth hormone or interleukin 2 (IL-2) and show a decreased receptivity and decreased binding of normal IL-2 than activated T cells of young individuals.[15-17] In addition to a qualitative and/or quantitative T cell deficiency state, overactive inhibitory mechanisms in the elderly may contribute to diminished immune responses. Excessive suppressor cell activity, increased sensitivity to suppression, and augmented autologous anti-idiotypic antibody responses have been described to occur with aging.[18,19] The sensitivity of lymphocytes to endogenous immunomodulators prostaglandin E, histamine, and hydrocortisone changes with aging.[20]

Other biochemical alterations of aged lymphocytes that have recently been described include: low activity of 5'nucleotidase,[21] alterations of LDH isoenzyme patterns,[13] and low basal levels of C-AMP and high levels of C-GMP,[22] although the last has not been confirmed by others.[23] Decreased antibody responses in the elderly have been shown to be associated with decreased T lymphocyte helper activity, increased suppressor cell activity, and with intrinsic B lymphocyte defects.[24-26] The progressive incidence of immunosenescence may be an important target for therapeutic intervention.

Vaccines and Adjuvants - Vaccines for the prevention of infectious disease have been used for a long period. Recently, synthetic peptide

vaccines have been introduced as a therapeutic manipulation. Synthetic peptide vaccines have the following potential advantages over conventional vaccines: decreased cost, increased safety because of elimination of the necessity to grow and attenuate or inactivate a virus or to purify a virus from blood products (as with preparation of Hepatitis B vaccine for humans), and increased stability.[27-29] The techniques to design and produce synthetic peptide vaccines are dependent on recent advances in biotechnology. To ensure that the selected peptide will induce antibodies which react with surface-exposed determinants of the native epitopes, computer programs have been used to predict surface orientation based on relative hydrophilicity of the individual amino acids of proteins with known amino acid sequence.[30-32] When amino acid sequences were unknown, the nucleotide sequence was used to determine amino acid sequence.[33,34] Short peptides, synthesized by high repetitive yield methods using automatic synthesizers,[35] have been coupled to either natural protein carriers (keyhole limpet hemocyanin [KLH] or bovine serum albumin) or to synthetic carriers (poly-DL-alanine-poly-L-lysine) and administered to animals with adjuvants (Freunds, aluminum hydroxide, N-acetylmuramyl-L-alanyl-D isoglutamine [MDP] or liposomes) to induce antibody responses.

Generalizations about the application of peptide vaccines have been made recently. Peptides with six or fewer amino acid residues were poorly immunogenic. Longer, soluble peptides, especially those with proline residues, proved to be most immunogenic. Effective peptides could correspond to regions of the protein that in crystal structure had α-helix, β-sheets, or random coil conformation and to locations in the intact protein that were not restricted to either the NH_2 or COOH terminus. In fact, it has been found that sites in the native protein accessible to peptide-induced antibodies are far more numerous than the sites recognized by the antibodies elicited during natural infection or by immunization with the intact purified protein. Thus, peptide vaccines can induce unique antibody specificities with protective potential as long as that part of the peptide sequence is exposed on the surface of the molecule to allow binding of antibody.[36,37]

Earlier research with tobacco mosaic virus and MS-2 bacteriophage demonstrated the antigenic and immunogenic potential of synthetic peptides (reviewed in 27). More recent investigations have demonstrated that neutralizing antibodies can be induced in animals with synthetic peptides of the VP1 virus polypeptide of foot-and-mouth disease virus (FMDV)[38] and a synthetic hemagglutinin polypeptide of influenza virus.[29,39] Protection in guinea pigs against FMDV was induced with a KLH-coupled peptide administered with either Freunds or alum adjuvant. Inhibition of *in vivo* growth of influenza virus with protection of animals has been induced with peptides.[29,39] Both a linear and a cyclic peptide which reproduced the native confirmation of the hepatitis B surface antigen (HBsAg) have proven immunogenic and capable of duplicating serologically important subdeterminants of HBsAg (the latter in the absence of linkage to a protein carrier), but active protection has not yet been reported with the vaccines.[37,40-42] Synthetic peptides of rabies virus glycoproteins have also been demonstrated to be immunogenic but none has been shown to protect animals from challenge with live virus.[29] Synthetic peptides of diphtheria toxin representing amino acid sequences found in the toxin loop which comprises the two functional segments of the toxin induced protective antibodies in guinea pigs.[43] The peptides were immunogenic when coupled to either a protein or to a synthetic carrier, multichain poly-DL-alanine-poly-L-lysine, and

when administered with either Freund's adjuvant or in aqueous medium with the synthetic adjuvant muramyl dipeptide (MDP). Type-specific protective immunity to Streptococcus pyogenes has been induced by immunization with synthetic peptides of the type 24 M protein.[44]

Recently, peptides have been modified to produce a stable secondary structure which may provide a means of augmenting peptide immunogenicity.[45] However, further research to define the best protein carrier, the optimum and safest adjuvant and the nature of peptides with protective potential are required prior to adapting this technology to the prevention of human infections.

Synthetic adjuvants such as muramyl dipeptide (MDP), a dipeptide component of the Mycobacterium cell wall, have been synthesized as N-acetyl-muramyl-L-alanyl-D-isoglutamine. It can replace whole mycobacteria in Freunds complete adjuvant (FCA) in generating antibody production or delayed hypersensitivity to an antigen injected simultaneously and can stimulate nonspecific resistance of the host.[46-50] MDP is at least equal in activity to FCA and has the advantages that it is non-immunogenic, devoid of toxicity in mice, and can induce antibodies to antigen in a completely aqueous medium.[47] It has recently been shown that covalent attachment of MDP to macromolecular antigens or synthetic peptides can induce even higher antibody responses.[51] Studies of the structure-biologic activity requirements of synthetic MDP have shown: a) an N-acetyl-muramyl structure is essential for full activity; b) L-Ala can be replaced by L-Ser but not by Gly and change in stereochemistry of L-Ala produces an immunosuppressor; c) D-Glu α-amide can not be replaced by D-Asp α-amide nor by γ aminobutyric acid; d) replacement of D-Glu by L-Glu leads to loss of activity; e) the two carboxyl functions of D-Glu can be substituted, but the dimethylamide is inactive, and replacement of the α-amide by Gly inactivates the molecule; f) lipophilic derivatives of MDP produced by replacement of the primary hydroxyl group at the C-6 position, are less rapidly excreted and display greater nonspecific resistance, especially when incorporated in liposomes; g) its N-acetylmuramyl-L-alanyl-D-glutaminyl-n-butyl ester derivative produces an adjuvant with comparable immunostimulatory activity but with markedly less pyrogenicity.[46-52] The mechanism of the adjuvant properties of MDP have been reviewed.[46-50] Recent reports have described that macrophages stimulated by MDP synthesize and secrete T cell-activating monokines.[53]

MDP has been employed as an adjuvant to induce antibody responses to the following infectious agents: a) Plasmodium falciparum administered with 6-O-stearoyl MDP in carrier liposomes induced protection in monkeys;[54,55] b) MDP potentiated the immune response of hamsters to influenza virus subunit vaccines;[56] and c) synthetic peptides of diphtheria toxin coupled to either proteins or to synthetic carrier molecules (poly-DL-Ala-poly-L-lysine) administered with MDP induced protective antibody responses.[57]

Nonspecific enhancement of host defenses against infection has been induced with MDP in animals. MDP has been shown to enhance resistance to Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans, Listeria monocytogenes, Salmonella typhimurium, Streptococcus pneumoniae, Trypanosoma cruzi and Toxoplasma gondii. The mechanism of increased resistance is unknown. Acylated derivatives of MDP containing linear C-18 and C-20 fatty acids show increased activity.[58] MDP is reported to activate macrophages[59] and analogues of MDP that produce the most

non-specific protective activity produce the greatest activation of the reticuloendothelial system.[60] Derivatives of MDP active in producing non-specific resistance also stimulate chemotactic mobility, phagocytic activity and superoxide anion production of polymorphonuclear leukocytes in mice.[60]

Serotherapy - Immunoglobulin therapy and its clinical application for the prevention or treatment of infectious diseases had its beginnings at the turn of the century. In the current practice of medicine, immunoglobulins are administered in the form of human immune serum globulin, special high-titered immune serum globulin and most recently, as modified immune serum globulin available for intravenous use.[61] Generally accepted uses of human immunoglobulin preparations include post-exposure prophylaxis of hepatitis A and B, chicken-pox in immunosuppressed patients, measles, prevention and treatment of tetanus and as replacement therapy in patients with various antibody deficiency syndromes.[62] Information has been rapidly accumulating over the past few years suggesting many other infections may be amenable to immunoglobulin therapy.

Infection with gram negative bacteria complicated by endotoxin shock and Group B streptococcal infection in neonates are two bacterial infections that still carry a high mortality despite appropriate antibiotic administration. Animal data has indicated that antibody to the core region of the lipopolysaccharide obtained from the "J5" mutant of E. coli protects against several different types of gram negative infection.[63] Recently a controlled study has shown that serum obtained from volunteers vaccinated with the "J5" mutant had a beneficial effect when infused into patients with documented gram negative infection.[64] Additional studies are needed to determine the relative contribution of IgG versus IgM in protecting against gram negative shock. Although similar controlled studies have not been carried out in infants with Group B streptococcal infection, animal data suggests that passive administration of antibody may enhance the survival of infants with this infection.[65,66] These findings support the observation that exchange transfusions enhance the survival of infants with Group B Streptococcal disease.[67]

Another well-studied application of immunoglobulin therapy is in the interrruption of transmission of hepatitis B virus from mother to infant. This is an especially important problem in the Far East where hepatitis B infection is endemic and 40-90 percent of infants born to carrier mothers become infected.[68,69] The efficacy of hepatitis B immunoglobulin in preventing the vertical transmission of hepatitis B from mother to infant was recently demonstrated in a double-blind placebo-controlled study.[70]

There are several other examples where immunoglobulin administration has been less well studied or where the results have not been clear cut. Echovirus meningoencephalitis, a usually fatal disease in patients with X-linked agammaglobulinemia, has been reported to respond favorably to immunoglobulin administration.[71] It has also been suggested that antibody therapy might be beneficial in the treatment of sepsis in preterm neonates or in adults with overwhelming bacterial infection who may have "selective immunoglobulin depletion".[72,73]

The most consistently successful applications of immunoglobulin therapy have been with the use of immunoglobulin preparations that have a high titer of antibody to the disease causing pathogen. However, these high titered preparations are often difficult to produce and limited in

supply. The technique of monoclonal antibody production offers a potential solution to this problem.[74] An unlimited supply of antiserum of defined potency could be produced against a particular pathogen. To date, mouse monoclonal antibodies have been produced to a wide variety of microorganisms and protection has been demonstrated with these antibodies in several animal models of infection.[75-78] While these studies are promising, the routine use of monoclonal antibodies in humans for in vivo diagnosis and treatment of infectious diseases may require the production of human monoclonal antibody in order to avoid sensitization to foreign proteins. Several human monoclonal antibodies have been produced to date, and two have been demonstrated to have a protective effect in animal models of tetanus and H. influenzae infection.[79,80] As the technique of human monoclonal antibody production becomes perfected, human monoclonal antibody preparations should supplement or replace current immunoglobulin preparations obtained from human volunteers.

Immunoregulation - The ideal situation would be one in which the specific defects in the immunosuppressed host are known, allowing administration of a specific biological response modifier (BRM). This BRM could either mimic the action of a depressed cytokine (natural biochemical messenger(s) of the immune system) level or antagonize the action of an abnormally high level of another cytokine. Additionally, a BRM could augment the activity of reduced numbers of immunoreactive cells perhaps by inducing the proliferation of such cells. Until disease associated specific immunologic defects are identified, we will probably have to rely on currently available agents to augment a less specific phase of the immune response.

Cytokines, such as interferon, have been recently demonstrated to augment and/or effect NK cell function as well as activate macrophages. Interferons, which were first discovered in 1957,[81] are highly active glycoproteins which are produced by various mammalian cells after infection with a virus or when exposed to a large number of other stimuli such as double stranded RNA, fungal extracts, bacteria or bacterial products, other microorganisms or mitogens.[82] Many detailed reviews have been written which summarize the biosynthesis, chemistry, mechanism of action and genetics of interferon production.[83-85] Their ultimate role in the therapy of infections is still uncertain. Although a wide variety of microorganisms have been shown to induce interferon synthesis, interferon has generally been used as a therapeutic agent in the treatment of viral infections. Since the first clinical trial in 1962 there have been over three dozen studies done in humans with various types of viral infection[86,87] and although some antiviral effect has been noted,the overall results have not lived up to the early expectations for these substances.

The most consistent and impressive results using interferon seem to be in the treatment of chronic hepatitis B infection or varicella in cancer patients. The most recent study using interferon in the treatment of chronic active or chronic persistent hepatitis B infection demonstrated that interferon either alone or in combination with adenine arabinoside suppressed viral replication, decreased the contagiousness of a significant proportion of patients and resulted in permanent disappearance of hepatitis B virus-associated DNA polymerase from a significant number of study patients.[88]

Varicella and herpes zoster infections are a major problem in immunosuppressed patients. One-third of children with cancer who develop primary varicella have viseral dissemination and 7 percent die.[89] When

started within 72 hours of the appearance of the exanthem, interferon reduced both the rate of new vesicle formation and the number of episodes of life threatening dissemination when compared to a placebo group.[90] The number of patients was too small to determine the effect on mortality. The use of human leukocyte interferon was similarly effective for the treatment of herpes zoster in patients with cancer.[91] For a summary of all the clinical trials up to 1980 the reader is referred to references 86 and 87.

The availability of large amounts of relatively pure interferon has previously been a problem which has complicated clinical testing of this material. Because of this problem, interest arose for using interferon inducers as a way to stimulate the in vivo production of interferon by the patient. The entire topic of interferon inducers has been excellently reviewed in the previous issue of this series.[92] Toxicity of many of these interferon inducers has been a significant obstacle in their development as therapeutic agents, although some of the low molecular weight inducers such as tilorone, benzimidaole ribosides or various pyrimidine molecules have retained antitumor or antiviral activity while demonstrating a lessened toxicity.[85,92,93]

Although the antitumor and antiviral effects of interferon inducers are felt to be secondary to endogenous production of interferon, these inducer molecules possess immunomodulating effects which can be separated from interferon effects.[85] In addition, interferon inducers have been used successfully against several different bacteria and parasites in animal models of infection.[94-97] Although interferon inducers have been more widely studied in man as antitumor agents, a few clinical trials have been performed to determine their efficacy as antiviral agents. While two studies were able to demonstrate a protective effect when the agent was administered early against Rhinovirus infection, a study of experimental influenzae virus infection in human volunteers failed to show any benefit.[98,99,100]

In a recent review, Oldham[101] has characterized the different classes of BRM agents which will be tested and evaluated by the National Cancer Institute Biological Response Modifiers Program. Some of these BRMs include immunoaugmenting agents such as BCG, methanol extraction residue or BCG fractions, C. Parvum, other bacterial products (muramyl dipeptides, FK156 and FK565,[102,103] polysaccharides and bestatin) and synthetic agents (such as the pyran copolymers). Some of the other agents which modulate the immune response include cimetidine, indomethacin, isoprinosine, lentinan and tuftsin. Levamisole and azimexon may be considered immunorestorative agents. Some of the more specific BRMs may be the biochemical messengers of the immune system including cytokines such as Interleukin-1 and Interleukin-2. These might be considered as therapeutic agents while in other cases drugs may be developed which agonize or antagonize the action of such cytokines. However, the complex nature of these substances obscure a clear cause and effect relationship for clinical use of these agents. Another interesting class of compounds are the thymic factors or agents secreted as thymic hormones or fractions of thymic hormones as polypeptides.[104]

MDP is an interesting example of a bacterial product serving as a regulator molecule. Does this sugar dipeptide which is derived either synthetically or from the bacterial cell wall of mycobacterial cells regulate (enhance or depress) immune response because it "mimics" a regulator molecule of the immune system? It has become increasingly

apparent that simple peptides will replace the need for macromolecules previously thought essential in several biological systems. For instance, the discovery of endorphins and the identification of specific opiate receptor sites may stimulate other investigations for synthetic congeners of natural substances endowed with adjuvant activity.[47] An increasing number of substances are capable of modifying immune responses. Their therapeutic use against infections is still uncommon. Several reasons for this exist but the major reason still appears to be the mechanisms of immunoregulation are not well understood and, depending on the route and the timing of injection, the immunostimulants can exhibit suppressive activities.[105]

Evaluation - There are several ways one could evaluate immunomodulating agents for treating infectious diseases.[106] Systemic host defenses include phagocytic cells (neutrophils and macrophages), humoral immunity, and cell-mediated immunity. Three general ways to modulate host defenses are biological manipulation, pharmacologic agents and radiation. Non-specificity of manipulation means a general inhibition or immunostimulation of a variety of immune responses which could lead to undesirable side effects. Specific manipulation of host defense would, therefore, be more desirable. While it is a well worn phrase that what we need is a better understanding of basic immunology, it really is true. For example, Fidler et al[107] recently reviewed the rationale for the design of a sequential test system for determining the potential value of biological response-modifying agents for treatment of cancer in general and metastasis in particular. Many of the studies that the Biological Response Modifiers Program of the National Cancer Institute perform are founded on the belief that host responses are diminished or absent and an awakening of a hosts response would be highly beneficial. For the monitoring of host defense(s) against infection, the basic assumptions of the BRM are certainly pertinent, yet, the problems of variable efficacy, inconsistent bioassays and paradoxic immunosuppression must also be addressed. For example, administration of an immunoregulatory agent, bestatin, has induced changes in myelopoesis and functions of blood phagocytes.[108] For a detailed explanation of these assays used by the BRM program, see reference 101.

Some of these undesirable inconsistent effects may be due to the incorporation of antigens other than those required for host defense enhancement. Therefore, the purest component capable of stimulating the immune response would be beneficial. Amongst these are several synthetic immunostimulants derived from the bacterial cell wall[50] which were recently reviewed by Dukor et al.[109] Studies to examine the discrepancies between bioassays and natural resistance factors in the host need to be emphasized. Is antibiotic therapy taking part in the healing mechanisms synergistically, additively, antagonistically or without relationship to the natural mechanisms of defense? The efficacy of antibiotic therapy and the possibility of interference with the induction of acquired resistance must, therefore, be analyzed in infected and non-infected animals. Time of infection with respect to treatment being simultaneous or delayed, continuous or discontinuous must be examined. Determination of infection rate and dependence of host defense with immuno-deprived animals is essential.

Immune modulation may have a price. Many questions still exist. Hopefully we shall find that host defense can be and should be manipulated along with antibiotic therapy.

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Section IV - Metabolic Diseases and Endocrine Function

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Chapter 17. Progress in Atherosclerosis Therapy: Hypolipidemic Agents

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Introduction - The localized deposition of plasma lipids, primarily cholesteryl esters, in the intima of the arterial wall accompanies formation of the atheromatous plaque or atheroma, the characteristic lesion of atherosclerosis.[1,2] Growth of the atheroma eventually leads to constriction of the coronary arterial lumen and ultimately results in coronary heart disease (CHD), the major cause of death in Western countries. In 1978, the economic impact of CHD in the United States was estimated to be $37 billion or 14% of the total cost of all disease.[3,4] These observations, coupled with compelling epidemiological evidence implicating hypercholesterolemia as a primary risk factor for CHD,[3,5] have provided impetus for the development of therapeutic approaches to the prevention and treatment of atherosclerosis based on the attenuation of plasma cholesterol levels. Recent advances made in research directed toward this objective are presented below. Recent reviews on the various aspects of lipid disorders,[3,4,6-9] the metabolism and function of high-density lipoproteins (HDL)[10-13] and low-density lipoproteins (LDL),[14-18] the therapeutic management of hyperlipidemic states,[18] and the metabolism of antihyperlipidemic drugs[19] should be consulted for further details.

Risk Factors - Epidemiologically-documented, major risk factors for the development of atherosclerosis and CHD include hypertension, smoking, diabetes mellitus, obesity, age, sex and hyperlipoproteinemia.[3,20-22] Less well established are positive correlations between CHD and dietary intake of saturated animal fat[23,24] or type A social behavior.[4,25] In contrast, plasma HDL levels are inversely correlated with CHD, a finding that has greatly stimulated research on HDL metabolism and function.[10,26] A general awareness of these risk factors likely accounts, at least in part, for the 29% decline in the age-adjusted death rate from cardiovascular disease (including CHD) recorded in the United States since 1960.[27,28]

Lipoproteins and Lipid Transport - The importance of the lipoprotein transport system to the regulation of plasma lipid levels in man is firmly established.[16] This system is responsible for delivering triglycerides (TG) primarily to adipose and muscle tissues and for continuously shuttling cholesterol between intestine, liver and extrahepatic tissues.[29] Exogenous and endogenous TG and cholesterol enter, are transported in, and depart the bloodstream complexed to specific proteins called "apoproteins" which are integral components of circulating lipoprotein particles or carriers. Each lipoprotein particle consists of a hydrophobic core containing TG and cholesteryl esters encased by a surface monolayer

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composed of polar phospholipids, apoproteins and small amounts of cholesterol. Lipoproteins are grouped into major classes according to their relative densities; each class also differs in lipid and apoprotein content. Plasma lipoproteins are directed to their sites of metabolism by interactions of their constituent apoproteins with enzymes and cell surface receptors. As discussed in the next section, the number and viability of lipoprotein receptors, which mediate the uptake and degradation of cholesterol-carrying lipoproteins, largely determine the efficiency of plasma cholesterol handling.

The major classes of lipoproteins known to participate in lipid transport are chylomicrons, chylomicron remnants, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL and HDL.[29] Chylomicrons are assembled in the intestine. VLDL and HDL are packaged in the liver and in the intestine. Chylomicrons and VLDL, the two triglyceride-rich carrier particles, are converted to the cholesterol-rich chylomicron remnants and IDL by lipoprotein lipase (E.C. 3.1.1.34, LPL), an enzyme activated by apoprotein C-II (apoC-II) and situated on the luminal surface of endothelial cells lining the capillaries in adipose and muscle tissues. Fulminant atherosclerosis is produced in man by elevated plasma levels of LDL, IDL and chylomicron remnants,[30] whereas, chylomicrons and VLDL appear to be neutral. As noted earlier, elevated plasma HDL levels are associated with a reduction in symptomatic atherosclerosis.[10,26]

Lipoprotein Receptors and Hypercholesterolemia - New insights into the critical role played by receptor-mediated metabolism of atherogenic lipoproteins, particularly LDL, in the regulation of plasma cholesterol levels have been achieved during the last five years. Extensive investigation has focused on the LDL receptor, a transmembrane glycoprotein of molecular weight 164,000 which avidly binds LDL.[31,32] LDL receptors are situated on the cell membrane surface and, prior to internalization, they become clustered in invaginated portions of the cell membrane called "coated pits",[33] which are sheathed with clathrin on their cytoplasmic side. Clathrin is composed of three proteins having molecular weights of 33,000, 36,000 and 180,000 daltons. Assembly of three of the largest protein units with three of the smaller ones forms a triskelion or basic building unit bearing three arms which radiate away from the building unit's center. Triskelions, in turn, are joined together to form a polygonal, ball-like structure composed of five- and six-sided figures which mimic the seams on a soccer ball.[34,35] Recognition of apoB, the major LDL apoprotein, by the LDL receptor initiates the binding interaction between LDL and its receptor. As the coated pit begins to fill with LDL, the cell wall is pinched off to form an endocytic vesicle; this event completes the process of receptor-mediated endocytosis.[36] The newly-formed vesicle carries the internalized LDL to lysosomes in the cytoplasm. Recent studies[17] suggest that the LDL receptor is recycled between the cytoplasm and cell surface an average of 150 times during its half-life of about 20 hours. Upon exposure of the vesicular contents to the lysosomal proteases, apoB is hydrolyzed to amino acids and cholesteryl esters are cleaved to cholesterol and fatty acids. The liberated cholesterol performs four important, regulatory functions in the cell. First, it suppresses the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (E.C. 1.1.1.34; HMG-CoA reductase), the rate-limiting enzyme in cholesterogenesis.[37] Second, it activates acyl-coenzyme A:cholesterol acyltransferase (ACAT), which catalyzes the conversion of cholesterol to cholesteryl esters. The resulting esters are stored as liquid droplets in the cytoplasm. Third, it suppresses the synthesis of LDL receptors

and thereby limits cellular LDL uptake and prevents over-accumulation of cholesterol in the cell.[38,39] Fourth, it suppresses squalene synthetase activity under conditions of minimal HMG-CoA reductase activity.

A genetic defect exists in the LDL receptor locus of individuals with familial hypercholesterolemia (FH). Inheritance of a single copy of the FH gene limits the number of viable LDL receptors in FH heterozygotes to one-half of normal, whereas, FH homozygotes inherit two copies of the FH gene and have few, if any, viable LDL receptors.[40] The atherogenic consequences of these genetic defects are profound. The diminished number or non-existence of functional LDL receptors results in either a half-rate of receptor-mediated LDL catabolism in FH heterozygotes or no receptor-mediated LDL catabolism in FH homozygotes.[16,41] As a result, plasma LDL-cholesterol (LDL-C) levels rise to average values of 300 mg/dL in FH heterozygotes and 700 mg/dL in FH homozygotes, values far above those (120 mg/dL) generally observed in normocholesterolemic adults.[14] Myocardial infarcts usually begin at age 30, peak between ages 40 and 50, and occur in 85% of all male FH heterozygotes before age 60. This prognosis is delayed by about 10 years in heterozygous FH females. FH homozygotes show clinical signs of coronary artery atherosclerosis before age 10 and generally succumb to a myocardial infarction before age 30.[41]

Approximately 45% of the plasma LDL pool in normal humans is catabolized daily, 30% by the LDL receptor-mediated or specific pathway and 15% by non-specific pathways. FH heterozygotes have a LDL fractional catabolic rate (FCR) of 30% with 15% being catabolized by the specific pathway and 15% by non-specific pathways. FH homozygotes have a 15% FCR for LDL, all of which results from catabolism by non-specific pathways.[41] Based on these observations, the non-specific pathways of LDL uptake and catabolism have been examined to determine if they hold the key to understanding how atheromas and xanthomas are formed in FH patients. In cell culture studies, various kinds of macrophages or "scavenger cells"[15] have been demonstrated to effect LDL uptake and catabolism by non-specific pathways which first require that LDL be altered in one of several ways. The enhanced negative charge attending acetylation of the lysine amino groups in the apoB component of LDL affords an altered LDL particle (acetyl-LDL) which is recognized by macrophage high affinity receptors. After internalization by receptor-mediated endocytosis, acetyl-LDL is metabolized in the normal way.[15] In the presence of normal plasma, a portion of the liberated cholesterol moves from the cell into the plasma where it is picked up by HDL; the remaining intracellular cholesterol is esterified by ACAT and stored as cholesteryl esters. Interestingly, in the presence of acetyl-LDL and in the absence of either serum or serum substitute, cultured macrophages amass large quantities of cholesteryl esters and are converted to foam cells,[42] a component of the atheromas and xanthomas which are the characteristic lesions of FH patients. Malondialdehyde,[43,44] a breakdown product of the prostaglandin endoperoxides (PGG_2, PGH_2), and cyclohexan-1,2-dione[45] also alter LDL in ways that render the altered LDL particles recognizable by macrophage receptors.

Other lipoprotein receptors possessed by macrophages include one type which specifically takes up β-VLDL[46,47] and another type which recognizes dextran sulfate-modified LDL.[48] The appearance of β-VLDL in the plasma of cholesterol-fed experimental animals correlates with a massive accumulation of cholesteryl esters in macrophages of many tissues, including those of the arterial walls.[49] A lipoprotein similar to β-VLDL

builds up in patients with familial dysbetalipoproteinemia and is associated with atherosclerosis.[50] An accumulation of LDL bound to sulfated glycosaminoglycans is found in human atheromas.[51] The structural resemblance of dextran sulfate to sulfated glycosaminoglycans confers interest to this observation. Hence, the artifically-altered forms of LDL recognized by macrophage receptors in vitro appear to relate to the altered forms of LDL detected in man.[41] The exact alterations responsible for rendering LDL recognizable by macrophages in vivo remain to be elucidated. Nevertheless, macrophages are throught to be one of the first cell types involved in the development of atherosclerosis in FH and other hypercholesterolemic patients.[41,49]

Animal Models - Efforts to develop improved animal models of atherosclerosis have continued. Of particular interest are recent papers describing the Watanabe heritable hyperlipidemic (WHHL) rabbit as a model of human FH.[52-58] Hypercholesterolemia, elevated plasma LDL levels, marked atherosclerosis and cutaneous xanthomas are manifested by these animals. The homozygous WHHL rabbits develop plasma cholesterol levels of 650 to 950 mg/dL (90% of which is contained in LDL),[53] are devoid of LDL receptors on membranes from cultured fibroblasts, liver and the adrenal gland[53,55] and fail to take up and catabolize LDL by the high affinity, receptor-mediated pathway both in vivo[56] and in their isolated hepatocytes.[57] Other recently reported animal models include strains of rats which develop spontaneous hypercholesterolemia,[59,60] the Yucatan miniature swine which is sensitive to diet-induced atherosclerosis,[61] and rabbits fed a wheat starch-casein diet.[62-64]

Hypolipidemic Agents - Although dietary intervention is often the first-line approach to the treatment of hyperlipidemia, many hyperlipidemic patients fail to respond adequately to diet alone and require the adjunctive use of one or more hypolipidemic agents.[18,65-67] These agents are grouped below in two classes: (a) nonabsorbable agents which elicit their hypolipidemic effects in the intestinal lumen by interfering with lipid or bile acid absorption, and (b) absorbable agents which elicit their hypolipidemic effects via mechanisms affecting lipoprotein levels.

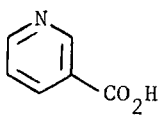
Nonabsorbable Agents - Inhibitors of Cholesterol Absorption - Dietary administration of the fat-like compound sucrose polyester (SPE) to African green monkeys for a two-month period proved hypocholesterolemic only in those animals having initial plasma cholesterol concentrations greater than 200 mg/dL, the relative response to SPE's action being directly related to each animal's degree of response to ingested dietary cholesterol.[68] The cholesterol-lowering effect of SPE was attributed solely to a reduction in plasma LDL levels; neither plasma HDL-cholesterol (HDL-C) nor TG levels were affected. In view of SPE's demonstrated hypocholesterolemic activity in man,[69] these results suggest that SPE may be therapeutically useful in individuals who are hyperresponsive to dietary cholesterol.[68] The poor tolerance displayed by experimental animals to the block copolymer Pluronic L-81 given in the diet has been surmounted by converting Pluronic L-81 to the corresponding benzoate (BEP).[70] When administered in an atherogenic diet to rabbits for two months, BEP lowered blood and tissue lipid levels, particularly plasma cholesterol, and prevented the development of atherosclerosis.[71]

Bile Acid Sequesterants - In spite of their unpleasant features, such as poor palatability, grittiness and gastrointestinal side effects,[72] as well as the necessity to use them in large doses, the bile acid sequesterants or resins are the most important class of drugs currently used to

lower plasma LDL-C levels.[67] The basis for their hypocholesterolemic activity stems from their ability to sequester bile acids in the intestinal tract for excretion in the feces. Studies with cholestyramine in man showed that sequestration of bile acids caused the liver to increase its LDL uptake by receptor-mediated endocytosis,[41] to convert the resulting intracellularly-liberated cholesterol to bile acids and to synthesize de novo cholesterol. The net effect of these combined actions was a two-fold increase in the high affinity FCR for LDL,[73] a result also observed in cholestyramine-treated rabbits.[74] The effects of low-dose cholestyramine therapy have been evaluated in children with Type IIa hypercholesterolemia. Daily cholestyramine doses of 8 g and 16 g lowered plasma cholesterol levels by 17% and 26%, respectively; both doses lowered plasma LDL levels by about 30%.[75] The results of a recent low-dose study with colestipol, another clinically-useful resin, led the investigators involved to suggest that patients whose cholesterol levels are only 15-20% above normal after dietary intervention may be adequately managed with minimal doses of colestipol.[76]

The increase in de novo cholesterogenesis evoked by the bile acid resins places a ceiling on their ability to lower plasma cholesterol levels.[77] In order to expand their therapeutic usage, these bile acid-binding drugs have been studied in combination with a variety of hypolipidemic agents having different mechanisms of action. Illustrative of such studies is that involving the evaluation of colestipol,[78] mevinolin (vide infra), a recently-discovered HMG-CoA reductase inhibitor,[79] and their combination in dogs. Administered at a daily dose of 700 mg/kg, colestipol reduced plasma LDL levels by 24%. A daily oral dose (10 mg/kg) of mevinolin elicited a 57% decrease in plasma LDL levels. When given at these same doses in combination, colestipol plus mevinolin produced a three-fold increase in liver LDL receptors, a two-fold increase in the FCR for LDL and an unprecedented 71% decrease in plasma LDL levels. These results clearly indicated that the actions of colestipol and mevinolin are synergistic. Combinations of colestipol with clofibrate,[80-83] fenofibrate[84] and nicotinic acid,[80,85,86] as well as combinations of cholestyramine with probucol[87] and nicotinic acid,[88] have been evaluated in hypercholesterolemic individuals. The most effective of these hypolipidemic combinations appears to be colestipol plus nicotinic acid which, in heterozygous FH patients, decreased plasma cholesterol and LDL-C levels by 45% and 55%, respectively,[80] and arrested the progression of angiographically-demonstrated coronary artery lesions.[86]

Absorbable Agents - Nicotinic Acid - Mechanism of action studies in hyperlipidemic patients were recently reported for nicotinic acid (1), which decreased plasma TG, TG in VLDL, transport of VLDL-TG, and plasma cholesterol levels by average values of 52%, 36%, 21% and 22%, respectively.[89] Kinetic modeling suggested that TG reduction resulted more from a reduction in lipoprotein size than in number. Nicotinic acid is apparently capable of concomitantly lowering VLDL and LDL and elevating HDL levels in plasma.[90] The kinetic and metabolic patterns in human volunteers have been determined for acipimox (2), an isosterically-modified analog of nicotinic acid.[91] Acipimox was rapidly absorbed and was excreted essentially unchanged in the urine. It was 20 times more potent than nicotinic acid in inhibiting lipolysis and was well tolerated.



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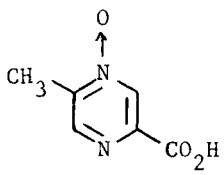

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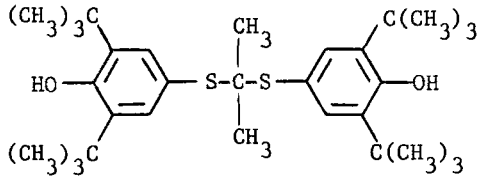
A significant reduction of plasma LDL levels and a significant elevation of plasma HDL levels were observed in Type IIa patients treated with acipimox; total plasma cholesterol levels were not altered.[92]

Probucol - Probucol (3) modestly lowered plasma cholesterol levels in some FH patients who previously failed to respond to clofibrate.[93]



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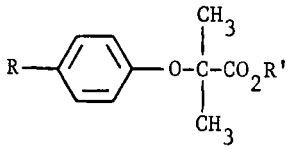
Because probucol lowers plasma levels of HDL, probably as a consequence of suppressed apoA-I synthesis,[94] and has been associated with electrocardiographic abnormalities, some investigators do not recommend its use in the treatment of hypercholesterolemia.[66,95]

Clofibrate - Clofibrate (4a) has been reported to increase plasma apoA-I and HDL levels in hyperlipidemic subjects[96] and to inhibit phosphatidyl choline synthesis, an effect suggested to play a role in its mechanism of



4

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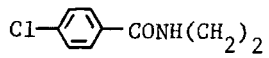
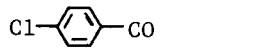
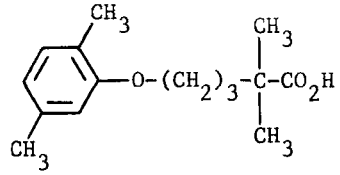
b,		H
c,		$(CH_3)_2CH$

action.[97] Recent clinical studies demonstrated that bezafibrate (4b)[98] and fenofibrate (4c)[99,100] two congeners of clofibrate, are more potent antihyperlipidemic agents than clofibrate in patients with primary familial hyperlipoproteinemias.

Gemfibrozil - No correlation between Lp phenotype and hypolipidemic response to gemfibrozil (5) was found in male volunteers with either Type II or Type IV hyperlipoproteinemia.[101] Treatment of male MI survivors with gemfibrozil corrected their dyslipidemias: total plasma cholesterol and TG levels were reduced by 17% and 54%, respectively; plasma HDL-C and apoA-II levels were increased by 16% and 20%, respectively; and plasma apoA-I levels were not altered.[102] More recently, gemfibrozil was demonstrated to elevate plasma levels of apoA-I, as well as apoA-II and HDL, in hyperlipidemic individuals.[103,104]



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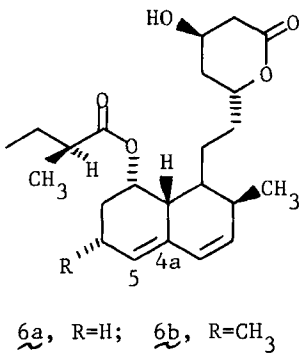
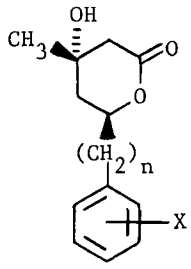
HMG-CoA Reductase Inhibitors - Compactin and Mevinolin - Reduction of plasma cholesterol levels in man by specifically inhibiting HMG-CoA reductase was first realized with compactin (6a, ML-236B = CS-500), a potent, competitive HMG-CoA reductase inhibitor isolated from Penicillium (P) citrinum[105] and P. brevicompactum.[106] An average 27% reduction of plasma cholesterol levels was achieved with orally-administered compactin (50-150 mg daily) in heterozygous FH and combined hyperlipidemic patients. Higher drug doses were required to achieve similar therapeutic results in a homozygous FH patient.[107] Cholesterogenesis in cultured skin fibroblasts from homozygous FH patients was also less susceptible to inhibition

by compactin than it was in fibroblasts from their heterozygous counterparts.[108] In heterozygous FH patients, compactin (30-60 mg daily) lowered plasma cholesterol levels by an average of 28%. Plasma levels of LDL and LDL-ubiquinone-10, but not ubiquinone per se, were also decreased.[109] Compactin has been reported to depress the rate of ubiquinone synthesis in cultured fibroblasts by as much as 70-80%.[110,111] Mevinolin (6b, MK-803),[79] a structurally-related HMG-CoA reductase inhibitor isolated from Aspergillus (A) terreus and Monascus ruber (as monacolin K),[112] has a K_i value of 0.6 nM and is two to three-fold more potent than compactin.[79] Given orally at doses of 6.25-50 mg twice daily in normal human volunteers, mevinolin reduced serum levels of cholesterol and LDL by 23-27% and 35-45%, respectively, and did not significantly alter serum HDL, VLDL and TG levels. No significant difference was found between the drug effects elicited by the lowest and the highest doses.[113] Plasma cholesterol levels and atherosclerotic severity were significantly diminished in mevinolin-treated rabbits maintained on an atherogenic diet.[114] Dihydro derivatives of compactin (trans-4a,5-dihydro 6a)[115] and mevinolin (trans-4a,5-dihydro 6b)[116] displaying slightly less HMG-CoA reductase inhibitory activities than their parent structures were isolated recently from P. citrinum and A. terreus, respectively. A series of mevalonate derivatives (7) with relatively modest HMG-CoA reductase inhibitory potencies has been described.[117]



6a, R=H; 6b, R=CH_3



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By virtue of their remarkable degree of inhibitory specificity, compactin and mevinolin have become valuable tools for probing those fundamental aspects of cell function involving HMG-CoA reductase. Exposure of cells containing this enzyme to compactin results in inhibition of the existing enzyme and a compensatory increase in enzyme levels. Addition of mevalonate, the product of HMG-CoA reductase action, blocks the increase in enzyme levels, an observation which suggests that one or more of the components participating in the multivalent feedback regulation of HMG-CoA reductase must be a non-steroid product (or products) derived from mevalonate.[118] A parallel in vivo result has been obtained in mevinolin-treated, cholesterol-fed mice.[119] Under conditions of diminished conversion of mevalonate to cholesterol and blocked mevalonate production, i.e., in the presence of LDL and compactin, [^{3}H]-mevalonate is incorporated into Δ^2-isopentenyl t-RNA, ubiquinone and dolichol.[120] Whether any or all of these natural products participate as feedback regulators is a topic of current investigation. In a series of related experiments, the normal S-phase burst of DNA synthesis attending cell growth in synchronized cells was prevented when HMG-CoA reductase was inhibited by compactin, an effect that was overcome by the addition of mevalonate.[121] Isopentenyl adenine and its 4'-hydroxy derivative zeatin were recently found to be effective in restoring the S-phase of cell growth.[122]

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Chapter 18. Disease Modifying Anti-Rheumatic Drugs

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Introduction - The scope of this review is limited to anti-rheumatic drugs defined as those drugs which have shown efficacy as disease-modifying anti-rheumatic drugs (DMARDs). In order to be classified as a DMARD, a drug need not necessarily effect a cure, but rather must retard or stop the underlying progression of the disease.[1] The term DMARD has replaced the term SAARD or slow-acting anti-rheumatic drugs which appeared in the literature for a brief period. Furthermore, only synthetic compounds are considered in our survey and natural products were deliberately excluded. Steroids and immunosuppressive therapies are not considered because there is little evidence that these forms of therapy are able to modify or retard the progression of arthritis.[2] Likewise, we have omitted the non-steroidal anti-inflammatory drugs or NSAIDs. These agents are highly effective in reducing the acute inflammatory response seen during exacerbations, but the underlying progression of the disease continues.[3] The DMARD therapeutic category attempts to address the chronic inflammatory response which is of major concern in rheumatology.[4]

There are only three classes of synthetic compounds currently marketed in the USA for which clinical evidence exists for their being classified as DMARDs.[5] These are antimalarials, gold salts and d-penicillamine. Therapies with each of these was found to be useful in rheumatology as a consequence of first being used for the treatment of other diseases. Astute clinical observation led to their development as DMARDs. The pharmaceutical industry has yet to market a DMARD as a product of its research and drug development programs. The unknown etiology of the disease combined with the dearth of experimental models which detect DMARDs has made the task formidable.[6]

PATHOGENESIS OF RHEUMATOID ARTHRITIS

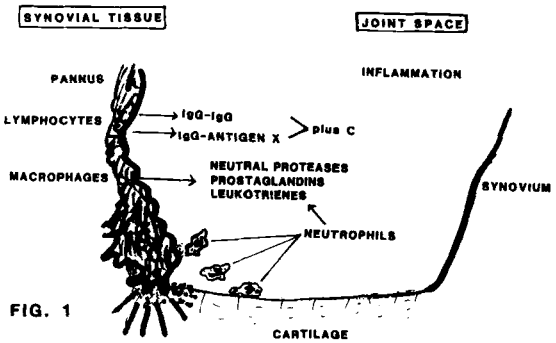

FIG. 1

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Figure 1 illustrates the two compartments involved in the pathogenesis of rheumatoid arthritis.[7,8] The NSAIDs are considered to exert their activity primarily in the joint space and reduce the inflammatory response through an influence on neutrophils and their mediators. The DMARDs, which have little direct effect on inflammation, probably modulate the cellular response occurring in the surrounding synovial tissue or pannus.

Investigations into the mode of action of the known DMARDs have been numerous but inconclusive. There are several extensive and recent reviews which address possible mechanisms.[9-11] Criteria have been proposed for fulfillment of a clinical definition of remission in rheumatoid arthritis.[12]

We will first review possible mechanisms of action of the known DMARDs, their clinical efficacy and side-effects. The severity and extent of side-effects associated with the currently available antirheumatic drugs represents the primary reason why their market has been limited. The section devoted to new compounds under consideration as DMARDs contains clinical candidates which may represent safer and more efficacious therapy.

The development of a drug which has the capacity to arrest or reverse the underlying rheumatic process and has a wide margin of safety would allow treatment to begin earlier, before there has been significant damage to the connective tissue (Figure 2).[1] Currently, treatment with DMARDs is not usually initiated until therapeutic attempts using several NSAIDs have failed.

The Effect of Drugs on the Course of RA

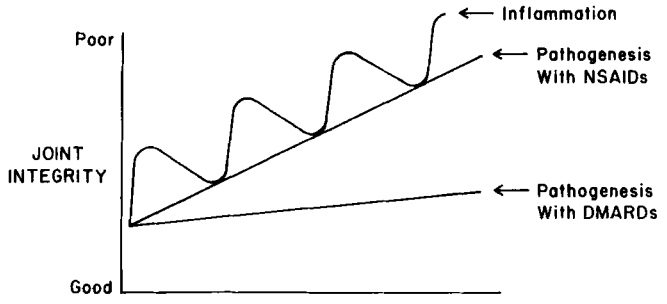


FIG. 2

Antimalarials - Although aminoquinoline antimalarials have been used to treat rheumatoid arthritis (RA) since 1951,[13] hydroxychloroquine is currently the only aminoquinoline marketed in the United States. The use of antimalarial agents as basic remission-inducing drugs was the subject of a recent symposium[14] and the clinical use of hydroxychloroquine has been reviewed by MacKenzie.[15,16] Several features of hydroxychloroquine, compared to other marketed DMARDs, commend its use. These include a low dropout rate due to side effects, a good safety record when used at a clinical dose of 4-6 mg/kg, proven efficacy of moderate degree, and low cost to the patient. Although hydroxychloroquine can cause retinal changes, risk can be limited by clinical observation for opthalmic changes.[17] Although retinopathy is rare in

patients taking the recommended low doses, ophthalmological evaluations at six month intervals are recommended since early retinal lesions are reversible. Limitation of daily dosage and ophthalmic monitoring can limit significant retinopathy even for prolonged therapy.[18,19] Clinical studies by Rynes have concluded that neither duration of treatment nor total cumulative dosage is important.[20,21]

Among the DMARDs, antimalarials have both the lowest dropout rate and best safety record. After a delayed onset of action, a high degree of disease suppression is seen in about 10 percent of arthritics and a moderate degree in 65 percent. Subjective benefit is usually not apparent until after six weeks of therapy and is measurable after three months.[22] Sixty percent or more of the effectiveness of hydroxychloroquine is obtained with six to nine months of continuous treatment. Diminution of erosive changes has not been confirmed statistically due to the lack of controls in most clinical studies. Among the DMARDs, the reduced potential for side effects by antimalarials permits earlier therapeutic intervention in the course of RA before there is joint destruction. Furthermore, NSAID therapy can be continued during treatment with hydroxychloroquine. It is of interest to note that the antimalarials are the only DMARDs that are also effective for the treatment of systemic lupus erythematosus.[18]

The diversity of actions reported for hydroxychloroquine are so numerous that the drug may be inhibiting several different systems which are relevant to its therapeutic profile.[23-31] Although direct inhibition of phospholipase A_2 and prostaglandins by hydroxychloroquine requires high concentrations, the drug *in vivo* may exert its activity indirectly via secondary messenger proteins such as macrocortin. Indeed, there is evidence that DMARDs have an indirect effect on cells and plasma proteins, and for this reason *in vivo* assays may facilitate their detection.[32,33] Although it is clear that a modification in the disease process of a chronic inflammatory condition will not be immediate, it may be possible to detect changes on cells or in plasma proteins relatively early. For example, Nagarkatti attempted to study the action of chloroquine on cell surface receptors *in vitro*, but found that human lymphocytes treated *in vitro* did not demonstrate any change in their capacity to bind the third component of complement (C3) and erythrocytes.[32] When chloroquine was administered orally, there was a significant decrease in the number of human peripheral lymphocytes with C3 and erythrocyte receptors. It was concluded from these studies that chloroquine is not acting directly on lymphocyte surface markers, but rather indirectly affecting factor(s) involved in the expression of the receptors. Likewise, it was shown that *in vitro* incubation with chloroquine has no effect on leukocyte adherence, whereas *in vivo* dosing and subsequent *ex vivo* evaluation results in a significant reduction in the ability of peripheral blood leukocytes to adhere.[33] It has been demonstrated that a plasma factor(s) is responsible for changes in leukocyte adherence.[34-36]

Gold The majority of rheumatoid arthritic patients who complete a course of injections of gold compounds experience measurable improvement.[37-39] Aurothioglucose, an oil suspension, and gold sodium thiomalate, an aqueous solution, are considered therapeutically equivalent. Although chrysotherapy appears to retard the progression of the proliferating pannus in RA,[40] the significant and consistent incidence of side effects has limited its use. Mucocutaneous reactions are most common with a reported[41] incidence of 15-30% but proteinuria and bone

marrow depression represent major toxicity problems. Approximately 15-20% of patients discontinue therapy because of side effects.[10]

Preliminary studies have indicated that auranofin, an oral gold preparation, may prove to be an important therapeutic alternative to injections of gold.[42] The effects of auranofin on humoral and cell-mediated immunity are greater than for injectable gold.[43] Extensive clinical trials have been pursued in order to establish a clear safety margin for auranofin over currently available chrysotherapeutic agents.

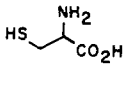
There are at least ten clinically effective gold compounds which have been investigated for anti-rheumatic activity; their potencies and toxicities are similar.[44] Each of these chrysotherapeutic agents contains ionic gold at the gold (I) oxidation level, and an ionic sulfur-gold bond. The importance of the thiol group in these compounds with respect to mechanisms of action is under investigation.[44]

Since RA is a disease of unknown etiology, the mechanism of action of gold salts remains the subject of extensive investigation and review.[45,46] The pharmacology of gold is thoroughly discussed in a recent chapter by Lewis and Walz.[44] More than 50 years of clinical use of gold in the treatment of RA has established that chrysotherapy is effective and can cause a modification or remission in the course of the disease.

D-Penicillamine and Other Thiol-Containing Agents - The use of penicillamine in the treatment of RA was pioneered two decades ago by Jaffee.[47] In a number of controlled studies, D-penicillamine was shown to be as effective as gold therapy as a remission-inducing drug.[48-50] Chrysotherapy usually precedes penicillamine in order of therapeutic preference because the high dropout rate seen with penicillamine detracts from its ultimate clinical value.[51,52] Skin rashes are the most common side effect, and complicate 44% of cases.[53] Bone marrow depression is a major side effect,[54] although thrombocytopenia is more frequent than leukopenia and is seen in approximately 15% of patients.

There has been considerable speculation that the efficacy of penicillamine in RA is due to its copper chelating and copper mobilizing properties.[9] Other studies have suggested an effect on collagen biosynthesis,[55] lymphocytes,[56] mononuclear phagocytic function,[57] complement,[58] antibody responses,[59] and cell-mediated immunity.[60] As is the case for the other DMARDs, the mechanism of action of D-penicillamine has not been defined.

Although the clinical value of penicillamine is limited by a high incidence of both toxicity and relapse during treatment, those patients able to tolerate the drug exhibit impressive improvement.[10] This fact has led investigators to examine additional thiol containing amino acids as potential DMARDs. Among the compounds which have been examined clinically are cysteine (1),[61] mercaptopropionyl glycine (2)[62] and captopril (3).[63] These compounds all elicit a spectrum of side effects not significantly different in frequency or severity from penicillamine. Additional clinical trials are ongoing with 2 being compared to penicillamine.[62]



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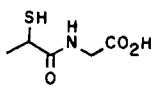
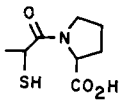
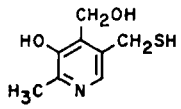


2



3

Two sulfur containing compounds which, unlike penicillamine, do not chelate copper but have shown a positive anti-arthritic effect, are 5-thiopyridoxin (4)[64] and pyrithioxine (5).[61] 5-Thiopyridoxin is a vitamin B6 analogue which does not antagonize B6. Pyrithioxine is marketed in Europe as a cerebral stimulant. Controlled clinical trials with 4 and open trials with dimer 5 show that these compounds possess the same efficacy as penicillamine. Interestingly, although side effects of the drugs are similar, patients who withdrew from penicillamine therapy because of toxicity seem able to tolerate 4 and 5.[64] In addition, some patients responded to pyrithioxine therapy after an ineffective course of penicillamine. More extensive laboratory and clinical studies will be required before 4 or 5 can be considered truly effective DMARDs.



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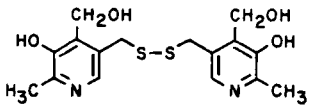

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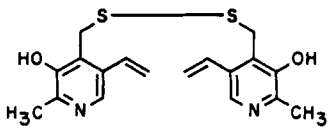
JPC-80 (6) also shows clinical efficacy as measured by clinical score, decreased erythrocyte sedimentation rate (ESR) and rheumatoid factor titers.[65] Additional studies with appropriate controls are awaited.



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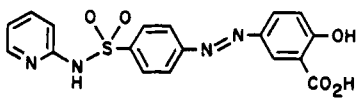

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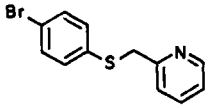
Salicylazosulfapyridine (Salazopyrin, 7), first described[66] in 1946 and used occasionally in ulcerative colitis, has been examined at various times for anti-rheumatic activity.[67,68] The most recent report of Salazopyrin therapy is an open trial conducted by McConkey in which 74 patients were given 0.5 g daily, increasing to 2 g per day for up to one year.[69] This regimen resulted in a significant improvement in clinical scores, and decreases in serum C-reactive protein concentrations and sedimentation rates. Side effects were limited and not severe. The anti-rheumatic mode of action remains unknown but is probably not attributable to the drug's salicylate content or its antibacterial activity. Randomized blind clinical trials are now underway in England.[70]

The 2-[(phenylthio)methyl]pyridine (8) and similar compounds are reported to inhibit the rat dermal as well as pleural reverse passive Arthus reaction.[71] They also inhibit both exudate volume and cell accumulation in the rat pleural carrageenin assay. This profile is different from that observed for both NSAIDs and steroids. Clinical studies have not been reported.



8

Arg-Lys-Asp-Val-Tyr

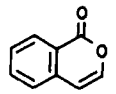
9

Immunomodulators - Levamisole, an antihelminthic agent with immunostimulatory activity, has been used experimentally for the treatment of RA since 1974.[72-74] The drug has little effect on inflammation,[75,76] but is able to restore defective cell-mediated immunity.[77] The degree of impairment of cell mediated immunity in RA correlates with the severity and activity of the disease.[78] The efficacy/side effect ratio was found to be better with 150 mg levamisole once weekly, than with 150 mg on three or seven consecutive days each week.[79]

The mode of action of levamisole is still unclear, although several hypotheses have been proposed,[80] and numerous effects on monocytes have been demonstrated.[81-84] The occurrence of sudden unpredictable agranulocytosis limits the uncontrolled practical use of levamisole as an anti-rheumatic, despite the fact that the drug appears to prevent progression of the disease.

A synthetic form of thymopoietin is now available as pentapeptide 9. This sequence contains the immunomodulatory activity of the 49 amino acid peptide.[85] In a controlled study of patients receiving 50 mg per week intravenously, 9 was shown to be more effective than levamisole and as effective as penicillamine in improving the clinical status of patients with RA.[86] There were small reductions in ESR and immunoglobulin levels; rheumatoid factor titers did not change. Although thymopoietin's mechanism of action is very likely related to its immunomodulatory properties,[87] it is probably not the same as that of levamisole.[86]

Potential DMARDs - The compounds included in this section reportedly demonstrated DMARD activity clinically, or elicited pharmacodynamic responses in chronic and systemic laboratory models of RA which distinguish them from NSAIDs. In the dextran-induced pleurisy assay in rats, 10 reduced total leucocytes by 40-45%, while the differential count showed a 4-fold increase in mononuclear cells.[88] In the carrageenin pleurisy model, 10 reduced the exudate volume by 60% and the total number of leucocytes by 75% at 4 hours. Venalot, a coumarin-rutin (11) preparation, significantly reduced paw volume and cornea-edemas in a rat erysipelas model.[89] Although the benzopyrones are predicted to be of value in the treatment of RA,[88] clinical evaluation of these agents has not yet been reported.



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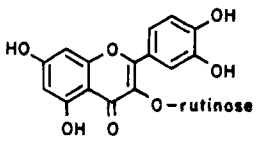
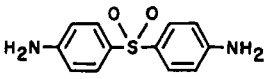


11

The leprostatic antibacterial agent dapsone (12) improved the clinical symptoms of rheumatoid patients in an open trial conducted by McConkey.[90] This result was accompanied by a gradual decrease in ESR and serum C-reactive protein levels, but titers of rheumatoid factor remained unchanged. Animal studies[91] showed that dapsone has anti-inflammatory activity and in vitro investigations demonstrated an inhibitory effect on complement activation and on selected lysosomal enzymes as well.[92] Although a subsequent controlled clinical trial[93] confirmed the anti-rheumatic activity of 12, its well documented toxicity involving blood dyscrasias prohibits serious consideration as an anti-rheumatic drug.



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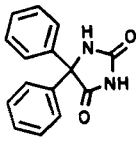

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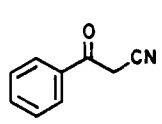
Phentoin (diphenylhydantoin, 13) was prescribed as an anti-epileptic for several rheumatoid arthritics who developed seizure disorders. Within six months, there was a modest reduction in ESR, no change in rheumatoid factor titers and an improvement in clinical score.[94] This drug has been shown to inhibit collagenase activity and immuno-reactive protein concentrations in vitro.[95] These results may define important aspects of the mechanism of anti-arthritic activity.[96] Nevertheless, the clinical improvement associated with phentoin remains to be assessed in appropriately designed trials.

Benzoylacetonitrile (14) represents a group of β-ketonitriles which inhibit the development of adjuvant arthritis in rats.[97] These compounds are inactive in the prostaglandin-mediated assays and display little or no ulcerogenic potential in rats at 800 mg/kg. Initial animal studies suggest that reticuloendothelial stimulation may be a mechanism of action.[97]



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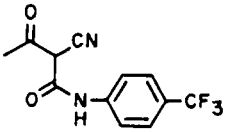

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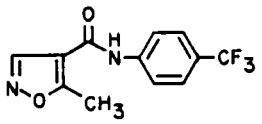

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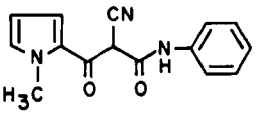

17

Two additional members of this structural class have been reported. Compound 15 is the active metabolite of Hoechst's new isoxazole 16 (HWA 486).[98,99] Pyrrolpropionitrile 17 has been patented[100] by Walker as an agent exhibiting both indomethacin and levamisole type activities.

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Chapter 19. Pharmacological Developments in Dermatology

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Introduction - A recent Health and Nutrition Examination Survey (HANES-1) estimated that 60.6 million Americans had one or more skin conditions that required medical treatment, suggesting an impressively high prevalence of dermatological disease.[1] Among the most frequent dermatological diagnoses of the ambulatory population are those diseases of the sebaceous glands, such as acne, dermatophyte infections, malignant and benign tumors, seborrheic dermatitis, atopic dermatitis and psoriasis.[1] Dermatology has had to be content to use drugs initially developed for other diseases, although the new discipline of dermatopharmacology has emerged to optimize delivery of existing drugs and, more importantly, to develop animal models of skin diseases and use them to identify new dermatologic agents. The field of immunodermatology has also been reviewed[2] stressing the importance of immunologic derangement in many skin disorders.

The scope of this review will include specific developments made concerning psoriasis, atopic dermatitis and acne, and include more general sections on inflammatory mediators likely to play a role in skin inflammation, topical antiinflammatories and vehicles of current use in topical therapy.

Psoriasis - Psoriasis is a common, multifactorial, genetically determined disease of unknown etiology that is characterized by a benign, unrestricted epithelial growth of skin.[3] It produces recurrent lesions that are often emotionally and physically debilitating to the patient.[4]

Epidemiologic population studies reveal either an autosomal dominant or polygenic inheritance.[5] This is further supported by histocompatibility complex (HLA) studies. It is often found that the HLA antigens B13, B17, B27, B37, Cw6, D11, and DR7 are increased in frequency relative to controls.[6]

The pathophysiology of psoriasis has been extensively reviewed.[3,4,7,10] Research has focused on intracellular mediators, e.g., cyclic nucleotides (cAMP and cGMP), prostaglandins (PGs), polyamines, autoantibodies to stratum corenum and protease-antiprotease complexes. An imbalance of epidermal cAMP and cGMP, which act as modulators of proliferation and differentiation and levels of which are maintained through prostaglandin regulation,[11-13] has been implicated in psoriasis. cGMP levels are elevated,[11] although conflicting values for cAMP levels in involved and uninvolved psoriatic epidermis have been reported.[14-18] A variety of studies demonstrated that epidermal cyclic nucleotide levels are influenced by prostaglandins. Epidermal cAMP levels are elevated by PGs in guinea pig epidermis and keratinocytes as well as human psoriatic epidermis.[19,20]

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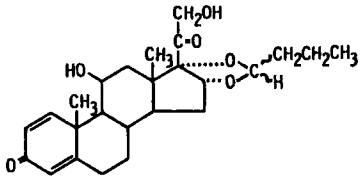
In guinea pig ears treated with topical vitamin A acid, a scaly, erythematous, proliferative, psoriaform dermatitis develops giving rise to increased levels of PGE and cAMP, suggesting that PGE synthesis and release stimulates cAMP levels.[21] Arachidonic acid (AA), 12-HETE and PGs in the F series are associated with elevated cGMP levels.[22,23]

The concentrations of AA and its metabolites from the lipoxygenase pathways are increased in involved, relative to those in uninvolved epidermis. Free AA and 12-HETE levels are markedly increased, whereas modest elevations are observed with PGE_2.[24] Recently, LTB_4 has been reported present in extracts of chamber fluid from psoriatic plaques.[25] This finding coupled to the report that intradermal injection of LTB_4 produces an inflammatory reaction in human skin[25] implicates LTB_4 as an inflammatory mediain psoriasis.[26,27]

Polyamines appear to be essential for cell differentiation.[28] Elevated levels of polyamines have been reported in involved and uninvolved skin of psoriatics.[29] The critical enzyme in polyamine biosynthesis is ornithine decarboxylase (ODC) which is also elevated in involved skin. Glucocorticoids reduce ODC activity to normal.[29]

A mainstay in the treatment of psoriasis is the application of occlusive emollients such as petroleum jelly or mineral oil.[30] Keratolytic agents such as salicylic acid aid in the removal of scale from plaques; furthermore, sulfur in combination with salicylic acid potentiates the keratolytic action.[30-33]



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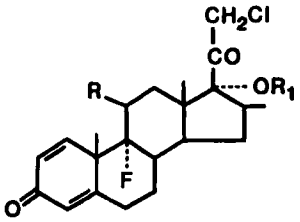
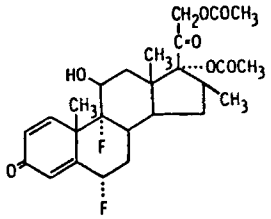


2, R= =O, R_1=COC_3H_7

3, R= -OH, R_1=COC_2H_5

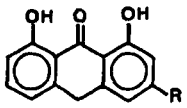


4

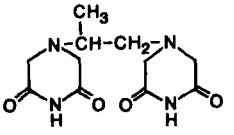
Glucocorticoids are still the most commonly used therapy for psoriasis.[5] These drugs have antiinflammatory, vasoconstrictive and antimitotic effects when applied to psoriatic plaques. Budesonide (1) was equiactive to fluocinolone acetonide in psoriasis.[34] Clobetasone butyrate (2) was more effective than hydrocortisone in psoriasis and reduced plasma cortisol levels less.[35] Clobetasol 17-propionate (3) was also an effective antipsoriatic by two application schedules.[36] Diflorasone acetate (4) normalized free AA levels and reduced 12-HETE content in psoriatic plaques.[37]

The anthralin products continue to be major topical noncorticosteroid antipsoriatic remedies. The synthetic anthrone (5) has replaced its naturally occurring relative, chrysarobin (6), as a treatment for psoriasis and structure specificity studies of the anthrones have been reviewed.[38] Mid-range ultraviolet light (UVB) and long wave ultraviolet light (UVA) combined with the psoralens (PUVA) have dramatic effects in the clearing of severe psoriasis and

the subject has been reviewed.[5] The use of systemic chemotherapeutic agents in psoriasis has been extensively reviewed.[39]



5, R = H
6, R = CH_3



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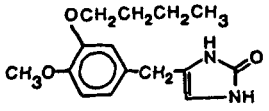

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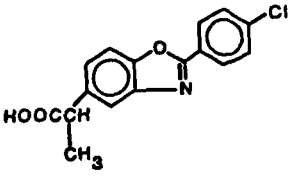


9

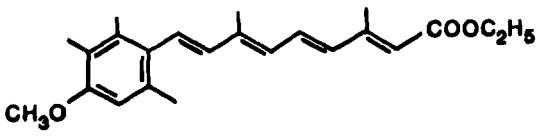
Razoxane (ICRF 159; 7), an antimitotic drug, was reported to be highly effective in cutaneous and arthropathic psoriasis.[40] No hepatotoxicity was reported but depressed neutrophil counts were observed.

The PDE inhibitor, Ro-20-1724 (8), significantly improved psoriasis when topically applied (1%) to the plaques.[41] This treatment was somewhat less effective than triamcinolone but it produced less systemic and topical toxicity.

The H_2 antagonist, cimetidine, was ineffective in the treatment of psoriasis and it was suggested that it may even worsen the condition.[42] Human growth hormone (HGH) levels are reported to be elevated in 70% of psoriatic patients.[43] Somatostatin, an inhibitor of HGH, was reported to induce remission or a reduction of disease activity in 75%-80% of patients.[44] The synthetic inhibitor of HGH, bromocriptin, was shown to be as effective as somatostatin in psoriasis.[45]

It has been suggested that there is a basic deficiency in the cyclooxygenase enzyme in psoriasis, and it has been reported that the cyclooxygenase inhibitor, indomethacin, may exacerbate the disease.[13] Hypothetically, this could lead to an overproduction of leukotrienes resulting in an accumulation of inflammatory leukocytes and a secondary increase in epidermal cell proliferation. Lipoxygenase inhibitors, therefore, may have beneficial effects in psoriasis. Benoxaprofen (9), reported to be a lipoxygenase inhibitor, markedly improved or cleared the disease in 8 of 13 psoriatic patients.[46]

One of the most promising approaches to the treatment of psoriasis is the use of systemic retinoids. Etretinate, (Ro 10-9359; 10), is extensively used in Europe and may become the treatment of choice in pustular psoriasis.[47-50]



10

Atopic Dermatitis (AD) - AD is a chronic inflammatory skin disease that occurs most frequently in persons with a personal or family history of

allergies (e.g. asthma or allergic rhinitis).[1,51,52] AD is characterized by abnormalities related to type I and type II immunity. IgE antibody is elevated in 80% of AD patients, and often the degree of elevation corresponds with the severity of the disease. The pathogenic significance of this finding is questionable. For example, normal serum IgE levels occur in AD, elevated levels occur in other diseases and serum IgE levels may remain elevated following clinical remission.[51] Nevertheless, local cutaneous reactions of IgE with antigen may contribute to the inflammation and pruritus. Circulating immune complexes containing IgE have also been described in patients with AD.[53]

There is evidence for a defective cellular immune system in AD.[51,54,55] There is an increased susceptibility to severe cutaneous infections with viruses, decreased skin sensitivity to several antigens,[52] increased spontaneous lymphocyte proliferation[56] and a circulating T-lymphocyte deficit.[55] A reduction of circulating suppressor/cytotoxic (OKT 8+) T cells but not helper/inducer (OKT 4+) cells has been reported and suggests active AD is associated with a selective reduction in the T8+ subset.[54] A predominance of T-lymphocytes has been shown in the skin infiltrates of AD[57] and immune mediated skin damage by autocytotoxic lymphocytes occurs resulting in skin fibroblast killing.[54]

Depressed monocyte and neutrophil chemotaxis and serum inhibition of monocyte chemotaxis has been found in patients with AD.[51, 58] The nature of the inhibitor of chemotaxis is not known.

The increase in sensitivity of suppressor T cells to released histamine in AD may be the explanation of the cellular immune deficiency and elevation in serum IgE.[59]

There appears to be an endogenous ß-blockade in keratinocytes,[60] lymphocytes and polymorphonuclear leukocytes from patients with AD[61] that is not attributed to abnormal or reduced numbers of ß-adrenergic receptors.[62] An imbalance in the cyclic nucleotide regulatory system may indeed explain many of the physiologic, pharmacologic and immunologic defects in AD.[51,52]

AD cannot be prevented or cured although partial control is achieved by topical steroids. No satisfactory systemic therapy is yet available. Topically applied[63] and oral[64] disodium cromoglycate (DSCG) were effective in AD; however, other reports fail to confirm this[65] and orally effective cromones related to DSCG were also without effect.[66-68] Neither H_1 nor H_2 histamine antagonists alone or in combination were effective in AD.[69] Immunologic intervention using levamisole and transfer factor was also unsuccessful.[70] The therapeutic value of reducing PDE using inhibitors such as Ro-20-1724 (8) is attractive in view of its ability to normalize IgE and histamine releasability.[71] Recently, aggressive PUVA therapy was locally effective in AD.[56,72] The mechanism by which PUVA therapy induces an improvement is not known but is unlikely to involve the immunological changes that it reduces.[56]

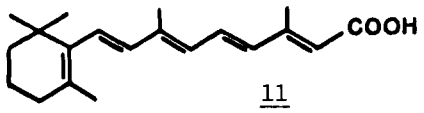
Acne - Acne vulgaris is the most common disease of the skin. In a recent survey, 68.1% or 15.5 million people between the ages of 12 and 17 exhibited facial acne.[73] Acne is characterized by a variety of lesions which may be noninflammatory (open and closed comedones) or inflammatory (papules, pustules, nodules).[74-76]

The basic cause of acne is unknown, however, considerable information on its etiology has accumulated in recent years.[74-77] It is a multifactorial disease involving sebum production, a keratinization abnormality and bacteria in the sebaceous follicle as essential contributing pathogenic factors. At puberty, circulating and tissue androgen levels are increased.[78] These androgens promote the size of sebaceous glands and their production of sebum[78] which is comedogenic.[79] Sebum production and severity of acne are closely related.[80-82] Hyperkeratinization in the sebaceous follicle initiates microcomedone formation and development of open or closed comedones.[75] The rupture of microcomedones and closed comedones leads to formation of papules, pustules and nodules resulting in an accumulation of inflammatory cells at the follicular wall.[83] A number of findings suggest a role for the anaerobic diphtheroid, Propionibacterium acnes (P. acnes), in the pathogenesis of inflammatory acne lesions.[83-93]

The retinoids include natural forms and synthetic analogs of vitamin A. Beneficial effects with oral vitamin A were first reported in 1943.[94] Subsequently, controversial studies followed on the use of oral vitamin A in the management of acne.[95-98] A recent report describes clinical improvement in inflammatory acne vulgaris with large oral doses of vitamin A (retinol) ranging from 300,000 to 500,000 International Units.[99]

Topical tretinoin (vitamin A acid, all-E-retinoic acid; 11) is probably the most effective topical agent in the treatment and prophylaxis of comedonal and inflammatory acne.[100,101] It reverses the primary event (abnormal follicular keratinization) in the pathogenesis of acne and prevents the formation of new comedones by increasing epithelial cell turnover and by reducing the cohesiveness of horny cells.[102-104] Consequently, inflammatory lesions which result from comedones are reduced.[104]

Ro 11-1430 (motretinide; 12), was compared to tretinoin in clinical acne studies. Both drugs were effective, however, topically applied tretinoin at 0.05% was superior to 0.1% Ro 11-1430 in reducing the number of comedones.[105,107]



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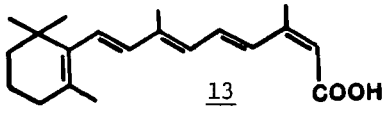

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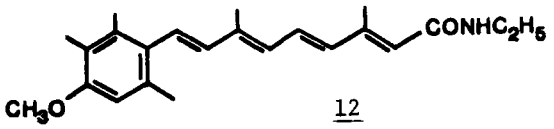


12

The most exciting new drug in the treatment of severe cystic acne is oral isotretinoin (13-Z-retinoic acid; 13). At 2 mg/kg/day or less, dramatic therapeutic effects with prolonged remissions of severe cystic acne were reported with oral isotretinoin.[108-113] The mechanism of action of isotretinoin is unknown, although its therapeutic effects in severe cystic acne are associated with a marked inhibition of sebum production.[114,115] Isotretinoin admin-

istered subcutaneously[116] or orally[113] to male hamsters produced a marked diminution in the size of flank organ sebaceous glands without any discernible effects on other androgen sensitive structures of the flank organ. Ro 10-9359 (10), orally effective in psoriasis, was less effective than oral isotretinoin in the treatment of severe cystic acne and in its ability to decrease sebum production.[117]

Since 1934, benzoyl peroxide (BPO), has been used as a topical agent for the treatment of acne.[104] The effectiveness of BPO is related to its antibacterial action since it has been shown to reduce the follicular population of P. acnes.[118,119] Various BPO preparations are available in lotion and newer more effective gel vehicles which improve its antibacterial effect in suppressing P. acnes and lowering free fatty acids.[120]

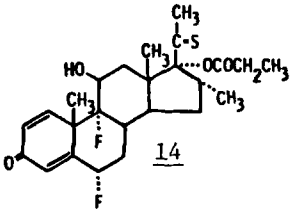
The efficacy and safety of systemic antibiotics for the treatment of acne vulgaris has been reported.[121-124] Recent reports support the use of topical antibiotics as effective agents in the treatment of mild to moderate inflammatory acne.[125-139] Antibiotics used in the treatment of acne have been shown to reduce P. acnes populations,[119,125] possess antiinflammatory properties,[140] and inhibit leukocyte chemotaxis.[141]

Oral zinc sulfate is effective in inflammatory acne.[142-145] The exact mode of action of zinc in acne is unknown; however, an interaction between zinc and retinol binding protein was suggested.[146] An oral antiandrogen, cyproterone acetate, reduced sebum secretion in man, and was shown to be effective in the control of recalcitrant acne in males.[147] In combination with ethinyl estradiol, cyproterone acetate had a pronounced effect in the treatment of persistent acne in adult females.[148] Benoxaprofen (9), in a limited trial, was reported to be beneficial in the treatment of nodular acne.[149] A recent study showed that salicylic acid at 0.5% in an alcohol detergent vehicle improved mild to moderate acne vulgaris.[150]

Topical Antiinflammatories - Topical antiinflammatory steroids are of palliative use only but are plagued by adverse side effects.[151-153] These include suppression of the hypothalamus-pituitary-adrenal axis due to percutaneous absorption, as well as dermal toxicity. Thus, there has been continued interest in the development of safer, potent non-fluorinated topical steroids with an enhanced ratio between their topical and systemic potencies.

Budesonide (1) was the most potent of a series of 1,4-pregnadienes possessing 16, 17 acetal substitutions.[154-156]

High topical and low systemic activity was reported for RS-35909 (14) [157] and for several 7-chloro and 7-bromo corticosteroid 17,21 diesters, especially the 17-benzoates and 21-acetates,[158-161] Sch 22219 (15) and Sch 23409 (16). In mice, Sch 22219 showed a greater dissociation of antiinflammatory activity from side effects than Sch 23409, although the reverse was true in rats.[162] Org 6216 (Rimexolone^{R};17)[163] and tixocortol pivalate (Pivalone^{R};18)[164] also possess potent local antiinflammatory activity with low systemic activity. Furthermore, (17) does not appear to produce skin atrophy in rats.[163]



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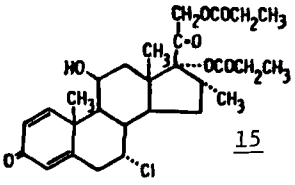

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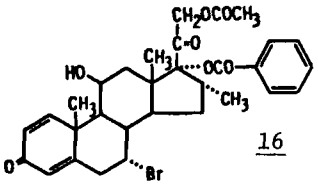

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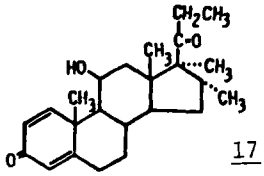

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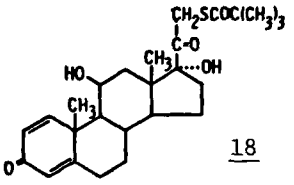
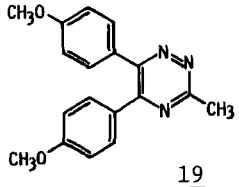


18

The antiinflammatory effects of glucocorticoids were reviewed.[165] Recently, they were proposed to be associated with the induction of phospholipase inhibitory proteins.[166,167] These proteins have been isolated from glucocorticoid-treated rabbit neutrophils (lipomodulin with Mr=40,000) and rat macrophages (macrocortin with Mr=15,000). Macrocortin may be a phosphorylated fragment of lipomodulin.[168]

Evaluation of topically applied nonsteroidal antiinflammatory drugs (NSAID) has attracted attention for several years.[169-177] Bufexamac (p-butoxy-phenylacethydroxamic acid) remains the only topical NSAID which is used clinically,[178] although its topical efficacy has recently been questioned.[179] Antirazafen (19) has recently been shown to possess potent topical antiinflammatory activity.[180] N-0164 (Na p-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl-propyl] phenyl phosphonate), a selective antagonist of PGs with an ability to inhibit thromboxane production, reduced experimental erythema when administered topically.[174,181]



19

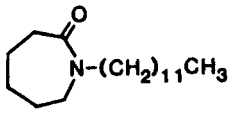
Inflammatory Mediators - The principal mediator(s) of subacute and chronic inflammatory dermatoses, their sequence of action and possible synergism are not clearly established. Both H_1 and H_2 histamine receptors are present in human skin and may contribute to the vasodilator and pruritic response induced by histamine.[182] The combination of H_1 (chlorpheniramine) and H_2 (cimetidine) receptor antagonists causes significantly greater suppression of histamine erythema than either drug given alone[183,184] although this synergism is controversial.[185] Histamine pruritus is largely mediated via H_1 activation.[186]

Recently, roles for several lipid mediators and peptides in dermatoses have been identified. PGs E_1, E_2 and PGI_2 enhance protein leakage from skin vessels induced by substances that increase permeability such as histamine, LTB_4 and bradykinin.[187,188]

Platelet activating factor (PAF-acether) is one of the most potent inflammatory mediators so far described for the induction of increased vascular permeability.[189,190] Intradermal PAF-acether produces an early wheal and flare response followed by a late response as well as a transient burning pain in man.[191-193] The increased vascular permeability in the skin of rabbits and guinea pigs may in part be due to release of cyclooxygenase metabolites[194] or lipoxygenase products.[195]

Several gut hormones that are present in peripheral nerves may have neurotransmitter roles in the pruritic response.[196,197] Vasoactive intestinal polypeptide (VIP) elicits pruritus, whealing and erythema in human skin. It is a very potent vasodilator[198] and histamine releaser[197] and may be involved in physiological control of normal blood flow in hyperemia in inflammatory conditions. In contrast, substance P is a weak vasodilator in the skin[199] but produces a wheal and flare reaction in man possibly by releasing histamine from skin mast cells.[199-201] Substance P is present in the skin in high concentrations[202] and may play a physiological role in causing pruritus. Met-enkephalin may be a sensory neurotransmitter in the skin.[203] Naloxone attenuates histamine-induced pruritus in man and this suggests a role for endorphins in itch.[204] Naloxone and related opiate antagonists may be of therapeutic value in a variety of pruritic conditions.[205]

Vehicles - It is well-known that agents in topical vehicles may influence the penetration of drugs through the stratum corneum into the skin.[206-209] Dimethyl sulfoxide,[210] alkyl methyl sulfoxides,[211] dimethylacetamide,[212] dimethylformamide[212] and N-methyl-2-pyrrolidone[213,214] are examples of such vehicles.



20

A new compound, N-0252 (1-dodecylazacycloheptan-2-one, AZONE^{R};20), was reported to increase the permeability of skin and enhance the penetration of antibiotics, glucocorticoids and 5-fluorouracil.[215] AZONE^{R} is colorless, relatively odorless and minimally irritating when applied to human skin.[215]

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Chapter 20. Mechanism of Action of Insulin, Glucagon and Somatostatin

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Introduction - Insulin, glucagon and somatostatin (SRIF) are polypeptide hormones synthesized and secreted by Islets of Langerhans of the pancreas and effect several major target tissues (liver, muscle, fat, GI tract). The metabolic effects of insulin and glucagon involve binding to specific membrane receptor sites with subsequent modulation of plasma membrane functions and numerous intracellular catabolic and anabolic processes. SRIF mainly alters metabolism indirectly by affecting the secretion of insulin and glucagon. Previous volumes in this series (9,14,16) have briefly addressed these hormones' action in discussions of diabetes mellitus, however, a detailed review of their molecular mechanisms has not been presented. Our intent is to fill this void by emphasizing certain aspects of these hormones' actions which have undergone rapid and important new advances. In the case of insulin, these advances have been mainly in the areas of insulin's second messenger and effects on membrane structure and function. For glucagon, effects on enzyme systems will be emphasized. We, therefore, refer the reader to selected reviews on the mechanism of insulin,[1-12] glucagon[13-16] and SRIF[17-19] for detailed background information.

Insulin Binding to Receptors - Insulin interacts with specific high affinity (Kd-1nM)[1] cell surface membrane receptors. The number of these receptors is small[20] and can be decreased by insulin exposure *in vitro*[21] or hyperinsulinemia *in vivo*.[22,23] Only minimal receptor occupancy by insulin has been shown to incur maximal physiological response,[24,25] although exceptions have been reported.[26] Insulin binding to receptors generally yields non-competitive dissociation kinetics and curvilinear Scatchard plots, which have been attributed to negative cooperativity or multiple classes of independent receptors.[27,28] Recent reports,[29-33] however, suggest no cooperative interactions for liver receptors and conversion of the receptor complex to a high affinity state subsequent to binding. These differences may relate to the varied incubation conditions and cell types employed.[34] The roles of cell membrane phospholipids for insulin binding to rat liver plasma membranes[35] and the role of lipid composition on binding[36] to turkey erythrocyte membranes have also been examined.

Insulin Receptor Structure - Insulin receptor identification and subunit structure has recently been deduced using ^{125}I affinity labelling and purification techniques combined with selective disulfide bond reduction.[37] Whether studied by photo-activatable insulin analogs,[8,38-42] crosslinking with disuccinimidyl suberate,[43-47] immunoprecipitation with anti-insulin receptor antibodies,[48] or radiation inactivation,[49] it is generally agreed that the insulin receptor complex has an apparent molecular weight of 300-350K and is composed of subunits of approximately 125K (α subunit) and 90K (β subunit) molecular weight.[38-42,44,45] The intact insulin receptor has a subunit structure β-α-α-β[4,50] with the major insulin binding site being the 125K α subunit.[42] The role of the 90K subunit in insulin binding is currently unresolved. Heterogenous

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receptor populations, ranging in molecular weight from 260K to 550K, as well as smaller fragments of 45-50K molecular weight have also been reported. These appear due to in vitro processing of the 330-350K form by released intracellular proteases.[4,42,45] Similar heterogeneous receptor populations and the cleavage of the β subunit have been observed following treatment of intact adipocytes with elastase or lysosomal proteases.[51] It is not clear if these receptor forms are native to the intact cell or are artifacts of in vitro membrane preparations.

Studies using mixed glycosidases, neuramidase, tunicamycin and tritiated monosaccharides have demonstrated the complex glycoprotein nature of the receptor complex, as well as the necessity of glycosylation for synthesis of functional receptors.[48,52-54]

Binding of insulin to the cell surface membrane receptor induces both an alteration in receptor subunit structure[49] and a decrease in the half-life of receptor subunits.[55]

Insulin Action on Membrane Transport - Insulin-sensitive transport systems include those for cations, anions, amino acids and glucose with the latter being understood in the greatest detail. Insulin binding rapidly activates glucose transport,[56,57] increasing the V_{max} of transport without having marked effects on the K_m.[58] Similar results have recently been observed in humans using the glucose clamp technique.[59] The major mechanism of insulin-activated glucose transport in adipose and/or muscle tissue appears to involve multi-step translocation of glucose transport units from an intracellular storage location to the plasma membrane and recycling of the transporters away from the plasma membrane during transport deactivation. Incubation of intact adipocytes or reconstituted fat cell liposomes with insulin increases the number of glucose transporters and stoichiometrically reduces the activity of either Golgi-rich or microsomal fractions.[60-62] Confirmatory results have been reported for diaphragm.[63] While an energy source is required for these effects,[61] they do not appear to depend on protein synthesis. Interestingly, agents known to have insulin-like effects on glucose transport in intact adipocytes also increased transport activity by plasma membrane but decreased the activity in Golgi-rich fractions.[62] Furthermore, neither the presence of the insulin receptor nor its internalization is absolutely required for migration of transport activity.[61] However, depletion of the intracellular glucose transporter pool has been suggested to account for diet-induced reduction of insulin stimulated glucose transport.[64]

Internalization and Intracellular Processing of Insulin and the Insulin Receptor - Following internalization of insulin-receptor complexes via adsorptive pinocytosis,[65,66] ligand-receptor complexes translocate to the Golgi,[67,68] lysosomes,[69,70] and intermediate density vesicles[71] and are intracellularly processed via an energy dependent, apparently saturable process.[72] Insulin is separated from the receptor complex,[8,73] initially degraded to intermediate products of molecular weight close to insulin but with greatly decreased biologic/immunologic activity[75-77] and subsequently degraded further.[8,77,78] A variable fraction of internalized receptors is processed via lysosomal proteolysis[8,45,42,79,80] with remaining intact receptors recycled to the plasma membrane.[8,79] The rate and magnitude of ligand-receptor endocytosis is dependent on cell type with adipocytes and hepatocytes internalizing a greater percentage of complexes than lymphocytes.[65,66,72,81]

Although the biochemical events involved in processing and recycling

are currently unknown, some understanding of the relationship between receptor down-regulation (a mode of decreased cell responsiveness to increased insulin levels) and intracellular events has been elucidated. Down-regulation in cultured hepatocytes appears to result from decreased cell-surface receptor number without concomitant alterations in receptor affinity or rates of receptor synthesis and degradation.[73,82] Total cellular insulin receptor number remained constant, suggesting that insulin exposure increased the percentage of receptors sequestered in a state inaccessible to insulin or translocated to an intracellular compartment.[82] Comparison of the rates of receptor turnover to insulin degradation showed that receptor-mediated insulin degradation exceeds the rate of receptor degradation by over 200-fold and that most of the internalized receptor complexes recycle to the cell surface following insulin loss.[73] Interestingly, insulin-induced receptor down-regulation in differentiated 3T3-L1 adipocytes results from alterations in receptor degradation with decreases in both total and cell-surface receptor numbers.[83]

Receptor degradation rate was inhibited by cycloheximide and puromycin, implying that protein synthesis is required for normal receptor turnover.[84] Mechanisms for receptor down-regulation similar to those obtained with 3T3-L1 cells were reported for the α and β-receptor subunits using IM-9 lymphocytes.[85,86]

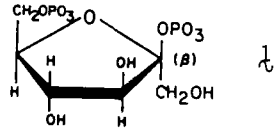
Effects of Insulin on Intracellular Enzymes - Our understanding of the molecular events for insulin's effects on a number of intracellular enzymes remains incomplete. Several of these enzymes, such as glycogen synthetase,[87,88] pyruvate dehydrogenase complex (PDH),[89] and pyruvate kinase,[90] appear to be regulated by phosphorylation-dephosphorylation reactions. Similar processes have been postulated for Ca^{+2}, Mg^{+2}-ATPase,[91] cAMP phosphodiesterase[92] and adenylate cyclase.[93] However, there is yet little agreement as to the precise mechanisms involved. Most interesting is the recent work of Seals et al., in which they have essentially reconstituted the insulin effect on PDH in adipose tissue.[94,95] These investigators added insulin-treated plasma membranes to mitochondria and observed that phosphorylation of what appeared to be the α-subunit of PDH was retarded. They postulated that a second messenger other than cAMP is released from the plasma membrane upon insulin addition and that this factor mediates the changes in PDH. Insulin-exposed particulate fractions from skeletal muscle,[87,96,97] and liver,[98,99] and exposure of heart mitochondria,[100] hepatoma cells,[101] and IM-9 lymphocytes[99] to insulin were reported to release one or more similar low-molecular weight substances, which affected certain insulin-sensitive enzymes. Fat-feeding of rats decreases generation of the low-molecular weight substances.[102] In nearly all cases, insulin's effect on generation of this substance appears to be biphasic with respect to the concentration of hormone,[98,103,104] and this has been attributed to the release of two antagonistic substances.[98,104-106] The first reports[105,107] of the separation of these two antagonistic activities have appeared and although the chemical nature of these substances remains elusive, it has been suggested that they are peptides[99,104] or oligoglycopeptides.[97] It should be noted here that added Ca^{+2} produces effects[94,95,108] similar to insulin on PDH and Ca^{+2} has frequently been suggested to be an important secondary messenger for insulin.[108-110] However, experimental results have failed to establish Ca^{+2} as a primary mediator for either insulin or glucagon,[111] though secondary changes in the intracellular Ca^{+2} concentration may ultimately prove significant. Study of the effects of insulin on inositol lipids may prove a fruitful area of research since

hydrolysis of these lipids appears to be intimately involved in a number of Ca^{+2}-mediated effects of hormones.[112,113]

Insulin might be mediating a number of the above effects on enzymes through its well-established ability to inhibit fatty acid mobilization. The resulting decreased fatty acid oxidation would, for instance, decrease the mitochondrial acetyl CoA/CoA and possibly NADH/NAD and thus result in less PDH in the active form.[114-118]

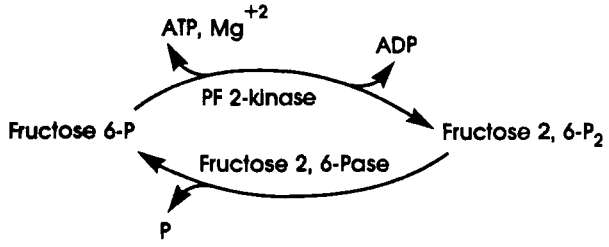
Glucagon: Regulation of Carbohydrate Metabolism - It is thought that glucagon regulates the direction and magnitude of cellular carbon flux by influencing the activity of three allosteric enzymes [L-isozyme of pyruvate kinase,[119-122] 6-phosphofructo 1-kinase (PF 1-kinase)[119,122,123] and fructose 1,6-bisphosphatase[119,122]] that are important sites for the regulation of gluconeogenesis and glycolysis. These enzymes are believed to be regulated by 1) direct effects of allosteric effectors, 2) phosphorylation of the enzyme, with phosphorylation modifying the influence of the allosteric effectors on the activity of the enzyme, and 3) in the case of pyruvate kinase, by the ability of the allosteric ligands to influence a conformational change, thereby making the enzyme more or less susceptible to phosphorylation by protein kinase.[119,124]

Even though extensive data exist that glucagon stimulates the cyclic AMP dependent protein kinase to catalyze the phosphorylation of all three enzymes,[122,125-127] the role of covalent modification in regulating their activity remains equivocal. More recent studies have focused on the role of fructose 2,6-bisphosphate (fructose 2,6-P_2, 1), a novel sugar phosphate, as an important physiological mediator of glucagon's effects on hepatic carbohydrate metabolism. In the absence of hormone,



fructose 2,6-P_2 and AMP are believed to act synergistically to activate PF 1-kinase and to competitively inhibit fructose 1,6-bisphosphatase, thus limiting the rate of gluconeogenesis by allowing substrate cycling to occur.[122,125,128,129] For fructose 1,6-bisphosphatase, fructose 2,6-P_2 binds to the catalytic site as well as the allosteric site for AMP with two separate groups of tyrosine residues being associated with each of these sites.[130,131] Fructose 2,6-P_2 is a potent allosteric activator of PF 1-kinase with a Ka of 0.05 μM and it is 100-2500 times more effective than fructose 1,6-P_2 in activating the enzyme.[122,128,129,132] In the presence of glucagon, fructose 2,6-P_2 levels are dramatically lowered relieving the inhibition on the bisphosphatase and decreasing the activation of the PF 1-kinase, thus promoting glucose synthesis.[125,128] This offers a possible mechanism for glucagon's regulation of this substrate cycle without invoking phosphorylation-induced activity changes in either enzyme. PF 1-kinase in liver[133] appears to be mainly under this allosteric control rather than under regulation by phosphorylation. However, glucagon does appear to be influencing the regulation of gluconeogenesis/glycolysis through cAMP-dependent phosphorylation processes that result in alterations of the synthesis and degradation of fructose 2,6-P_2. Recent results indicate that a cycle between fructose

6-P and fructose 2,6-P_2 occurs in liver in which the synthesis of fructose 2,6-P_2 is catalyzed by PF 2-kinase and the hydrolysis is catalyzed by fructose 2,6-Pase.[122,125,128,134] A number of reports[125,128,135,136] have shown that the addition of glucagon to



hepatocytes leads to inhibition of PF 2-kinase and to a decrease in the level of fructose 2,6-P_2. 6-Phosphofructose 2-kinase appears to be a bifunctional enzyme having both phosphohydrolase and phosphotransferase activities,[137] with both activities being regulated by glucagon through cAMP-dependent phosphorylation. The enzyme can also be inactivated by phosphorylase kinase in the presence of Ca^{+2} and calmodulin and be activated by phosphorylase phosphatase[138] setting up the possibility that glucagon-induced changes in glycogen synthesis and glycolysis are coordinated in this manner. In diabetes, hepatic levels of fructose 2,6-P_2 were also found to be very low,[128,139] probably secondary to the decreased levels of glucokinase and the glucagon-mediated decrease of PF 2-kinase activity.[139] Insulin administration to diabetic rats restored the levels probably by virtue of its ability to reverse the above changes.

Recent studies indicate that glucagon may also control the non-oxidative segment of the hepatic pentose pathway through alterations in the levels of fructose 2,6-P_2.[140] However, serious questions have been raised about the physiological significance of fructose 2,6-P_2 in controlling muscle carbohydrate metabolism.[128,141]

Another hypothesis is that the primary site of glucagon's effects on gluconeogenesis is located within the mitochondria rather than the cytosol.[142,143] Mitochondria derived from glucagon-treated rats show enhanced rates of pyruvate metabolism[143,144] and it has been suggested that the production of a more alkaline matrix supports faster rates of pyruvate transport into the mitochondria.[143,145] Glucagon also decreases the quantity of exchangeable Ca^{+2} within the cell but it is not known whether the levels of fructose 2,6-P_2 are sensitive to changes of Ca^{+2}.[146,147] Glucagon may also be influencing the rate of gluconeogenesis through activation of pyruvate carboxylase secondary to its well known activation of fatty acid oxidation.

Glucagon: Regulation of Fatty Acid (FFA) Metabolism - In going from the fed to the fasted state, the liver turns from the utilization of carbohydrate and the biosynthesis of FFA to the consumption of FFA for energy with the production of ketones as by-products. While increased adipose-tissue lipolysis, brought on by a relative insulin deficiency, is necessary for this changeover to be expressed *in vivo*,[148] the changes in liver are induced by glucagon.[148-150] Glucagon action is thought to be mediated through its control of the cytoplasmic malonyl-CoA concentration.[16] Malonyl-CoA (the first committed intermediate in fatty acid biosynthesis) is a potent inhibitor (K_i 1-2 μM) of carnitine palmi-

toyl-transferase-I (CPT-I),[151,152] an enzyme required for the uptake of long chain FFA into mitochondria where they are oxidized. Malonyl-CoA reduces FFA oxidation by liver homogenates[153,154] and isolated mitochondria[151-153,155-157] presumably by slowing the entry of FFA into the β-oxidative pathway. Glucagon treatment lowers malonyl-CoA in isolated hepatocytes,[158-162] a system which displays a tight inverse relationship between malonyl-CoA levels (or lipogenesis) and the rate of FFA oxidation.[158-161,163-165] Glucagon's depression of malonyl-CoA occurs mainly as a consequence of its inhibition of glycolysis[159] and through a partial suppression of the enzyme responsible for malonyl-CoA synthesis, acetyl-CoA carboxylase.[159,162,166] Insulin may have a direct, opposite effect.[162]

While the "malonyl-CoA hypothesis" has gained wide acceptance, recent reports have documented a reduced sensitivity of CPT-I[152,167-170] and FFA oxidation[152,155-157] to malonyl-CoA in fasting. In some cases, the decrease in sensitivity is of sufficient magnitude to raise questions about the physiological significance of the further decline of malonyl-CoA concentrations. As such, glucagon manipulation of CPT-I characteristics and not the concentration of malonyl-CoA *per se* might be primary. Both the magnitude and the significance of the changes in malonyl-CoA sensitivity have been debated.[152,171,172]

In addition to effects relative to malonyl-CoA, glucagon may influence the rate of FFA oxidation by decreasing the activity of the esterification pathway,[162,165,173,174] by elevating liver carnitine and long chain acyl-CoA,[174,175] by stimulating mitochondrial carnitine/acyl carnitine transport,[176,177] and by increasing CPT-II activity.[178]

Somatostatin (SRIF) - SRIF produces a wide variety of antisecretory effects, including the inhibition of insulin and glucagon secretion.[17-19,179] The mechanism of these effects has not been conclusively elucidated, though apparently it does not involve α-receptors, phosphodiesterase, Na^+-K^+ ATPase, or effects on hormone biosynthesis or glucose metabolism.[179] Specific high affinity SRIF binding sites have been identified in pituitary[17-19,179] and pancreas,[179-184] with SRIF addition resulting in decreased cAMP levels and adenylate cyclase activity.[179,185] SRIF may also be inhibiting hormone secretion by interference with cyclic nucleotide action. It inhibited the activation of cAMP-dependent protein kinase in islet tissue[179] and has been reported to induce phosphorylation of mast cell proteins[186] and to be a potent activator of phosphoprotein phosphatase.[187] SRIF has also been postulated[17-19] to act by altering the intracellular status of Ca^{+2}. It inhibited Ca^{+2} influx into pituitary cells[179,188] and disrupted glucose-induced pancreatic insulin secretion by activating and deactivating K^+ and Ca^{+2} permeability, respectively, as well as inhibiting adenylate cyclase.[189,190] However, since SRIF has been shown to inhibit insulin secretion in the absence of extracellular Ca^{+2},[179,189,190] the precise role of Ca^{+2} in SRIF action remains unclear.

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Chapter 21. Structure-Activity Relationships of Calmodulin Antagonists

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Introduction - Calmodulin (CM) is a widely-distributed, Ca^{2+}-binding protein having a molecular weight of approximately 17,000. Since its discovery in 1970 as an activator of a Ca^{2+}-sensitive form of phosphodiesterase, CM has been shown to regulate a variety of Ca^{2+}-dependent enzymes and processes [1,2]. Considerable evidence now suggests that CM is the principal mediator of the effects of Ca^{2+} in most eukaryotic cells. For discussions of the biochemical properties and the physiological functions of CM, the reader is referred to any of several excellent reviews. [3-9]

Since CM plays a fundamental role in cell biology, agents that inhibit its activity should have important pharmacological effects. An understanding of the mechanisms by which these agents alter CM activity may help to explain their pharmacological actions or may suggest new approaches for modifying various physiological or pathological processes. In addition, the development of selective CM antagonists may provide a useful means for further studying the biological roles of CM.

In the mid 1970's Weiss and co-workers reported that the phenothiazine antipsychotics inhibited the CM-induced activation of phosphodiesterase. [10,11] Subsequent studies have shown that a vast array of compounds belonging to diverse chemical and pharmacological classes can inhibit the actions of CM in a variety of biochemical systems. [8,12,13] Table 1 lists the classes of agents that have been shown to inhibit one or more actions of CM. The purpose of this review is to briefly summarize the general mechanisms by which these agents modify the activity of CM and to examine in detail the structural factors that enable drugs to interact directly with CM. Several aspects of the pharmacology of CM have been reviewed elsewhere. [8,12,13,52,53]

Mechanisms of Pharmacologically Altering CM Activity - Most if not all of the effects of CM result from the activation, or occasionally inhibition, of specific enzymes. [3-6] Figure 1 illustrates the general mechanism by which CM regulates most Ca^{2+}-dependent enzymes. Neither Ca^{2+}, nor CM alone is active. However, each CM molecule can bind up to 4 Ca^{2+} ions. [3-7,50,54] The binding of Ca^{2+} alters the conformation of CM, [3-7] increasing its helical content [54-56] and exposing hydrophobic regions. [57,58] In this conformation, the Ca^{2+}-CM complex can bind to regulatory sites on target enzymes and through an unknown mechanism alter their activities. [3-6,59] Although controversial, some evidence suggests that various CM-sensitive enzymes may require different stoichiometric amounts of Ca^{2+} and CM for activity. [5,6,47,60-63]

There are several mechanisms by which drugs might act to inhibit the actions of CM. [8,52] Agents might act by: (A) decreasing the concentration of available Ca^{2+} and preventing the formation of the active Ca^{2+}-CM complex, (B) binding to CM and altering its ability to bind Ca^{2+}, (C) binding

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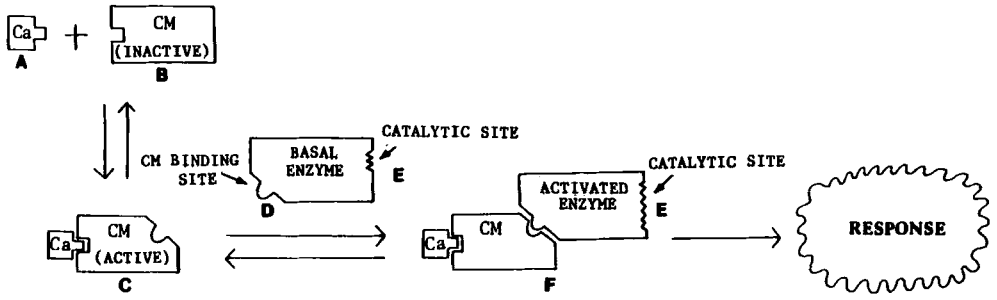
to the Ca^{2+}-CM complex and modifying its activity, (D) binding to the CM-recognition site on the CM-sensitive enzyme and thus preventing the interaction of the Ca^{2+}-CM complex with the enzyme, (E) interacting, either

Table 1 - Classes of Agents that Inhibit the Actions of Calmodulin

Pharmacological Class	Chemical Class	Representative Agent	*Probable Mechanism	**Selected References
α-adrenergic antagonist	benzyl-β-chloroethylamine	phenoxybenzamine	C	14,15
	ergot alkaloid	dihydroergotamine	C	14
β-adrenergic antagonist	alkylaminonaphthyl ether	propranolol	C	16,17
antianxiety agent	benzodiazepine	diazepam	C,D	18,19
antiarrhythmic-antianginal agent	1,4-dihydropyridine	felodipine	A,C,E	20,21
	dimethoxyphenylacetonitrile	verapamil	A,C,E	21
antidepressant	dibenzazepine	imipramine	C	11,22
	dibenzocycloheptadiene	amitriptyline	C	11
antidiarrheal	diphenylalkylpiperidine	loperamide	C	23
antihistamine	phenothiazine	promethazine	C	11,22
antimalarial	aminoacridine	quinacrine	C	16,17,22
	cinchona alkaloid	quinine	C	17
antipsychotic	butyrophenone	haloperidol	C	11,12,24
	benzocycloheptapyridoisoquinoline	butaclamol	C	24
	dibenzodiazepine	clozapine	C	12,24
	diphenylbutylpiperidine	penfluridol	C	11,12,24
	phenothiazine	trifluoperazine and analogs	C	11,12,22
	thioxanthene	chlorprothixene	C	12,22,24
cancer chemotherapeutic agent	anthracycline	adriamycin	C	25
	vinca alkaloid	vinblastine	C	15,26
local anesthetic	benzoic ester	tetracaine	C	16,17,27
	cinchoninamide	dibucaine	C	16,17,27
	phenylacetamidine	phenacaine	C	16,17,27
	phenylamide	lidocaine	C	27
neuropeptide	opioid peptide	β-endorphin	C	12,28-30
	insect venom peptide	melittin	C	30,31
smooth muscle relaxant	diphenylpropylamine	prenylamine	C	32
	naphthalenesulfonamide	W-7 and analogs	C	32-34
miscellaneous agents	Ca^{2+} chelator	EGTA	A	8,11
	CM-binding protein	calcineurin	C	35-40
	polyoxyethylene detergent	Triton X-100	C	41
	chlorinated ethane insecticide	DDT	C,D	42
	modified CM	chlorpromazine-linked CM	D	43
	metal ion	Mg^{2+}	B	44-47
	methylxanthene	theophylline	E	48-50
	miconazole-analogue	R-24571	C	51
	β-aminoethyldiphenylpentanoate	proadifen (SKF-525A)	C	16,17

*Probable mechanism refers to letters shown in Figure 1, as discussed in the text.
**References are for each chemical class, not only the representative agent.

Figure 1 - Mechanisms for the Activation and Inhibition of CM-Sensitive Enzymes



competitively or non-competitively, with the catalytic portion of the CM-sensitive enzyme and altering its activity, (F) interacting with the ternary Ca^{2+}-CM-enzyme complex.

Agents that act by decreasing the concentration of Ca^{2+} (Mechanism A) include chelating agents (e.g. EDTA and EGTA) and the so-called Ca^{2+}-channel blockers (e.g. verapamil and nifidipine), which inhibit the influx of extracellular Ca^{2+} (see Chapter 9 and references 64,65). Since they do not interact directly with CM, these agents display little specificity for CM-regulated systems. For example, the Ca^{2+}-chelators inhibit all biochemical actions of Ca^{2+}, not only its interaction with CM. It should be noted that there is some evidence that certain Ca^{2+} channel blockers might interact with CM itself. [20,21]

Little is known about agents that interact with CM and alter its ability to bind Ca^{2+} (Mechanism B). Such agents might act by binding directly to the Ca^{2+}-binding sites on CM or by binding to some other site on the molecule and inducing conformational changes in the Ca^{2+}-binding regions. Although a variety of di- and trivalent cations can interact with Ca^{2+} binding sites on CM, [50,66] and to varying degrees mimic the actions of Ca^{2+}, [66,87] it is less clear whether these agents can antagonize or potentiate the effects of Ca^{2+}. Several studies have shown that Mg^{2+} inhibits the activation of CM-dependent enzymes by competing with Ca^{2+} for ion binding sites on CM. [44-47] However, La^{3+}, which enhanced the Ca^{2+}-mediated binding of CM to rat striatal particles, had no effect on the activation of adenylate cyclase by Ca^{2+} and CM. [67] Recently, several metals including Hg, Cd, Zn, Co and Sr have been shown to inhibit the CM-induced activation of phosphodiesterase, although the mechanism underlying this effect has yet to be determined. [88] Results of studies examining the NMR spectrum of CM suggest that the binding of phenothiazine antipsychotics by the Ca^{2+}-CM complex (see below) alters the Ca^{2+} binding domains of CM. [68,69] However, the significance of this is unclear since the binding of the drugs themselves requires Ca^{2+} [8,12] and the inhibitory effects of the drugs cannot be overcome by increasing the concentration of Ca^{2+}. [11]

By far, the largest group of CM antagonists consists of those agents that interact directly with the Ca^{2+}-CM complex (Mechanism C). Accordingly, this group of compounds will serve as the focus of this review.

Thus far, little attention has been given to agents that act at the CM-binding sites on CM-sensitive enzymes (Mechanism D). However, recent findings suggest that these may provide useful sites for pharmacological intervention. CM that had been irreversibly linked to chlorpromazine by

ultraviolet irradiation (see below) inhibited the activation of phosphodiesterase by native CM. [43] The chlorpromazine-CM complex, which was unable to activate phosphodiesterase, apparently competed with CM for a regulatory site on the enzyme, suggesting that it may be possible to develop a new class of CM antagonists that are directed at the CM-binding sites on CM-sensitive enzymes. Since there is some evidence that various CM-sensitive enzymes may have somewhat different CM-binding sites,[5,6,60-62] agents directed at these sites might display greater selectivity than agents that interact with CM itself.

Like the Ca^{2+}-chelators, agents that interact with the catalytic portions of CM-sensitive enzymes (Mechanism D; e.g. methylxanthine phosphodiesterase inhibitors) should be considered as indirect CM antagonists, since they do not interact with CM or its binding sites. These agents are relatively non-specific because they can inhibit the non-stimulated form of the enzyme as well as the CM-stimulated form. [48-50] In addition, they can inhibit the activation of the enzyme by agents other than CM, or inhibit CM-insensitive forms of the enzyme. [48]

No agents that interact specifically with the ternary Ca^{2+}-CM-enzyme complex (Mechanism F) have been described, although agents that act on the enzyme or CM itself might remain bound after the ternary complex is formed.

It is, of course, possible that certain agents may act by more than one of the mechanisms described above. For example it has recently been suggested that the antipsychotic agents trifluoperazine and penfluridol inhibit the CM-induced activation of Ca^{2+}-transport ATPase by interacting with both CM and the enzyme itself.[59,70,89] The benzodiazepine antianxiety agents not only bind directly to CM, [18] but also interact with the CM-sensitive protein kinase of rat brain membranes. [19] The antiarryhthmic and antianginal agent nimodipine inhibits the CM-induced activation of phosphodiesterase by interacting with both CM and with the catalytic portion of the enzyme. [21]

Reversible Binding of Drugs to CM - Of the drugs that interact directly with CM or the Ca^{2+}-CM complex, the phenothiazine antipsychotics have been studied most extensively. These agents bind to two distinct classes of sites on CM: a class of specific, high-affinity, Ca^{2+}-dependent sites; and a class of non-specific, low affinity, Ca^{2+}-independent sites. [18,71-73] The Ca^{2+}-dependent sites appear to be the pharmacologically important sites since there is an excellent correlation between the Ca^{2+}-dependent binding of various drugs and their anti-CM potencies. [8,18] There are 2-3 Ca^{2+}-dependent binding sites per CM molecule, with the most potent phenothiazines displaying dissociation constants in range of 1-10 uM. [12,18] Several other classes of drugs also bind to these same Ca^{2+}-dependent sites on CM. Ligand binding studies have shown that the diphenylbutylpiperidine and butyrophenone antipsychotics, [12,18] tricyclic antidepressants,[12,18] benzodiazpine antianxiety agents, [12,18] neuropeptides, [28] and naphthalenesulfonamide smooth muscle relaxants [32] bind to CM in a Ca^{2+}-dependent manner and can compete with phenothiazines for sites on CM. The various agents do, however, exhibit markedly different affinities for the protein. [12,18] Interestingly, the more potent agents show certain structural similarities which may enable them to bind to similar receptor sites on CM. [13,22] It should be emphasized that the binding studies just described do not completely rule out the possibility that a particular agent may bind to several sites on CM, some of which might differ from the phenothiazine binding sites.

Among the agents that interact with the Ca^{2+}-CM complex are several naturally-occurring proteins and peptides. These include a Ca^{2+}-binding protein called calcineurin, [39] a heat stable protein, [35] myelin basic protein, [38] histone, [38] β-endorphin, [28-30] dynorphin, [29,30] substance P [29] and the bee venom peptide melittin.[30,31] Although the physiological significance of the interaction of these endogenous compounds with CM is not yet known, they may function as endogenous modulators of CM activity and may thereby provide potential sites of drug action. [8]

Irreversible Binding of Drugs to CM - Although the binding of the phenothiazines and related compounds to CM can be reversed by removing Ca^{2+} or by dialyzing against an excess of competing drug, [18,71-73] several agents have been shown to interact irreversibly with CM. Upon irradiation with ultraviolet light or treatment with peroxidase-hydrogen peroxide, the phenothiazine antipsychotics chlorpromazine and trifluoperazine bind irreversibly to CM, presumably through a free radical mechanism. [12,74] Like the reversible binding of these agents to CM, the irreversible binding is enhanced by Ca^{2+}. The Ca^{2+}-dependent binding is saturable with one Ca^{2+}-dependent drug binding site per molecule. The irreversible binding of the phenothiazines to CM results in the irreversible inactivation of CM. [74] CM that had been irreversibly linked to chlorpromazine or trifluoperazine by ultraviolet irradiation was unable to activate phosphodiesterase, [12,74] although the drug-CM complex did inhibit the activation of phosphodiesterase by native CM. [43]

Earl et al. [14] reported that the α-adrenergic antagonists phenoxybenzamine and dibenamine also inhibited CM irreversibly, probably by binding directly to CM in a Ca^{2+}-dependent manner. These agents generate ethyleneimmonium and carbonium ion intermediates that might bind irreversibly to electron-rich functional groups on CM. [75] Several alkylating agents that are not α-adrenergic antagonists displayed little anti-CM activity. [14,15]

Along with these studies showing that certain drugs can irreversibly inactivate CM, some evidence suggests that it may also be possible to irreversibly activate CM-sensitive enzymes. Andreasen et al. described a procedure for preparing azido-CM that retained most of the biological activity of native CM. [76] Upon irradiation with U.V. light, the azido-CM formed covalent linkages with several CM-sensitive enzymes and CM-binding proteins and irreversibly activated the CM-sensitive ATPase of human erythrocytes.

Factors Influencing the Interaction of Drugs with CM: Structure-Activity Studies - The specific structural factors that enable drugs to bind to CM are poorly understood. It has been suggested that the abilities of various drugs to interact with CM may be closely related to their abilities to partition between a lipid phase and an aqueous phase, [24,27,34,77] or to their abilities to stabilize membranes. [77-79] These findings are consistent with those showing that hydrophobic regions of CM are involved in the binding of drugs. [57-59,80] According to proposed models, the binding of Ca^{2+} induces conformational changes in CM, exposing a hydrophobic domain which can then bind lipophilic drugs or serve as an interface for the binding of CM to its target enzymes. [57-59]

However, there is also evidence suggesting that other forces besides hydrophobic interactions may be involved in the binding of drugs to CM. Many highly lipophilic drugs are only weak inhibitors of CM and certain hydrophilic drugs are very potent CM antagonists. [13,22] Studies on the pH-dependence of the binding of trifluoperazine to CM suggest that ionic

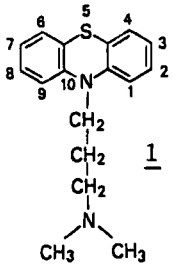
forces may play an important role in this interaction. [12,13] Finally, recent structure-activity studies have shown that various classes of CM inhibitors exhibit certain structural similarities. [13,22] In many cases, slight modifications in chemical structure can greatly alter the ability of a compound to bind to CM and inhibit its activity. Such structural specificity is suggestive of specific drug-receptor interactions and not simple hydrophobic bonding.

To more precisely determine the structural factors that enable drugs to interact with CM, it is necessary to systematically evaluate the structure-activity relationship of representative chemical classes of CM antagonists. To date, only a few such studies have been performed. Their results are summarized below.

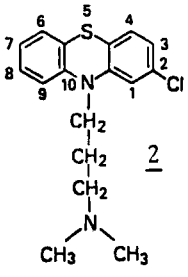
Several studies examining the structure-activity relationships of the phenothiazines have been reported. Modifications of the promazine structure (1) included ring substitution, [11,13,22,23,77,81] length of side-chain, [22] structure of side-chain amine, [22,82] oxidation state of sulfur, [11,22,81] and attachment of an additional aromatic ring [82] (2-11).

Within a series of ring-substituted promazine derivatives, good correlation was found between octanol/buffer partition coefficients and IC_{50} values for inhibition of CM activity, indicating that the hydrophobicity of the phenothiazine nucleus may be an important determinant of potency. By contrast, no such correlation was found among a series of promazine analogs in which the side-chain was modified, although varying the nature and position of the side chain amino group did influence potency. [22] A likely explanation for these findings is that both hydrophobic and electrostatic interactions play a role in the binding of phenothiazines to the Ca^{2+}-CM complex. [13,22]

Although only limited information is now available, the structure-activity relationships of the dibenzazepines (12-14) appear to be similar to those of the phenothiazines. [11,12,22]

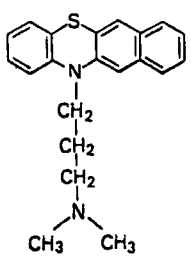


1

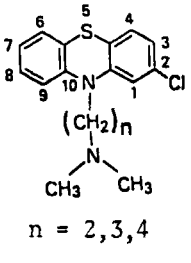


2

The thioxanthene antipsychotics (15) contain an exocyclic double bond that holds the side chain amino group in a relatively fixed position. These compounds were slightly more potent CM inhibitors than their analagous phenothiazines. [12,22,24] Although geometric isomers can exist across the double bond, there was little evidence of stereospecificity in the interaction of these agents with CM. [12,18,24] The implica-

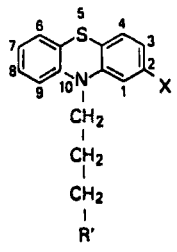


3



n = 2,3,4

4



5-11

X	R	No.
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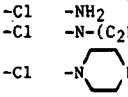
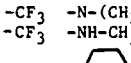
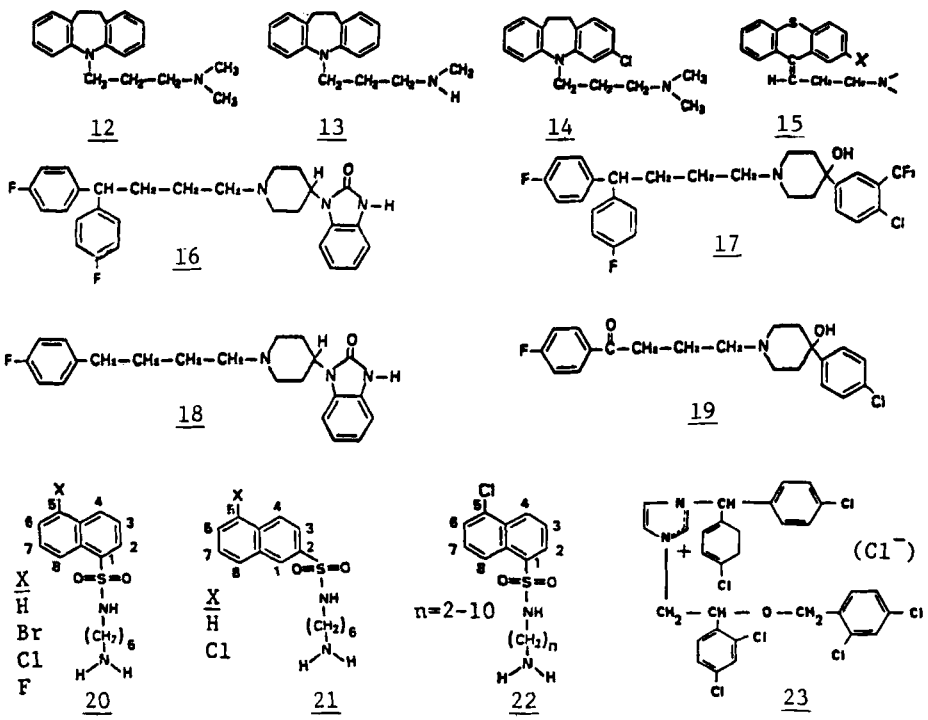
-Cl	$-NH_2$	6
-Cl	$-N-(C_2H_3)_2$	7
-Cl		8
$-CF_3$	$-N-(CH_3)_2$	9
$-CF_3$	$-NH-CH_3$	10
$-CF_3$		11



tions of this lack of stereospecificity have been considered elsewhere.[8,12,13,24]

The diphenylbutylpiperidine antipsychotics pimozide (16) and penfluridol (17) are among the most potent CM antagonists.[13,22,24] Both aromatic rings appear to be necessary for high potency, since the compound R-6033 (18), a pimozide analog lacking one phenyl group, was only 1/6 as potent as pimozide.[22] Similarly, the butyrophenone antipsychotic haloperidol (19), which contains only a single aromatic ring, is a relatively weak CM antagonist.[12,13,22]

Hidaka and his co-workers have studied the structure-activity relationships of the naphthalenesulfonamide class of CM antagonists.[33,34,83,84] The effect on potency of ring substitution and modifying the length of the side chain[34,83,84] and position of the sulfonamide function[33,34] have been examined. Within a series of 1-naphthalenesulfonamides, good correlation was found between octanol/buffer partition coefficients and anti-CM potencies. However, the significance of this correlation was reduced when data from the corresponding 2-substituted series was factored in,[34] indicating that the position of the amino group as well as hydrophobicity may be important in determining potency.

Compound R 24571, calmidazolium (23), is a derivative of the antimycotic agent miconazole and appears to be an especially potent and selective CM antagonist. It is about 500 times more potent than trifluoperazine in inhibiting the activation of several CM-dependent enzymes, but displays little affinity for several neurotransmitter and hormone receptors to which other CM inhibitors bind.[51] In addition, R 24571 has been shown to be more potent in preventing the activation of Ca^{2+} transport ATPase by CM than in preventing the activation of the enzyme by other agents.[59]

Although it is not yet clear whether the polypeptide class of CM inhibitors bind to the same sites on CM to which the phenothiazines bind, their structure-activity relationships suggest that both hydrophobic and electrostatic interactions are involved in their binding to CM. Of several peptides that bound well to CM,[29] or displayed anti-CM activity, [30] all contained a region of basic amino acid residues in close proximity to several hydrophobic residues.

General Structure Characteristics of CM Inhibitors - Although the various CM-binding drugs belong to different chemical classes, many exhibit certain structural similarities. The essential features of these agents are a large hydrophobic region consisting of at least two aromatic rings, either adjacent to each other, or joined at one or two positions, and a side chain amino group that is at least 4 atoms removed from the aromatic rings. [13,22]

The geometric conformation of the ring structure does not appear to be an important determinant of anti-CM activity because compounds having different ring structures, such as the diphenylbutylpiperidines, phenothiazines, thioxanthenes, dibenzazepines, and naphthalenesulfonamides, can all interact with CM. A more important factor appears to be the hydrophobicity of the ring structure; substituents that increase hydrophobicity increase anti CM potency, whereas those that decrease hydrophobicity decrease potency. [13,22,34]

The type of amino group does not seem to be critical since primary, secondary, tertiary, piperazinyl and imidiazole amines inhibit CM. All that seems necessary is that the amino group carry a positive charge and that it be separated from the hydrophobic region of the drug molecule by 4 or more atoms, although the distance required for optimal activity is not yet known. [13,22,34] This issue is complicated by the fact that amino groups at the end of long, flexible alkyl chains could assume a variety of different positions to allow binding to CM, or the long alkyl chains might undergo hydrophobic interactions with CM. Interestingly, the amino-acridine derivative quinacrine, which has a very hydrophilic side chain, and the thioxanthene antipsychotics, which have their side chain amino groups held in fixed positions, are potent CM inhibitors, [22,24] suggesting that the position of the amino group, rather than the hydrophobicity of the side chain, determines potency.

This model for the interaction of drugs with CM applies only to agents that interact with the phenothiazine binding sites. It is possible that drugs having somewhat different structural characteristics might bind to different sites on CM. Agents such as DDT and Triton X-100 have structures that differ from the proposed model. At present, it is not known whether these agents bind to the same sites on CM that the phenothiazines do. [41,42] Results of NMR and fluorescence spectroscopy studies have provided some evidence for the existence of several types of drug binding sites on CM. [15,20,21]

Effects of Drug Binding on the Conformation and Activity of CM - The mechanism by which drug binding alters the activity of CM is not completely understood. NMR [20,68,69,80,86] and fluorescence spectroscopy [15,21,29] data indicate that the binding of drugs induces changes in the conformation of CM. Studies examining the binding of ^{125}I-labelled CM to membrane sites have shown that drug binding decreases the affinity of CM for its target enzymes.[62,67] These data suggest that drug binding alters the conformation of the Ca^{2+}-CM complex and decreases its ability to interact with recognition sites on CM-sensitive enzymes.

The location of the phenothiazine binding sites on CM is not known. It has been suggested that the drugs may bind to a hydrophobic region of CM that serves as an interface for the binding of CM to its target enzymes. [57-59] Results of ^1H NMR studies indicate that methionine and phenylalanine residues near Ca^{2+}-binding domains II, III and IV of CM may play an important role in binding phenothiazines. [80,86] Other results suggest that acidic residues on CM are involved in binding basic drugs. [12,13] These data suggest that drug binding sites on CM may consist of a hydrophobic region, containing methionine residues, in close proximity to an acidic residue such as aspartic or glutamic acid. Head et al. reported that a cyanogen bromide-cleavage fragment of CM, containing residues 77-124, was able to bind phenothiazines in a Ca^{2+}-dependent manner. [85] The recent findings that certain drugs can bind irreversibly to CM should aid in further identifying drug binding sites on CM. [14,70]

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Section V - Topics in Biology

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Chapter 22. Tyrosine-Specific Protein Kinases

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Introduction - Recently, a new class of protein phosphotransferases has been identified. The enzymes which comprise this group can be distinguished from other protein kinases by a number of criteria, most notably, their specificity for phosphorylation of tyrosine residues. The importance of these phosphotransferases is not merely a consequence of their tyrosine-specificity, but the evidence that all of the enzymes in this class appear to be associated with the regulation of cellular morphology and growth control.

Tyrosine phosphorylation was first identified in association with the transforming proteins of several oncogenic viruses.[1-3] "Transforming" proteins are those polypeptides which are responsible for the initiation and maintenance of the transformed phenotype (see review 4). The viral genes which encode these transforming proteins are highly homologous to genes found in normal eukaryotic cell DNA (see reviews 4 and 5). It is believed that these viral transforming or "onc" genes were acquired from normal cell chromosomal DNA by genetic recombination. The proteins encoded by several of these cellular homologues of viral onc genes have been identified in uninfected cells. These normal cell proteins are structurally and functionally similar to their viral counterparts.[4] It is speculated that these normal cellular proteins might regulate cellular events involved in growth control and that aberrant expression of the viral homologues of these cellular gene products is responsible for a loss of normal cell growth regulation.

A second group of tyrosine-specific protein kinases has been identified in normal cells. This group contains the membrane receptors for many cellular growth factors including epidermal growth factor (EGF), insulin, and platelet derived growth factor (PDGF). Interaction of each of these factors with their respective cellular receptor results in activation of tyrosine-specific kinase activity associated with the receptor molecule.

The precise mechanism whereby these tyrosine kinases interact with other cellular proteins to exert their biological effects is presently unknown. In this review, we will summarize the current state of knowledge on these two groups of tyrosine kinases.

Viral Transforming Proteins - With one exception,[1] tyrosine-phosphorylation has been identified in conjunction with a single group of viruses, designated retroviruses. Retroviruses possess an RNA genome, yet replicate via a double-stranded DNA intermediate which becomes stably integrated into the cell (see review 6). Retroviruses

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which are highly oncogenic and induce tumors after a very short latency period each encode a single gene product which is responsible for the initiation and maintenance of the transformed state. Thus far, seventeen different "onc" genes have been identified in association with oncogenic retroviruses (see review 5). Tyrosine-specific protein kinase activity is not associated with the gene products of all "onc" genes. The transforming proteins which have been identified as tyrosine-specific protein kinases are the products of the src, fps, fes, abl, ros, and yes genes. Although each of these represent unique gene products, analysis of the amino acid sequence of these transforming proteins has revealed a high degree of amino acid homology. The extensive nucleotide and amino acid homology which exists between the avian sarcoma virus fps gene and the feline sarcoma virus fes gene suggests that the fes gene is the feline homologue of the avian fps gene.[7,11] Eighty-two percent of the amino acids of the transforming protein of Y73 ($pp90^{yes}$)[8] and Rous sarcoma virus ($pp60^{src}$)[9,10] are homologous. The strongest degree of homology is found in a 133 amino acid sequence in the carboxyl-half of these proteins where 93% homology is found. The Fuginami sarcoma virus transforming protein, $pp130^{fps}$, shows greater divergence from $pp60^{src}$ than $pp90^{yes}$ yet there is a high degree of homology in the carboxy-terminal 280 residues of both proteins.[11] These results strongly suggest that the 3' regions of the src, yes, and fps and fes genes originated from a common cellular progenitor sequence. The domain of $pp6^{src}$ encoded by this 3' region of the src gene has been shown to possess tyrosine-specific kinase activity.[12]

The best characterized oncogenic retrovirus is Rous sarcoma virus (RSV). Since RSV is the only replication-competent transforming virus, it has been amenable to genetic and biochemical studies which were technically difficult to perform on the replication-defective viruses. The transforming gene of RSV, termed src, encodes a 60,000 dalton phosphoprotein, denoted $pp60^{src}$.[13-15] When isolated from RSV-transformed cells, $pp60^{src}$ contains both phosphoserine and phosphotyrosine.[3,16,17] At least one of the serine residues is phosphorylated by a cAMP-dependent protein kinase.[18] Phosphorylation on tyrosine is believed to result from autophosphorylation.

In Vitro Protein Kinase Activity - The first evidence that $pp60^{src}$ possesses phosphotransferase activity involved an unusual enzyme assay. Immunoprecipitates containing $pp60^{src}$ bound to its specific immunoglobulin molecule were incubated with γ-[^{32}P]ATP. Under these conditions the antibody molecules served as substrates of $pp60^{src}$-mediated protein kinase activity and a tyrosine residue in the variable region of the IgG heavy chains was phosphorylated.[18,19] The antiserum used in this assay (TBR serum) is obtained from rabbits bearing tumors induced by RSV.[13]

Following this report, further evidence was sought and found which clearly demonstrated that the enzyme activity identified in this immune complex assay is intrinsic to $pp60^{src}$. The most conclusive evidence included the following findings: 1) The protein kinase activity of $pp60^{src}$ from mutant viruses containing temperature-sensitive defects in the src gene is thermolabile.[18,20,21] 2) The $pp60^{src}$ protein copurified with the tyrosylphosphotransferase activity through extensive chromotography procedures.[22-25] 3) The $pp60^{src}$ protein translated in an in vitro system programmed with purified viral RNA is active as a protein kinase.[26,27] 4) The $pp60^{src}$ expressed in E. coli

cells carrying a plasmid which contains a cloned copy of the src gene functions as a tyrosine-specific protein kinase.[28,29]

The immune-complex kinase assay for pp60^{src} proceeds almost instantaneously at 0°C in the presence of Mn^{++} or Mg^{++} ions and either ATP or GTP.[18,19] Other nucleotide triphosphates can also serve as phosphate donors including CTP and some deoxyribonucleoside triphosphates.[36] The rate of this reaction is independent of concentration of the protein reactants.[19,36]

Tyrosine-specific protein kinase transforming proteins encoded by the fps, fes, yes, ros, and abl genes are also active within an immune complex.[2,30-34,37] Since all of these proteins are chimeric proteins containing a portion of the viral structural protein precursor, Pr76 (see reviews 4 and 5), they can be immunoprecipitated by antibodies directed against this structural protein. Immune-complex bound transforming-proteins encoded by the above genes are able to phosphorylate RSV-specific TBR-IgG as well as exogenous substrates such as casein. The cation requirements and phosphate donors for several of the viral tyrosine-specific protein kinases as well as the growth hormone stimulated kinases are shown in Table 1. Phosphorylation of pp60^{src} and the other viral transforming proteins also takes place within the immune complex. The tyrosine acceptor sites of the src, fps, yes, fes proteins (shown below) have similar amino acid sequences.

pp60^{src} GluAspAsnGluTyrThrAlaArgGlnGly[9,10,16,38]

pp90^{yes} GluAspAsnGluTyrThrAlaArgGlnGly[8]

pp140^{fps} GluAspGlyValTyrAlaSerThrGlyGly[11]

pp85^{fes} AlaAspGlyIleTyrAlaAlaSerGlyGly[7]

It has not been rigorously demonstrated that this reaction occurs via autophosphorylation. Although no tyrosine phosphotransferase activity other than pp60^{src} has been detected in immune complexes, autophosphorylating activity was not found associated with the E. coli-produced pp60^{src} protein.[28,29] The latter evidence also suggests that tyrosine phosphorylation of pp60^{src} is not essential for its enzymatic activity. Another line of evidence also supports this conclusion. By chemical mutagenesis of cloned copies of the src gene, a phenylalanine residue was substituted for the tyrosine acceptor site on pp60^{src}.[39] This form of pp60^{src} is active as a protein kinase and able to cause in vitro transformation. This evidence suggests that tyrosine phosphorylation is not necessary for the functional activity of pp60^{src}. One cannot rule out from the above result that phosphorylation of pp60^{src} on tyrosine causes subtle differences in the enzymatic activity of pp60^{src} which are over-ridden by the excess amounts of the protein in transformed cells. Tuyrosine phosphorylation could be involved in regulating the functional activity of the normal cellular src protein.

Most of the viral transforming proteins are phosphorylated on serine as well as tyrosine. It is not known whether this modification regulates the functional activity of these proteins.

Soluble phosphotransferase reactions have also been performed using partially purified preparations of pp60^{src}. pp60^{src} has been

purified by both immunoaffinity chromatography and more standard chromatographic procedures.[22-25] When prepared by the latter method, a 52K cleavage product of pp60^{src} is generated. This modified protein has lost a portion of the amino terminus of pp60^{src} but retains kinase activity. Several proteins have been shown to serve as substrates *in vitro* including casein, actin, tubulin, and histones H4 and H5. The cation requirements and phosphate donors of the soluble phosphotransferase reaction are very similar to those of the immune complex assay. Maximum enzymatic activity was found to occur at pH 6.5-6.8 and the Km for ATP is 30M. The reaction is sensitive to ionic strength; 0.1M KCl, 0.1M NaCl, 50 mM KPO_4 or 50 mM $NaPO_4$ inhibit casein phosphorylation.[25]

There is evidence which suggests that the functional activity of the transforming proteins is contained within a distinct domain of the molecule and can function independently of the remainder of the molecule. 1) A fragment of pp60^{src} from the carboxyl-half of the molecule is able to phosphorylate IgG in the immune complex protein kinase assay.[12] 2) Deletion mutants have been constructed which contain varying portions of the *v-abl* gene.[40-42] A small fragment of the gene representing 1000 nucleotides from the 5' end of the gene was found to be both necessary and sufficient for oncogenic transformation. In addition, naturally occurring isolates of retroviruses which contain varying portions of the *fps* gene have been characterized.[43-45] While these isolates do not define the narrowest limits of activity, they do indicate that portions of the *fps* gene are not necessary for oncogenesis.

Table I. Properties of Tyrosine Kinases

Gene Product	P Donor	Cation
src	ATP,GTP[18,19,23,24]	Mg, Mn
fps	ATP[30]	Mn>Mg[30]
yes	ATP,GTP[34]	Mn>Mg[34]
abl	ATP[2]	Mn.Mg[2]
ros	ATP[34]	Mn>Mg[34]
fes	ATP[113]	Mn>Mg[113]
EGF	ATP,GTP[85,93]	Mn>Co>Mg[85,93] Mg>Mn[94]
PDGF	ATP,GTP[102]	Mg>Mn[103] Mn>Co>Mg[102]
insulin	ATP[99,100]	Mn>Co>Mg[99,100]

The only non-retrovirus transforming protein which has an associated tyrosine-kinase activity is the middle T antigen of polyoma virus. In the presence of ATP and Mg^{++} immunoprecipitates containing this antigen allow phosphorylation of two species of middle T (56 and 58K).[1] There is presently no definitive evidence that would indicate whether the kinase activity is intrinsic to the middle T protein(s) or

resides in an associated cellular enzyme. Efforts to obtain kinase activity associated with middle T synthesized in an *in vitro* translation system or when cloned and expressed in *E. coli* have been negative.[46] Studies on mutant virus (Py-1387T) and subcellular localization studies indicate an association between the insertion of middle T into membranes and its ability to function as a protein kinase.[35,48] These results suggest the possibility that middle T may be associated with a cellular tyrosine-specific kinase within the plasma membrane.

In Vivo Protein Kinase Activity - Tyrosine phosphorylation is a rare event in eukaryotic cells. Phosphotyrosine accounts for only .01-.03% of the total phosphoaminoacids in proteins.[47] Since this level increases 7- to 10-fold after transformation by RSV and this increase is temperature-sensitive in cells transformed by viruses containing a mutation in the *src* gene, it would appear that pp60^{src} functions as a protein kinase *in vivo* and that tyrosine phosphorylation is involved in the events mediating transformation by pp60^{src}.[47] It is therefore crucial to identify the substrates which are phosphorylated by pp60^{src} *in vivo* and understand the biological consequences of these phosphorylation events. Although pp60^{src} has been reported to be localized in various regions of the cytoplasm, the majority of this protein appears to be tightly associated with the cytoplasmic face of the plasma membrane.[12,49-51] Specialized regions within the plasma membrane like adherence placques and gap junctions show specific staining with antibody directed against pp60^{src}.[50,52] There is not strong evidence that any portion of pp60^{src} is exposed on the external surface of the cell. It is therefore speculated that pp60^{src} exerts its biological effects at the cytoplasmic face of the plasma membrane; however, mutant forms of pp60^{src} which have an altered association with the membrane, yet still retain the ability to transform cells *in vitro*, have been described.[53,54] While the requirement for membrane association has not been clearly demonstrated, it would appear that many of the phenotypic alterations of transformation do not require involvement of the nucleus. Enucleated transformed cells have been shown to display many of the phenotypic properties of RSV-transformed cells.[55]

Many approaches have been employed to identify proteins containing elevated levels of phosphotyrosine after RSV-transformation. The number of substrates identified by these methods is quite large and it is likely that the search has not uncovered all protein targets.[56-67] Indeed, by a somewhat crude analysis of total cell proteins separated on SDS-polyacrylamide gels, elevated levels of phosphotyrosine were found in proteins from every molecular weight range.[63,64] Individual protein species have been resolved by two-dimensional separation of proteins.[56,57,61] It is possible to increase the resolution of phosphotyrosine-containing proteins by treating the gels in alkali before autoradiography.[61] This technique was designed to take advantage of the alkali lability of phosphoserine and phosphothreonine relative to phosphotyrosine. Another recently described set of reagents for the detection of phosphotyrosine containing proteins are monoclonal and polyclonal antibodies to phosphotyrosine or analogues of phosphotyrosine.[68] These reagents are able to bind to the transforming proteins of several retroviruses, the growth-hormone receptors, and several candidate substrates of these phosphotransferases.

The identity of some of the protein substrates has been determined. Three of the substrates are glycolytic enzymes (phosphoglycerate mutase, enolase and lactate dehydrogenase),[67] at least one is a cytoskeletal protein (vinculin)[60] and one protein (pp50) is associated with pp60^{src} in a short-lived protein complex.[3,58,59,69,70] The functional significance of the tyrosine phosphorylation of these proteins is an important and complex question. The fact that expression of the abl transforming protein in E. coli results in the tyrosine-specific phosphorylation of multiple bacterial proteins raises the question of whether some (if not most) of the phosphorylation events in the virus transformed cells have no biological significance.[71] This is especially important in view of the fact that only 1-10% of the total cellular population of these substrates are phosphorylated on tyrosine.[57,58,60,67] Resolution of these questions awaits demonstration of a functional change in a substrate after tyrosine phosphorylation.

It is also important to note that all proteins which contain elevated levels of phosphotyrosine after RSV transformation may not be directly phosphorylated by pp60^{src}. It is possible that pp60^{src} could activate other cellular tyrosine kinases. Direct demonstration of pp60^{src} phosphorylation of candidate substrates in vitro would not necssarily prove that pp60^{src} is responsible for the phosphorylation in vivo since certain tyrosine kinases might have overlapping substrate specificities. Indeed, there is considerable (but not complete) overlap in the major phosphotyrosine-containing proteins observed after transformation by yes, fps, src, and abl.[62,72,73] This suggests that transformation mediated by each of the different oncogene products involves similar phosphorylation events and that these kinases share cellular protein substrates.

One candidate substrate of pp60^{src} phosphorylation is a cellular protein of M 50,000 (pp50) which is associated with pp60^{src} in a short-lived protein complex. The functional role of this complex is unclear, however, the localization and the kinetics of the turnover of this complex suggest that it may be involved in some aspect of the processing of newly synthesized pp60^{src} before its association with the plasma membrane. It has been demonstrated that pp90 and pp50 bind to pp60^{src} shortly after its translation on soluble polysomes.[74] pp90 and pp50 are complexed with pp60^{src} in the cytosol fraction of the cell; membrane-associated pp60^{src} is not bound to pp90 or pp50.[74,75] The transforming proteins of Y73, Fujinami and PRCII viruses are also associated in a complex with pp90 and pp50.[73] This suggests that these cellular proteins may have a common functional interaction with the transforming proteins which possess tyrosine-specific protein kinase activity.

Normal Cell Protein Kinases - As mentioned earlier, normal cells contain genes homologous to the retrovirus transforming genes. The protein products encoded by the cellular c-src, c-fps, c-fes and c-abl genes have been identified and partially characterized. The c-src and c-fps gene products are both phosphoproteins which possess tyrosine-specific protein kinase activity.[76-78] Phosphotransferase activity has not been found associated with the c-abl gene product.[79] Two possible explanations have been proposed to account for the oncogenicity of the virus-encoded gene products. 1) The oncogenicity of the viral transforming protein may be a dosage phenomenon. The continual overexpression of cellular protein homologues which play an important role in cellular growth control and metabolism could be responsible for

oncogenic transformation. Indeed, twenty-to-fifty-fold higher levels of the v-onc gene products are found in virally transformed cells compared to the levels of the c-onc proteins in uninfected cells.[76,77] 2) Changes in the amino acid sequence of the virus-encoded protein could alter the functional expression of the protein. This change could be very subtle and difficult to assay in vitro but cause dramatic changes in cellular metabolism in vivo, i.e. change the substrate specificity of the phosphotransferase activity.

Differences in the nucleotide sequence between cellular and viral src genes have been detected but it is not known whether these changes are responsible for oncogenic properties of $pp60^{v\text{-}src}$. This question could be addressed by constructing hybrid genes containing various mixtures of the viral and cellular genes as performed with the ras gene from normal and bladder tumor cells (see review 5). This type of reconstruction has not been reported for any of the oncogenes which encode tyrosine-specific protein kinases. It has also not been reported that transfection with either the c-fps, c-src, c-yes or c-abl DNA induces cellular transformation (as has been reported for the c-mos and c-ras genes (see review 5).

Therefore, the issue is not yet resolved as to whether overexpression of any of the c-onc genes which encode tyrosine-specific protein kinases can induce oncogenic transformation or whether mutations in the c-onc genes are required to confer oncogenicity on these genes.

Growth Factor-Activated Protein Kinases - Tyrosine-specific protein phosphorylation appears to be involved in the action of at least three mitogenic hormones: EGF, PDGF and insulin. Tyrosine-specific protein kinases are rapidly activated following binding of these hormones to specific plasma membrane receptors on target cells. In their specificities for divalent cations and phosphate donors (Table I) as well as in their low Km for ATP, rapid kinetics at 0°C, and membrane association, these kinases resemble the virally coded tyrosine kinases. By analogy with the viral tyrosine kinases, it seems likely that these hormonally-activated kinases are important in the changes in metabolism and growth with which they are associated. (For reviews of the biology of these factors, see references 80-83).

Hormonally-activated tyrosine phosphorylation was first described in studies of the interaction of EGF with its receptor in plasma membranes prepared from A431 human carcinoma cells.[84-87] This unusual cell line was useful in that it possesses on the order of 100-fold more EGF receptors than typical EGF-responsive cells such as fibroblasts. A tyrosine-specific protein kinase activity present in these membranes was found to be stimulated several-fold by EGF, and to copurify with the EGF receptor. These early studies, which have been reviewed,[82,88] provided evidence that the EGF receptor of M_r 170,000 is itself an EGF-stimulated protein kinase able to phosphorylate itself or exogenous substrates on tyrosine. This hypothesis has recently been confirmed by a series of experiments in which A431 cell membranes were affinity-labeled with ^{125}I-EGF and ^{14}C-fluorosulfonyl benzoyl adenosine (FSBA).[89] Both reagents specifically labeled the 170 Kd EGF receptor, and attachment of ^{14}C-FSBA to the receptor correlated with inactivation of kinase activity. This indicated that the same molecule contained both the EGF-binding site and the kinase ATP-binding site.

The observation that EGF stimulated tyrosine phosphorylation in A431 membranes *in vitro* was quickly extended to living A431 cells. Within one minute of exposure to EGF, levels of phosphotyrosine in total cellular protein rose several-fold; these levels remained high for many hours in the presence of EGF, but declined to control levels upon removal of EGF from the culture medium. When A431 cell protein phosphorylation was analyzed using alkali-treated two-dimensional gels, EGF was found to stimulate phosphorylation on tyrosine of an 81 Kd protein and a 39 Kd protein.[90] This 39 Kd protein is the same 39 Kd protein phosphorylated on tyrosine in response to viral transformation, and is phosphorylated on the same tryptic peptide in response to either RSV or EGF.[91,92] In addition, EGF stimulates phosphorylation phosphorylation of its receptor in living A431 cells. This stimulation involves increased phosphorylation on serine and threonine as well as on tyrosine.[90]

Recent studies on EGF-induced tyrosine phosphorylation have focused on normal cells which are growth-responsive to EGF; A431 cells not only have abnormally high numbers of EDG receptors, but they are also abnormal in that they are tumor cells and are growth-*inhibited* by EGF. Nevertheless, the effects of EGF *in vitro* on tyrosine phosphorylation of membranes from normal cells are essentially the same as those observed using A431 membranes; the responses in these membranes are of lower magnitude, since normal cells have far fewer EGF receptors. EGF-stimulated phosphorylation of a 170 Kd protein is observed *in vitro* in membranes from fibroblasts, NRK cells, placenta and liver.[93-96] In the case of liver, this 170 Kd protein has been purified and shown to be the EGF receptor and to have tyrosine kinase activity, as described above for the A431 cell EGF receptor.[96] The effects of EGF on tyrosine phosphorylation in intact normal cells, however, are quite different from those observed in A431 cells, and are discussed below.

Parallel investigations concerning the mechanisms of action of PDGF and insulin have resulted in reports that both of these hormones activate tyrosine kinases when they bind to their receptors *in vitro* or in living cells. In partially purified preparations of insulin receptor from rat liver, mouse 3T3-L1 cells, or human placenta, binding of insulin to the alpha subunit of its receptor stimulates tyrosyl phosphorylation of the beta subunit of the receptor and of exogenous substrates.[97-100] As with the EGF receptor, binding of insulin to its receptor in living cells results in increased phosphorylation of the receptor on serine and threonine as well as on tyrosine. PDGF-stimulated tyrosine phosphorylation has been observed in plasma membranes prepared from human fibroblasts, human glial cells, and 3T3 cells.[101-103] The major PDGF-dependent phosphoprotein in these preparations is a 180 Kd protein thought to be the PDGF receptor based on its size and single-chain structure, as well as by analogy to the EGF system.

Several recent studies have addressed the role of tyrosine phosphorylation in the growth response of normal cells to EGF and PDGF. Such investigations have been technically difficult in normal cells as compared with A431 cells, where the EGF response is amplified perhaps 100-fold over the phosphorylation response in fibroblasts. Nevertheless, it has been possible, using the alkaline two-dimensional gel analysis of Cooper and Hunter[61] and the one-dimensional gel/phosphoamino acid analysis technique of Martinez et al.[63] to detect

PDGF-stimulated tyrosine phosphorylation of several proteins of about 40 Kd; the phosphorylation of two of these proteins is also stimulated by EGF.[104,105] Tyrosyl phosphorylation of one 43 Kd protein in chicken embryo fibroblasts is induced by PDGF or by sarcoma viruses. The 81 Kd and 39 Kd proteins phosphorylated on tyrosine in response to EGF in A431 cells are not generally phosphorylated in growth-stimulated fibroblasts, although some phosphorylation of the 39 Kd protein may be observed following PDGF treatment of certain 3T3 sublines.[104-106] Thus, it seems doubtful that phosphorylation of these proteins is involved in EGF- or PDGF-induced mitogenesis. Again, in contrast to the EGF response in A431 cells, EGF induces negligible increases in whole cell phosphotyrosine in 3T3 cells. Thus, gross differences in tyrosine phosphorylation are not needed for the stimulation of transport, glycolysis, macromolecular synthesis, and growth by EGF. PDGF induces a greater, although transient, increase in whole-cell phosphotyrosine. The greater magnitude of the PDGF effect is presumably due to the greater number of PDGF receptors than EGF receptors on these cells, and the transcience of the response is probably due to down regulation of PDGF receptors.

Such studies of normal cells have raised several questions about the role of tyrosine phosphorylation in growth and transformation. The relatively low levels of tyrosine phosphorylation induced by EGF and PDGF suggest that the vast majority of tyrosine phosphorylations induced by viruses are not responsible for the many similar metabolic events induced by both growth factors and viral transformation. Some virus-specific phosphorylations may be involved in transformation-specific events, while others may be meaningless. Recent studies by Schlessinger et al. suggest further complexities.[107-108] These workers found that CNBr-treated EGF, which retains binding activity, can stimulate tyrosine phosphorylation in A431 membranes and can induce early EGF-induced changes in living A431 cells or fibroblasts. Nevertheless, this EGF derivative is not mitogenic. Although it may be premature to conclude that the CNBr-EGF-activated tyrosine phosphorylation response in A431 membrane preparations also occurs in living fibroblasts, these results suggest that EGF-induced tyrosine phosphorylation may be necessary, but not sufficient, to stimulate DNA synthesis and growth.

Another area in which the relationship of growth factors, tumor viruses, and tyrosine phosphorylation is being explored is the field of transforming growth factors (TGF's). A variety of tumor cells including those induced by RNA tumor viruses produce and secrete polypeptides, referred to as TGF's, which are capable of reversibly inducing the transformed phenotype in target cells. The best-studied TGF's are able to bind to the EGF receptor and stimulate tyrosine phosphorylation, although they are antigenically distinct from EGF.[109,110] It has been difficult to imagine how similar polypeptides, acting through the same receptor and inducing the same changes in tyrosine phosphorylation, can have different biological effects: TGF, but not EGF, can induce growth in soft agar. A recent study in which ^{125}I-TGF was cross-linked to membranes of target cells suggested that the factor bound both to the EGF receptor, and to a separate 60 Kd protein which may bind TGF but not EGF.[111] Thus, the EGF-like effects of TGF's may be produced by binding to the EGF receptor and stimulating tyrosine phosphorylation, while growth in soft agar may be induced by binding of TGF to a separate receptor. It is not yet known whether this putative TGF receptor has any role in regulating tyrosine

phosphorylation. It is of interest that TGF's are produced by cells infected with RNA tumor viruses whose transforming proteins are not tyrosine kinases. Thus, even these viruses may rely on tyrosine phosphorylations to induce some aspects of the transformed phenotype.

Conclusions - Independent investigations of two different types of agents which alter cellular growth behavior have uncovered multiple enzymes which possess tyrosine-specific protein kinase activity. It is likely that future searches will reveal a greater number of similar enzymes. Indeed, Wong and Goldberg[112] have identified at least seven tyrosine-specific phosphotransferases in normal liver tissue using the tyrosine-containing eight amino acid peptide, angiotensin, to probe cell fractions for kinase activity. The function of these multiple tyrosylphosphotransferases is open to speculation. There is no experimental evidence which suggests any functional interaction between these enzymes, i.e. by a cascade-type mechanism. For instance, infection with the avian sarcoma viruses Y73 or FSV does not stimulate phosphorylation of the normal cell src gene product. However, sufficient information has not been obtained to rule out this possibility. It is possible that each tyrosyl phosphotransferase controls one or more specific events which occur in response to stimualtion of cellular growth. Further multiplicity would be required if these kinases are regulated independently during growth and development. Preliminary screening of the expression of the various normal cellular "onc" genes and their gene products have revealed tissue-specific differences in some of these proteins, however, no clear pattern has emerged.[114-116]

The preliminary characterization of the viral and cellular tyrosine-specific protein kinases in this chapter provides evidence that these enzymes play a crucial role in the regulation of cellular growth control. Further understanding of the mechanisms involved in these events awaits identification of the biologically relevant substrates of these kinases and elucidation of the functional changes elicited by these phosphorylation events.

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Chapter 23. Oncogenes

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The nature of the biochemical changes which discriminate tumor cells from normal cells has been the focus of much interest in the last two decades. Three independent lines of research have focused attention upon changes in cellular DNA as being involved in oncogenic transformation: 1) Most, if not all, chemical mutagens are chemical carcinogens, an observation which suggests that certain DNA mutations can be oncogenic. 2) The transformed phenotype is heritable, either as a result of mitosis or as shown in somatic cell hybrids. 3) Cells can be oncogenically transformed by certain types of viruses which encode proteins that are responsible for the initiation and maintenance of the transformed state. Recent investigations, buttressed by such findings, have led to the discovery of onc genes or oncogenes, i.e., genes whose polypeptide products are capable of causing oncogenic transformation. In this review, we shall discuss oncogenes and their potential roles in certain types of human cancers.

VIRAL ONCOGENES

Retroviruses - RNA tumor viruses, or retroviruses, have been useful agents for studying the molecular events accompanying transformation. The original tumor-derived isolates were found to be able to transmit neoplastic diseases of the same types as the tumors from which they were isolated.[1] In the last thirty years, many more isolates, both oncogenic and nononcogenic, have been made from normal tissues and from tumors of a wide range of vertebrate species.[2] The genome of replication-competent retroviruses contains three genes important to the replication process: 1) gag (group-specific antigens) encodes four structural polypeptides which compose the virion core;[3] 2) pol (polymerase) encodes the viral reverse transcriptase (RNA dependent DNA polymerase); 3) env (envelope) encodes the virion envelope glycoprotein (and associated nonglycosylated envelope protein) which is responsible for viral infectivity and host range. Following attachment of a virion to an uninfected cell, the viral RNA molecule enters the cell cytoplasm and is copied into a double-stranded DNA molecule by means of reverse transcriptase.[4] An important structural feature of this DNA is the presence of both its 5' and 3' termini of long terminal repeat (LTR) segments of several hundred nucleotides. The LTR is an amalgam of sequences from the 5' and 3' termini of the viral RNA molecule and contains signals for the efficient promotion and termination of RNA transcription from the viral DNA template. Subsequent to reverse transcription, the linear DNA molecule circularizes and integrates into the host cellular DNA by covalent attachment at apparently random locations.

Discovery of src - The focus assay which quantitates the appearance of foci of morphologically transformed cells following infection by certain retroviruses, has permitted the division of retroviruses into two major biological groups:[5] 1) transforming retroviruses, isolated only from tumors, cause morphological transformation in vitro, are generally replication-defective or helper-dependent (Rous sarcoma virus (RSV) being a notable exception), and are oncogenic in vivo with relatively short latent

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periods. 2) Nontransforming or helper-independent retroviruses, isolated from tumors or normal tissue, do not induce morphological changes *in vitro*, are generally replication-competent, and either are nononcogenic or induce lymphoid tumors with relatively long latent periods. The recognition of these two classes of viruses as well as nontransforming or transformation-defective variants of transforming retroviruses suggested the existence of a viral gene(s) responsible for the initiation and maintenance of the transformed cell phenotype.

The first virus for which an oncogene was demonstrated definitively was RSV. From the viral stock of this replication-competent transforming retrovirus could be isolated mutants that were transformation-defective (td) or temperature-sensitive for transformation but not for virus replication (ts). These mutants suggested the existence of a functional viral oncogene, known as *src* (sarcoma).[6] Since *src* was not required for viral replication, its polypeptide product was considered unlikely to be a virion structural component. Therefore, two approaches were taken toward identifying this putative *onc* protein. Cell-free translation was performed on RSV *RNA* in search of a translational product not encoded by td RSV. Also, antiserum from rabbits transplanted with RSV-induced tumors was used to immunoprecipitate radiolabeled proteins from extracts of cells transformed by RSV. Both approaches revealed a 60,000 dalton phosphoprotein, pp60*src*, whose expression was found to be ts in cells transformed by ts RSV mutants.[7] The *src* gene has been sequenced and found to contain a single open-reading frame of nearly 1.6 kilobases (kb), from which a sequence of 526 amino acids (approximately 60,000 daltons) was predicted.[8]

Other Viral Oncogenes - The definition of other viral oncogenes (conventionally referred to according to three letter abbreviations such as *ras*, *myc*, *mos*, etc.[9]) was not as straightforward as with *src*, due to the fact that the other transforming retroviruses were replication-defective and many lacked conditional mutants for genetic analyses. Besides the above-mentioned approaches on the polypeptide level, i.e., cell-free translation of viral RNA and the use of antiserum to virally-induced tumors, a combination of nucleic acid hybridization techniques (including recombinant DNA and heteroduplex analyses as detailed below) helped resolve the location of these other oncogenes on their viral genomes. Such transforming sequences could be assayed for biological activity by the DNA transfection assay (see below). Furthermore, hybridization of these transformation-specific viral oncogene sequences to cellular genomic DNA of a wide variety of species detected cellular genes whose polypeptide products are expressed in cells from many species. Details of the relationship between viral oncogenes and their related cellular genes will be detailed below (see "Proto-oncogenes"). As a result of a combination of the above approaches, a total of sixteen distinct viral oncogenes have been identified to date. Some of the viral oncogenes have been recognized as a result of a single transforming retrovirus isolate from a single species, while others represent multiple isolates. For instance, *ras*^{H} (sometimes referred to as Harvey *ras* or *has*) is represented by two independent isolations from rat and one from mouse.[10] The *myc* oncogene has been found in four independent avian isolates.[11] Most interestingly, the same viral oncogene has been identified across class barriers among three avian (*fsv*) isolates and two feline (*fes*) isolates.[12] Among viral oncogenes, two have been identified as members of a gene (*ras*) family, *ras*^{H} and *ras*^{K} (also referred to as Kirsten *ras* or *kis*). While their nucleotide coding sequences are only 75% homologous, their polypeptide products share 85% of their amino acids, and both are approximately 21,000 daltons in molecular weight, serologically cross-reactive, and functionally related.[13] As more viral oncogenes have continued to be defined, certain general structural principles of

their organization have been noted. Some oncogenes, such as src and ras, encode polypeptides whose sequence lacks viral structural components. Such onc proteins, which are totally unrelated to virion proteins, are representative of type I oncogenes. Other onc proteins contain sequences derived partially from viral structural proteins and are representative of type II oncogenes, or "fusion" proteins of the general structural gag-X.[14] The contribution of the gag genetic information to the function of such onc proteins is unknown.

Onc Protein Biochemistry - In light of the ability of onc proteins to mediate cellular transformation, these polypeptides have been the object of intense study. Since RSV was the first transforming retrovirus whose oncogene was identified and since ts mutants of src were available, pp60^{src} has been by far the most widely studied onc protein. Analysis of phosphoamino acids by thin-layer chromatography demonstrated that the polypeptide contained one major phosphoserine residue and one major phosphotyrosine residue.[15,16] The latter residue is very striking, since phosphorylation at this residue appears to correlate with the transforming phenotype of the protein: pp60^{src} in cells infected by ts RSV is not phosphorylated at tyrosine at the non-permissive temperature but is phosphorylated at the permissive temperature.[17] This tyrosine phosphorylation is mediated by a cyclic AMP-independent protein kinase. In contrast, the serine phosphorylation is mediated by a cyclic AMP-dependent protein kinase; this may be significant, since the substrates of the cyclic AMP-dependent protein kinases often are regulated by their phosphorylation state. In immunoprecipitates, pp60^{src} was found to phosphorylate the heavy chain of IgG on a tyrosine residue.[16] That this enzymatic function is specific to pp60^{src} rather than a copurifying contaminant was demonstrated by recombinant DNA technology: E. coli were engineering to produce pp60^{src}, and this pp60^{src}, free of all eucaryotic proteins, was capable of phosphorylating IgG at tyrosine.[18] Additional in vitro and in vivo data have suggested that pp60^{src} possesses autophosphorylating activity at the tyrosine residue. The ts RSV has been used to try to identify natural cellular targets for pp60^{src}. The analysis of phosphoamino acid content of cells has shown that transformation by RSV is associated with a 10-fold elevation in phosphotyrosine levels, suggesting that tyrosine phosphorylation activity is critical to the transforming function of pp60^{src}. Following a shift from nonpermissive to permissive temperature, a 35,000 dalton protein was found to be phosphorylated on a tyrosine residue in mutant-infected cells.[19] Furthermore, the purified nonphosphorylated form of this protein could be phosphorylated in vitro by pp60^{src} at the same tyrosine residue. Similarly, vinculin has been suggested to be a possible substrate of pp60^{src} in vivo. This is provocative, since vinculin is associated with cellular adhesion plaques, and pp60^{src} has been localized to adhesion plaques by immunoflorescence analysis.[20]

With the precedent set by pp60^{src}, other onc proteins have been tested for protein kinase and autophosphorylation activity.[7,21] Several onc proteins have been shown to be tyrosine kinases, while other onc proteins lack such activity. The specific onc proteins themselves are phosphorylated on different amino acids, including serine and threonine. Type II fusion proteins can be phosphorylated in either their gag or their onc sequences. The ras^{H} and ras^{K} p21 molecules, lacking tyrosine kinase activity, possess auto-phosphorylating activity specific for a single threonine residue at protein 59. Moreover, unlike other onc proteins which utilize ATP as a phosphate donor, p21^{ras} specifically binds guanine-containing nucleotides as phosphate donors.[22] The fact that this guanine nucleotide binding activity is unique to p21^{ras} among viral onc proteins suggests that ras may have a mechanism of action distinct from other oncogenes. In this regard, the association of ras gene expression with certain human tumors is

provocative (see "Activated Oncogenes").

PROTO-ONCOGENES

The observation that the passaging of slowly leukemogenic viruses through rodents sometimes gave rise to acutely malignant viruses was the first direct experimental indication that normal cells might harbor genetic information capable of causing malignant transformation, as hypothesized by several workers.[23,24] It appeared that these usually slow-acting leukemia viruses had transduced genetic information from the host animal, thereby enabling the viruses to become acutely transforming. It is now known that the onc-specific sequences of acutely oncogenic retroviruses do have cellular homologues, termed proto-oncogenes, from which they were derived.[2,4,6,10,14,21]

Methodologies - Two highly relevant methodologies became available during this period of investigation into the cellular origin of viral oncogenes. The first involved the use of restriction endonucleases with recombinant DNA cloning technology. Using these enzymes, it became possible to cut DNA molecules at specific sites into fragments of defined size. These fragments could be electrophoresed in agarose gels, visualized by ethidium bromide staining, transferred onto nitrocellulose paper, and hybridized to radiolabeled nucleic acid probes of known specificity.[25,26] Newly defined DNA fragments, whose length could be quantitated using size markers in gels, then could be cloned into bacteriophage or plasmid vectors which enable the amplification of the DNA fragments in bacteria and the isolation of large quantities of homogeneous DNA. Furthermore, the presence of defined restriction endonuclease sites in the cloned DNA permits the easy isolation and manipulation of specific fragments of the clone.[27] The second important methodology was the calcium phosphate precipitation technique of Graham and van der Eb.[28] This procedure, also termed DNA transfection or gene transfer, involves exposure of recipient cell cultures, such as NIH3T3, to donor DNA. This DNA, which is presented to the cells as a calcium phosphate precipitate, can be large fragments of cellular DNA or small, defined restriction endonuclease fragments of viral DNA. This ensures that any phenotypic change observed is genetic in origin, i.e., attributable to the polypeptide products encoded by the transfected DNA. NIH3T3 cells are a flat, non-neoplastic mouse cell line which is able to stably integrate donor DNA with a relatively high efficiency. The integration of donor DNA is assayed efficiently by nucleic acid hybridization techniques. In the case of oncogene (viral or cellular) transfection, scoring for foci or transformed cells is relatively easy on the mouse monolayer background and quantitative (with single-hit kinetics) with respect to the DNA inoculum.[29] Several methodologies which became essential to the defining of cellular oncogenes initially were applied to viral oncogenes, as discussed above. Restriction endonuclease mapping coupled with DNA transfection and focus assays allowed delineation of the functional onc gene regions of these viruses. This was done by taking differently-sized restriction endonuclease fragments (spanning the cloned viral genome) and transfecting them onto NIH3T3 cells to assay for focus formation.[3,5,29] In addition, heteroduplexing studies gave further physical delineation of the onc gene region. This technique involves nucleic acid hybridization between two molecules and electron microscopic visualization of the homologous regions.[30]

Discovery of Cellular Genes - The identification of the src gene region of RSV provided the first tool to explore the origin and distribution of this transforming sequence in normal vertebrate cells. Using uncloned src-specific DNA as a radiolabeled probe, molecular hybridizations were performed

against DNA and RNA prepared from a variety of normal uninfected vertebrate cells.[31] This experiment gave the initial evidence that there were actively transcribed cellular sequences, highly conserved in vertebrates, which were homologous to the viral src gene. The identification of RNA transcripts homologous to the src gene and the subsequent identification of a 60,000 dalton protein in normal cells closely homologous by size, enzymatic function, and peptide composition to the viral pp60^{src} gave further support to the cellular origin of this gene.[32] Similar molecular hybridization studies were performed with other isolated viral oncogene sequences to determine their existence and distribution as proto-oncogenes in normal cellular DNA. It now is apparent that these proto-oncogenes are highly conserved evolutionarily.[2,6,10,21] Many have been found to be transcribed actively in normal cells, during certain stages of mouse enbryogenesis, and preferentially in particular tissues.[33] In many cases, a protein product closely homologous to the viral oncogene product has been identified. All of this taken together would suggest that these proto-oncogenes play some essential role in cellular metabolism or differentiation.[34]

Cloning of Proto-oncogenes - Subsequent to the identification of these proto-oncogenes in normal cells, many groups began screening recombinant DNA libraries of different species. A recombinant library consists of fragments of DNA, representative of the entire genome of the organism used, cloned into bacterial vectors.[26] By using viral oncogenes as probes, it was possible to isolate individual clones which represented their cellular proto-oncogene homologs. The first proto-oncogenes to be cloned were homologous to the viral (v-) oncogenes of two rodent retroviruses, v-mos[35] and v-ras.[36] The normal mouse cellular gene equivalent to v-mos, called c (cellular)-mos, was the first proto-oncogene to be cloned and shown to have biological activity. C-mos was shown to be colinear with v-mos by restriction endonuclease and heteroduplexing analyses. Proof of biological activity was accomplished by transfection studies using NIH3T3 cells. The c-mos clone alone did not give foci when transfected. However, ligation of an LTR, the potent viral promotor, to the c-mos clone resulted in transformation following transfection.[37] Neither c-mos nor c-ras^{H} was found to be flanked by retroviral sequences. These findings corroborated results observed in the RSV system which provided firm evidence that the oncogene of acutely transforming retroviruses did arise by recombinational events with normal cellular sequences: infection of chickens with td RSV resulted in the isolation of transforming RSV as a result of recombination with the chicken c-src gene.[38] The mos system has suffered, until very recently, from the lack of an identifiable viral onc protein product. Furthermore, c-mos transcription in vivo has never been observed. Consequently, investigations into c-mos biology have been impossible. This has not been the case with the second cloned proto-oncogene, c-ras^{H}, whose p21^{ras} gene product is found to be expressed at low levels in normal cells.[10] The cloning of the c-ras^{H} gene provided the first example for the existence of interrupted proto-oncogenes. Heteroduplexing studies combined with restriction enzyme mapping showed c-ras^{H} to have 3 regions of noncoding information (introns) interrupting the coding sequences (exons) and to be disposed over 2 kb of cellular DNA.[36] Other c-onc genes have several exons which are disposed over as much as 25 kb.[2] Similarly to c-mos, transfection of the c-ras^{H} alone failed to give foci. However, ligation of the viral LTR to the cellular clone resulted in transformation upon transfection. The transformed cells showed elevated levels of a 21,000 dalton protein which was homologous to p21^{ras}. In addition, there were elevated levels of an mRNA of a size sufficient to encode such a protein. This was the first example of a spliced cellular onc gene shown capable of being activated in vitro. In addition, the activation of c-ras showed definitively for the first time that elevated levels of a normally

expressed cellular protein can cause transformation. These experiments indicated that quantitative changes in the expression of a cellular gene could be involved in neoplasia.[36]

ACTIVATED ONCOGENES

Neoplasms and Oncogenes - As discussed above, carcinogenesis has been hypothesized to be a result of dominant genetic alteration. The finding that elevated expression levels of normal cellular proto-oncogenes are sufficient to cause transformation represents a quantitative model for such an alteration. In search of a qualitative genetic alteration, the DNA transfection assay has been the cornerstone for testing the nature of potential genetic changes in tumors. Several workers reasoned that if neoplasia were the result of dominant genetic changes involving potential cellular cancer genes, then it should be possible to induce transformation of normal (NIH3T3) cells by transfection of tumor cell DNA. Prototype experiments indicated that while transfection of high molecular weight (30 kb) DNA from normal cells did not induce cellular transformation, fragmentation of these same DNA's to smaller (1-5 kb) sizes did induce transformation. This suggested that potential transforming genes of normal cells could become activated to oncogenes when dissociated from their surrounding regulatory sequences.[34] The first case in which it was shown that the DNA of some neoplasms contained activated oncogenes involved the transfection of DNA derived from 3-methylcholanthrene transformed mouse cells.[39] Transfection of this DNA induced foci, while normal mouse cellular DNA did not induce foci. This result quickly was followed by similar findings utilizing DNA from either chemically-induced or spontaneously occurring tumors from a variety of species, whose normal cellular DNA did not induce foci. Activated oncogenes now have been detected by transfection of DNA from chicken B-cell lymphoma and nephroblastoma, rodent neuroblastoma and glioma, human promyelocytic leukemia, and human bladder, colon, pancreas, gall bladder, lung and mammary carcinoma.[34] Secondary transfection assays, utilizing the primary transformed cells as a source for donor DNA gave similar efficiencies of transformation (0.05-1.0 transformants per microgram DNA), as expected for serial transfer of a transforming gene. It was possible to further analyze these oncogenes by determining the sensitivity of their transforming capabilities to digestion with different restriction endonucleases. By use of this phenotypic analysis of the DNA, it was possible to determine whether the same enzyme abrogated the biological activity of the activated oncogenes from different tumors.[34,39]

Ras Genes - The first breakthrough in identifying these transforming sequences occurred with the molecular cloning and isolation of discrete transforming sequences from a human bladder carcinoma cell line.[40] This was achieved by the serial transfer of transforming sequences in transfection assays and the monitoring for the presence of the responsible human sequences in the transformed NIH3T3 cellular DNA. This monitoring utilized a radiolabelled probe made to cloned human repetitive DNA to demonstrate the presence of discrete human sequences in these transformed cells. In addition to showing that the transformation event was mediated by human DNA, this also allowed the identification and subsequent cloning of the human sequences. Nucleic acid hybridization demonstrated that these transforming sequences were present in normal human cells. It naturally followed that a comparison could be made between this gene and the available cloned proto-oncogenes. This analysis demonstrated that the bladder oncogene was homologous to the human c-*ras*^{H} proto-oncogene.[41] At the same time as these experiments with the human bladder carcinoma were occurring, similar experiments were being performed utilizing DNA from

human lung and colon carcinomas. The activated oncogenes in both these human cancers not only were similar to each other by restriction enzyme inactivation analysis, but were also homologous to the second member of the ras gene family, c-ras^{K}.[42] The c-ras^{K} gene recently has been found to be activated in several other solid tumors, including those of the gall bladder, pancreas, and bladder.[43] These coincident findings unified for the first time the potential role of proto-oncogenes and dominant genetic changes in human oncogenesis.

The bladder oncogene/c-ras^{H} homology has been the focal point for the determination of the nature of the DNA changes which relate to the activation event. Just prior to the isolation of the bladder oncogene, the human equivalent to c-ras^{H} had been cloned.[44] This made possible a detailed comparison between the two genes. The ras gene family, as has been discussed more fully in earlier sections, encodes a phosphoprotein of 21,000 daltons termed p21^{ras} (the cellular p21^{ras} is not phosphorylated).[45] Initial studies of the NIH3T3 cells transformed by the bladder oncogene revealed an interesting result: it was discovered that there was only a small (less than three-fold) increase in ras-specific mRNA relative to normal cells.[41] A similar finding resulted from protein studies using monoclonal antibodies to p21^{ras}. However, a comparison of p21^{ras} immunoprecipitates from normal and bladder gene transformed NIH3T3 cells revealed a difference in the electrophoretic migration of the proteins in SDS-gel electrophoresis. These results indicated that a qualitative change had occurred in the protein concomitant with oncogene activation. Genetic localization of the region which was responsible for the genetic alteration, followed by DNA sequencing of this region in the cloned bladder oncogene and human c-ras^{H} gene, delineated the change at the nucleotide and amino acid level. It was found that a single base change (a point mutation from G to T) corresponding to amino acid residue 12 converted the normally present glycine residue to a valine residue, thus affecting the function and structure of p21^{ras}.[46,47] This finding of a qualitative change in p21^{ras} being responsible for its transforming capability, along with the previously described finding (see "Proto-oncogenes") that increased levels of expression of c-ras^{H} are sufficient to cause transformation, strongly suggests that, at least for the ras^{H} gene, there are two independent mechanisms which can be operative in causing transformation. In this regard, it is noteworthy that the p21^{ras} encoded by the activated ras^{K} gene from human colon or lung tumors demonstrates an altered electrophoretic mobility suggestive of a similar qualitative change.[42]

Other Activated Oncogenes - Efforts to elucidate the function of cellular oncogenes are of primary importance to understanding cancer. To this end, the identification and analysis of proteins associated with these genes is an area of intensive investigation. The use of sera from tumor-bearing animals in immunoprecipitation assays has allowed the detection of some transformation-associated proteins in these tumors.[34] In addition to p21^{ras}, specific proteins have been found associated with the activation of the mammary carcinoma transforming gene (86,000 daltons)[48] and of the rat neuroblastoma and glioma transforming genes (185,000 daltons).[49] A tumor-specific transplantation antigen has been detected in association with the transforming gene of a chemically-induced mouse sarcoma.[50] The continued investigations into various transforming genes of different tumors has led to some general conclusions. It is apparent that different transforming genes are activated in different types of tumors. However, it also is apparent that the same transforming gene, e.g., c-ras^{K}, can be activated in independent tumors of the same differentiated cell type. Implicit in these observations is the fact that these transforming genes apparently can become activated across species and tissue boundaries. It

should be noted at this point that only 50% of the tumors so far tested have activated oncogenes.[34] The reasons for this are unclear. However, one possibility is that these tumors contain transforming genes of a type that are unable to induce transformation of NIH3T3 cells, either because of changes these heteroploid cells have already undergone, or because of a specificity for transformation by these genes for a particular cell type, which NIH3T3 cells do not represent. Investigations into alternative assaying systems, to be used alone or in conjunction with the NIH3T3 transfection system, are in progress. Since positive assay results have been obtained, two systems worth mentioning are the Chinese hamster embryo fibroblast system and the athymic nude mouse.[51,52]

Virally-induced Oncogenes - The above-described tumors in which activated oncogenes have been detected presumably were spontaneous or chemical in origin. In addition, it has been found that there are activated oncogenes in tumors induced by weakly oncogenic, nontransforming retroviruses, including avian leukosis virus, murine leukemia virus, and murine mammary tumor virus.[4] The long latent period of tumors induced by these viruses suggests an indirect mechanism of leukemogenesis, unlike that of transforming retroviruses and their direct viral oncogene mechanism.[2] This indirect mechanism was substantiated by nucleic acid hybridization experiments. The DNA in the NIH3T3 cells transformed by the virally-activated oncogenes was hybridized to a radiolabelled probe of the retrovirus which induced the tumor. In no case was hybridization detected in any of the transformed cell lines.[53] The molecular biology of retrovirus infection was hypothesized to bear upon oncogene activation. As mentioned above, the integrated retrovirus genome is flanked by an LTR at both its 5' and 3' terminus. The 5' LTR provides the promoter function while the 3' LTR provides the mRNA polyadenylation signal. Given the duplicated structure of the two LTRs, the 3' LTR could provide promoter signals for adjacent cellular sequences.[3,4] This hypothesis was demonstrated by the ability of a radiolabelled LTR probe to detect novel nonviral mRNA transcripts in retrovirus-induced tumors.[54]

The most closely studied system of virally-induced tumors is the chicken bursal lymphoma induction by avian leukosis virus. The viral genome has been found to integrate in the vicinity of the avian *myc* oncogene.[55,56] Moreover, high levels of expression of *myc* mRNA have been found in many of these virally-induced tumors. Many tumors contained viral genomes either 5' or 3' to *myc* and in either of the two transcriptional orientations, suggesting an indirect effect of the viral LTR upon *myc* transcription. When DNA from these virally-induced lymphomas was transfected onto NIH3T3 cells, it was found that the activated oncogene in the transformed cells was not *myc*.[57] These data suggest that there is a mechanism of at least two steps operative in these virally-induced tumors: 1) viral integration directly or indirectly leads to very high levels of *myc* transcription, and 2) a distinct oncogene becomes activated, perhaps as a result of *myc* transcription. Since the development of cancer seems to be a multistage process, the virally-induced leukemia model merits further study. This indirect mechanism of oncogenesis may be useful for understanding the sequential steps of gene expression in tumor development.

Conclusion - The manner in which normal cellular genes become activated oncogenes is an area of great importance, since there is finally the potential to draw together the areas of chemical carcinogenesis and oncogenes. It has been known for a long time that chemical carcinogens act as chemical mutagens for DNA. The finding of a point mutation in the bladder oncogene which results in its acquiring transforming activity correlates well with such a pathway. In addition, some chemical carcinogens have been associated

with DNA rearrangements such as deletions and translocation. Studies have shown that the myc gene is associated with translocated chromosomes, an abnormality often found in human cancer cells.[58,59,60] Furthermore, the myc mRNA in these cells can be quantitatively or qualitatively altered relative to myc mRNA in normal cells. The abl oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukemia.[61] Such relocating of proto-oncogenes may be the signal which allows an alteration in their expression. In addition to the possibility of translocations resulting from chemical carcinogens, there are recent data implicating the myc gene as being involved in a translocation event that is a consequence of normal antibody gene rearrangements.[62] Therefore, it appears that cellular oncogenes can be involved in several different processes which could affect their expression. A major reservation which one must have in mind when interpreting these data is that the biological activity of oncogenes may be a consequence, rather than a cause, of tumorigenesis. Nevertheless, oncogenes represent some of the most promising molecular markers for cellular transformation. The fact that specific oncogenes are activated in lymphoid tumors of distinct stages of differentiation and that the same oncogene can be activated in independent tumors of the same histology argue for specificity in the mechanism of action of oncogenes.

In conclusion, several experiments in the last two years have provided the first connection between oncogenes and human cancer. The findings of ras gene activation and of myc and abl gene translocation represent two of the most promising leads in this regard. Future work in this field should address some of the following questions:

1) Are additional proto-oncogenes activated beside ras? Can other assay systems be developed for detecting activation?
2) Aside from proto-oncogenes, what other types of genes have been detected as activated genes?
3) What is the mechanism of action of onc proteins? With which cellular targets do these proteins interact?
4) What are the molecular events associated with oncogene activation (particularly of ras^K)?
5) How does myc and abl gene expression change concomitant with translocation?

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Chapter 24. In Vitro Mutagenesis: Powerful New Techniques for Studying Structure-Function Relationships in Proteins

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Introduction - The linear sequence of amino acids uniquely determines the exquisite three-dimensional structures of proteins and their wide diversity of biological function--as catalysts, hormones, transport agents, cell surface receptors, structural elements, transducers of chemical energy into work. To be able to generate, at will, any sequence of amino acids will therefore allow rational study of the relation between protein structure and function. This objective has become possible only recently through development of reliable methods for chemical synthesis of oligodeoxynucleotides and DNA sequence analysis; these advances, along with rapidly developing cloning technology, have made possible a large variety of genetic manipulations that alter the structures of genes and of gene products. Not only do these techniques now allow one to generate essentially any variant of a protein to study how three dimensional structure and biochemical function depend on amino acid sequence, they further ensure that, once the DNA of the structural gene has been prepared, the protein can be produced ever after in any quantities desired.

In vitro mutagenesis of a cloned structural gene of a protein can introduce deletions, insertions and single or multiple amino acid substitutions either randomly within a pre-selected region or at a single, specific site. In addition to these mutagenic techniques, the in vitro synthesis of entire structural genes has been accomplished.[1-4] Together, these techniques now allow the relatively rapid construction of a protein with any predetermined amino acid sequence. In short, one can specify a particular structure and determine its function. Analogous approaches have, over the years, greatly enriched our resources of useful pharmaceuticals. The procedures characteristic of this approach are those of site-directed, specific mutagenesis.

For structure-function studies of proteins, a second powerfully complementary approach should also be emphasized. This approach takes advantage of the ability of biological systems to produce a very large number of random structural variants; these can then be screened for those variants that have a particular function. In this case, one specifies a particular function and then determines which structures, of many millions that can be easily tested, have that function. The procedures useful in this approach are those of random mutagenesis, perhaps directed toward a particular region of the protein.

This review will focus particularly on those aspects of mutagenesis that have a direct bearing on the study of protein function. These techniques have also been used to address a large variety of genetic problems as well as to probe structure-function relationships; the more general subject has been reviewed extensively.[5-8]

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Amino Acid Substitutions - The roles of particular amino acid residues in catalysis, ligand binding, folding and in determining other properties of a protein can be investigated by the study of the effects on these properties of both specific and non-specific substitutions. Mutant proteins can be generated by methods that fall into four major categories: (a) deliberate, site-specific substitutions, (b) segment directed, random mutagenesis, (c) non-directed, random mutagenesis, and (d) suppression of nonsense codons generated by an appropriate mutagenic procedure.

a. Site Specific Mutagenesis - Unique, predetermined base changes at any desired specific site in a structural gene, leading to any desired amino acid substitution in the corresponding protein, can be readily accomplished by oligonucleotide-directed mutagenesis; this is the most specific and generally applicable form of site-directed mutagenesis. First developed in the single stranded phage $\phi\chi$ 174,[9,10] this method has since been used to produce several point mutations in this phage,[11-15] as well as in M13[16] and genes cloned into M13.[17-19] The technique has been extended also to double-stranded circular DNAs.[20-22]

As outlined in Figure 1, this method involves priming in vitro DNA replication with a chemically synthesized oligodeoxynucleotide that has been designated to have a sequence that is largely complementary to the "wild-type" DNA template in the region where the mutation(s) is(are) to be introduced. The synthetic nucleotide contains, however, one, or more,

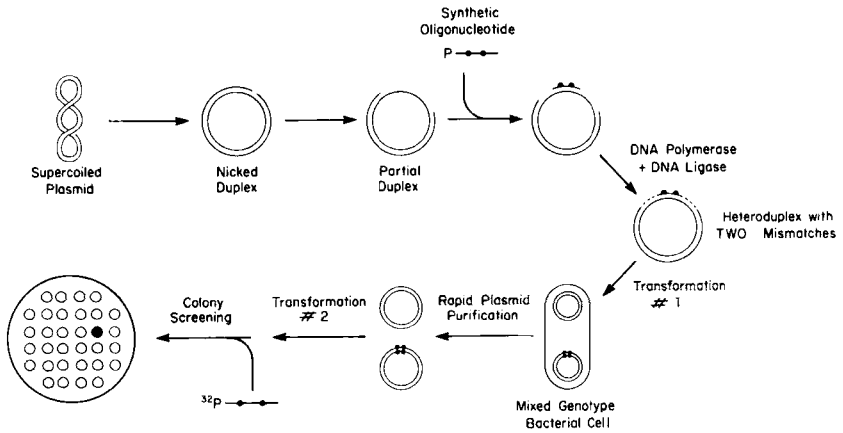

FIGURE 1: Overall scheme for oligonucleotide-directed mutagenesis of double stranded circular plasmid DNA. Supercoiled plasmid circles are nicked in one strand and rendered partially single stranded by treatment with exonuclease. The gapped circles are hybridized with a homologous oligodeoxynucleotide carrying, by design, some mismatches. In vitro DNA synthesis, primed in part by the oligodeoxynucleotide, leads to heteroduplex plasmid circles. Molecular cloning and in vivo DNA replication generates homoduplexes, some of which have the DNA sequence of the primer oligodeoxynucleotide. Colony screening, with the same oligodeoxynucleotide labeled with ^{32}P as a hybridization probe, allows identification of the desired mutant colony regardless of its phenotype.

bases that are not complementary to the wild-type template; the mutation originates from these mismatched bases. Annealing of the synthetic oligonucleotide to the wild-type circular DNA, which must of course be at least

partially single stranded, can be accomplished under conditions of sufficiently low strigency that a heteroduplex forms, in spite of some mismatching of bases. The site-specificity of this duplex formation is, nevertheless, exceedingly high. For example, in a 15 base oligodeoxynucleotide there will normally be 13-14 complementary base pairings and 1-2 mismatches at the site where the mutation is being introduced. Binding of the synthetic oligonucleotide to the template DNA anywhere else than at the desired site will generally involve no more than 9-10 pairings by design. The heteroduplex region thus formed now serves as an initiation site for in vitro DNA synthesis that occurs in the presence of the four deoxynucleotide triphosphates and is catalyzed by the Klenow fragment of DNA polymerase I (which requires a double stranded region to begin DNA replication). After ligation, the heteroduplex is introduced into an appropriate bacterial host and multiplies as a result of normal semiconservative, in vivo replication into two types of homoduplexes; the mutant homoduplexes have a sequence that reflects the synthetic, mutagenic oligonucleotide; the other homoduplexes have the original, wild-type sequence.

An essential feature of any procedure for producing specific mutants is the necessity of identifying mutant colonies in a background of wild-type colonies. To be generally applicable, the procedure should allow identification of mutants at the level of DNA and not rely on some phenotypic difference between mutant and wild-type that may be difficult to predict and to assay. In this regard, oligonucleotide mutagenesis has the distinct advantage that mutants can be found by screening bacterial colonies with the same synthetic oligonucleotide, now labeled with ^{32}P, that was originally used to introduce the mutation.[19-22] In this case, the hybridization is carried out under conditions of high stringency such that even a single base mismatch between oligonucleotide probe and template can be readily distinguished from perfectly matching hybridization. This allows clear discrimination between wild-type and mutant colonies of bacteria randomly spread on a Petri dish.[21,23]

We have recently demonstrated that these procedures for oligonucleotide directed mutagenesis applied to the structural gene of an expressed protein can be used to produce mutant proteins with amino acid substitutions introduced deliberately and precisely, and we have shown that these sequence changes can be generated with a negligible background of non-specific mutations.[20] Both single and double base changes, leading to single and double amino acid substitutions, were created in the structural gene for β-lactamase in the double stranded circular plasmid pBR322. Specifically, synthetic 15 and 16 base oligomers, respectively, were used to mutate the wild-type sequence, 5'-AGC ACT-3' (-ser70 thr71-) to 5'-A$\overset{*}{C}\overset{*}{C}$ T$\overset{*}{C}$T-3'; (-t$\overset{*}{h}$r s$\overset{*}{e}$r-)(a double base, and double amino acid mutation) and to 5'-A$\overset{*}{C}$C ACT-3': (t$\overset{*}{h}$r thr)(a single base, single amino acid mutation).[21] Both of these mutants are catalytically inactive. (The serine residue at position 70 is known to be involved in catalysis[24-29] and all lactamases of this family have a conserved ser-thr dyad at residues 70-71.[29]) In these cases mutants were recovered at the level of several mutants per thousand colonies. Though seemingly a low yield, this represents a few mutant colonies on each Petri dish and oligonucleotide screening at the DNA level is sufficiently rapid and reliable that at least several mutants can be easily recovered from each successful mutagenesis. (We have also obtained the fourth possible combination of serine-threonine residues in this region, (-ser70-ser71-); this protein shows a significant level of catalytic activity and was obtained by spontaneous mutation as discussed later.)

β-Lactamase has recently proved a popular subject for studies of mutagenesis. In an elegant use of oligonucleotide directed mutagenesis, a primer was used to create a ser→arg substitution at the second position in the leader sequence.[30] In this "piggyback" procedure, the original template had an ochre codon TAA at residue 4; the oligonucleotide primer corrected this sequence to CAA (glu), thereby allowing protein expression, and also introduced the mutation of ser→arg at the nearby site; at the same time a new Eco RI site was introduced in this region which facilitated screening for mutants. Another mutant of β-lactamase, thiolactamase, with a cysteine residue at position 70 was prepared by a specific reconstruction of the DNA sequence.[31] This approach depended on the location of a restriction site in this region of the genome. In fact, the ser 70→cys mutant has lost this restriction site, which was useful in identifying mutants. The thiolactamase shows catalytic activity.

As noted earlier, single-strand templates serve very effectively as subjects for oligonucleotide directed mutagenesis, and generally give a higher yield of mutants than do double-stranded plasmids. This approach has been used in creating a substitution mutant, with cysteine in place of serine, in the ATP binding site of tyrosyl tRNA synthetase.[19] This substitution was achieved by mutating the structural gene attached to M13 and was accompanied by an unusually efficient protein synthesis allowing easy isolation of the mutant protein. The major effect of the ser→cys substitution was manifest in Km for binding ATP which was raised from 0.9 to 4.1 mM; Vmax was reduced from 1.4 to 0.9 sec^{-1}.

Oligonucleotide directed mutagenesis has also been used to study[22] the role of positively charged amino acids in the amino-terminal region of the outer membrane prolipoprotein of E. coli. Substitutions that reduce or eliminate the positive character of the amino terminus of the signal peptide substantially alter the processing and secretion of the prolipoprotein.

b. Segment Directed Mutagenesis - The techniques so far described focus on the creation of a mutant protein with a predetermined structure; such approaches are useful to learn the properties and functions of a protein with a prespecified structure. An alternate approach is to generate, by nonspecific mutagenesis, a large number of structural variants, to select those with particular properties and thus to determine the structures of those variants that manifest the desired properties. Such random mutagenesis can be allowed to take place anywhere in the structural gene of interest or, to focus more closely on the role of a particular domain of the protein, can be restricted to a region, such as the leader sequence or a loop of a catalytic domain.

Several methods are available to restrict the action of a non-specific mutagen to a particular segment of DNA. In general they depend on the in vitro generation of a single stranded gap or loop in the desired region of a double stranded DNA molecule, followed by a mutagenesis technique that operates only on single stranded DNA.

i. Deletions Target a Region of DNA for Mutagenesis - To target a particular region of DNA for subsequent mutagenesis, deletion mutants have proved useful; they are relatively easy to obtain and have been extensively used in diverse genetic studies. In deletion loop mutagenesis[32,33] the wild-type DNA and the deletion mutant DNA, both cloned in a bacterial plasmid in the same manner, are used to form circular heteroduplexes that contain a looped-out single stranded region of DNA from the wild-type

strand; this looped-out single stranded region corresponds to the segment that was absent in the deletion mutant (see Fig. 2a).

A gapped heteroduplex can also be constructed by annealing two linear molecules derived from the same plasmid if the shorter of the two is open at the site of the deletion.[34] Again, the deletion defines the region of DNA to be mutagenized (see Fig. 2b).

ii. Specific Nicks Target Regions of DNA for Mutagenesis - In a related approach, single strand gaps can be introduced into a double stranded DNA molecule (see Fig. 2c). In one procedure, the site is specified by a "nick"[35,36] introduced at a site recognized by a specific restriction endonuclease.[37] An alternate approach used a single strand of a suitable restriction fragment in the presence of Rec A protein to generate a displacement loop that is then susceptible to the action of S1 (single strand specific) nuclease[38] (see Fig. 2d). The "nick" introduced by these procedures can now be converted to short, single stranded gaps by limited

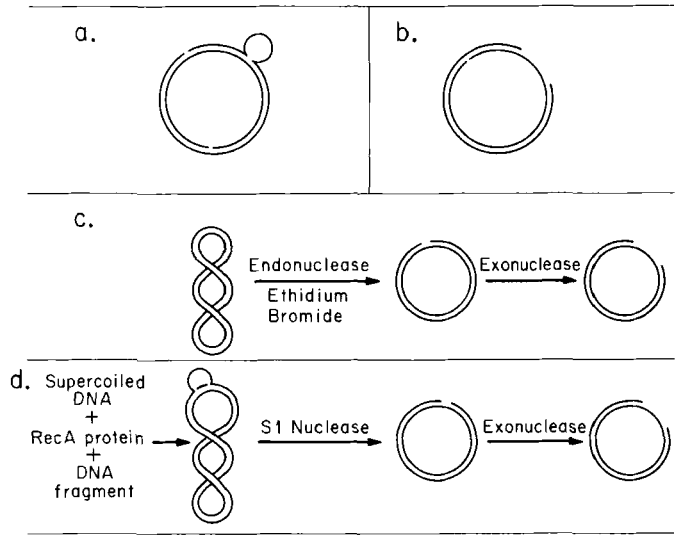

FIGURE 2: Specific segments of DNA can be made single stranded in various ways. These single stranded regions then can be subjected to the action of a single strand specific mutagen (a,b,c,d) or used to introduce mutations during in vitro enzymatic DNA synthesis (b,c,d).

a) Deletion loop

b) Deletion gap

c) Nicking at a specific site followed by gap expansion.

d) Nicking in a specific region followed by gap expansion.

digestion with an exonuclease;[38] alternately, specifically nicked molecules can serve as substrates for nick translation.[39]

After formation of single stranded gaps by the types of methods just discussed, a variety of techniques are available to mutagenize the single stranded regions. For example treatment with sodium bisulfite selectively converts cytosine to uracil residues in the single stranded DNA but leaves intact cytosine residues in the double stranded DNA. This procedure generates single or multiple G-C to A-T transitions.

Procedures are also available for misincorporation of non-complementary nucleotides during enzymatic repair of the single stranded region.[40,41] For example, an error prone DNA polymerase has been used[41] to incorporate mismatched nucleotides at a specific site on the $\phi X174$ genome. The site on the single strand phage template was specified by a restriction fragment used to prime *in vitro* DNA synthesis. When the DNA sequence in the region of interest is known, as in this case, base substitutions can be introduced at a particular site; the method can also be used to generate a "library" of mutations directed to a given region of the DNA template.

One can also achieve misincorporation simply by omitting one of the four deoxynucleotide triphosphates normally present during enzymatic gap repair; under certain conditions, misincorporation of the three remaining nucleotides occurs at roughly equal frequency. Use of excess DNA ligase traps the misincorporated base.[40]

α-Thionucleotides[42,43] have been shown to be incorporated into DNA in lieu of the normal nucleotides by DNA polymerase I of *E. coli*.[44] These thionucleotide analogues prevent normal proofreading during *in vitro* DNA synthesis with both *E. coli* DNA polymerase I and T4 DNA polymerase;[45] they also prevent digestion by *E. coli* exonuclease III.[46] These analogues have also been used for misincorporation of non-complementary, excision resistant bases into circular DNA molecules that contained single stranded gaps in specific regions.

Both types of misincorporation reactions are reported to be efficient. The nucleotide omission method, however, produces substitutions that are not predictable *a priori* and may introduce multiple base substitutions when the single stranded segment of DNA being "misrepaired" is longer than a few bases.[40]

Again, β-lactamase has served as the subject of many of these approaches. Two different single stranded fragments of the DNA of the β-lactamase gene were used to direct the formation of D-loop structures in the presence of Rec A protein;[38] subsequent digestion with S1 nuclease produced single stranded nicks distributed throughout the length of the single stranded D-loops. The nicks were then enlarged to gaps and these then mutagenized with bisulfite and repaired enzymatically. Mutants were selected by screening for loss of ampicillin resistance; virtually all such mutants have base substitutions in the regions spanned by either one or the other of the two single stranded fragments used to direct mutagenesis; they were a 369 base pair Taq I fragment that includes the initial 135 base pairs (45-N-terminal amino acids) of the β-lactamase structural gene and a 128 base pair PvuI-PstI fragment from the center of the gene.

c. Random Mutagenesis - The mutagenic strategies discussed above can be usefully complemented by approaches involving random mutagenesis. For example, the construction of a catalytically inactive mutant enzyme by site-specific mutagenesis provides a unique opportunity to study the structural requirements for reactivity.[21] Such an inactive mutant, produced for example by a change in one of the residues at the active site that is essential for catalytic activity, can serve as a subject for testing what changes, if any, will lead to a restoration of activity. Such changes could either be at the active site itself or might, more interestingly, occur elsewhere in the protein in a fashion that compensates for the original, inactivating mutation. Such compensatory sequence changes at a secondary site, should they be observed, would provide infor-

mation on residues that can play a key role in catalysis or in protein folding and may be distant, in terms of primary sequence, from the site of the original mutation. (The mutations at positions 211 and 175 of the α subunit of tryptophan synthetase constitute a well-known example of second site reversion: the substitution of glycine 211 by glutamate abolishes catalytic activity, but activity is restored by a tyrosine to cysteine mutation at position 175.)[47]

Such an approach has been applied to the double mutant of β-lactamase (ser 70→thr; thr 71→ser).[21] The normal level of misincorporation of bases, in the absence of techniques to enhance mutagenesis, in the replication of plasmid DNA in E. coli is of the order of $1/10^6$ at any locus. Accordingly, one might expect to recover catalytically active revertants of an inactive double mutant that have changes at a single amino acid residue; changes in two or more sites are very much less likely; thus phenotypic screening for catalytically active revertants is unlikely to be overwhelmed by reversions to the original wild-type enzyme. When applied to the inactive double mutant of β-lactamase, this procedure gave rise to an active revertant (thr71→ser) that had recovered the normal serine residue at 70, apparently essential for catalytic activity, but retained the mutant serine (in place of threonine) at residue 71.

Random point mutations, both spontaneous and induced by mutagenic agents can be useful in identifying sites essential for function, and also for the isolation of large numbers of nonsense mutations as in the case of the lac repressor protein.[48] Random mutagenesis is also well suited to the generation of proteins with altered characteristics when selective pressures are applied to mutagenized populations of cells; this approach produced a mutant β-lactamase with an increased affinity for cephalosporins.[49]

d. Suppression of Nonsense Mutations - Nonsense mutations can be obtained in large numbers at many different sites or at a specific site or region by adaptation for this purpose of any one of a number of mutagenic strategies, including any of those described above. Proteins with altered amino acid sequences can then be obtained when the mutant gene is expressed in an appropriate suppressor strain.[50] In this way a single amber mutant can be used to introduce different amino acid substitutions by using different suppressor strains. As an example of this approach, many mutants of the lac repressor protein have been produced[48-50] and characterized[50,51] yielding important insights into the nature of protein-DNA interactions.

Major Restructuring - Today's ability to manipulate the DNA of structural genes allows one not only to create proteins that are relatively minor variants of the parent; it also allows one to create almost totally redesigned structures. For example, to establish the relationship between structural domains and the contribution of these domains to function, various segments in the structural gene can be progressively deleted. Such an approach has been used[52] to delete from the structural gene for alanyl tRNA synthetase segments that code for the carboxyl terminal region of the protein.

Another approach to the elimination of C terminal regions involved the use of chain terminating mutations;[53,54] the objective in this case was to study the role of the carboxyterminus in the processing and secretion of β-lactamase into the periplasmic space. Drastic alteration in the sequence in a region of a protein, without altering the length of the polypeptide chain, has also been accomplished in this system by the introduction of double frameshift mutations.[55]

Other major restructurings involve use of recombinant techniques to produce fused or hybrid proteins so that, for example, the products of exogenous genes can be expressed, and even sometimes secreted, by bacterial cells. Examples abound, but two involving β-lactamase may serve to illustrate the strategy. In one, the gene for rat proinsulin was fused to the gene for the leader sequence of β-lactamase with consequent transport of the proinsulin into the periplasmic space of E. coli.[56,57] In a second example, the gene for β-lactamase, devoid of its own leader sequence, was fused to the leader sequence of α-amylase from B. amyloliquefasiens;[58] in consequence, β-lactamase from gram negative E. coli was efficiently secreted by gram positive B. subtilis.

Conclusion - As we hope this review demonstrates, many techniques have recently been developed that allow a variety of quite novel approaches to studies of the relationship between the linear sequence and the function of proteins. Much of the discussion has focused on procedures for manipulating the DNA to achieve proteins with altered function. Largely because it is still early days, few specific cases have been discussed in which insights have been gained into the functional roles of particular structural domains, or even single residues of proteins. However, toward solution of these problems, the new methodologies allow, for the first time, a truly systematic approach; their application should now proceed rapidly and allow one to address such intriguing questions as: What are the essential structural features for a protein to function effectively as a catalyst, hormone, or receptor? How are the various structural aspects of an antibody related to its ability to distinguish self from non-self and thereby, for example, to initiate the destruction of infectious agents such as bacteria and viruses, or the altered cells that form tumors? What regions of a protein located on the surface of a cell carry the information essential to the interactions between cells that play a central role in the development from a single cell (the fertilized egg) of a complex organism such as man? From these studies will come striking new insights into the mechanisms by which proteins successfully carry out their myriad functions as well as the ability to design proteins with specific, novel, and useful properties.

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Chapter 25. Early Biochemical Events Leading to Mast Cell and Basophil Degranulation

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Introduction - Immediate hypersensitivity or anaphylactic reactions are caused by the release of vasoactive mediators from mast cells or basophils. Significant progress has been made over the last few years in understanding the early membrane associated immunoglobulin E (IgE) mediated biochemical events that lead to mast cell and basophil degranulation and mediator release. This chapter will review how the binding of IgE to specific receptors triggers a series of biochemical reactions. These reactions represent well-defined biochemical targets for novel anti-allergic drugs. Such drugs, by acting at an early stage of mast cell degranulation, should prevent the release of a wide spectrum of mediators. In contrast, most current available anti-allergic drugs inhibit the activity or the synthesis of a single class of mediators.

Mast Cells, Basophils and Mediators - A number of differences exist between tissue-localized mast cells and circulating basophils. For example, turnover of mast cells is very slow (months to years) whereas that of basophils is much faster (approximately 10 days); recently it has been shown that human mast cells do release and basophils do not release prostaglandin D_2 (PGD_2) and platelet activating factor (L. M. Lichtenstein, person. commun.). However, both cell types contain granules that are released after the cells have been stimulated. A number of mediators are released with the granules (see Table 1) and these have been discussed in detail in a recent review article.[1] Most mediators are preformed and stored in the granules; however, activation of mast cells also induces the synthesis and subsequent release of specific mediators such as prostaglandins and leukotrienes, the oxidative products of arachidonic acid. Heparin is present in mast cells and basophils only,[2,3] whereas prostaglandins[4,5] and leukotrienes[6-8] are released by a number of additional cell types. Histamine, the mediator most commonly associated with allergic reactions, is largely contained within mast cells and basophils, but it is also present in cells without granules.[9,10] Mediators such as histamine or leukotriene C_4 (LTC_4) have an immediate effect; others, such as chemotactic peptides or LTB_4 have a delayed effect presumably because they attract other cell types (polymorphonuclear leukocytes and mononuclear cells) that in turn exert their inflammatory effect.[1]

The origin of human mast cells and basophils is not known. Recent experiments in rats and mice suggest that mast cells are derived from thymus, bone marrow or mesenchymal cells.[11-17] Cell transfer experiments with an inbred strain of mice (W/W^v) that is deficient in mast cells[17,39] will contribute significantly to our understanding of the ontogeny of these cells and their biological functions.

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Table 1[x]

MAST CELL DERIVED MEDIATORS

Preformed, rapidly eluted under physiologic conditions
- Histamine
- Eosinophil chemotactic factors
- Neutrophil chemotactic factors
- Superoxide
- Arylsulfatase A
- Exoglycosidases
- Serotonin

Preformed, firmly associated with the granule under physiologic conditions
- Heparin
- Chymotrypsin/trypsin
- Peroxidase
- Superoxide dismutase
- Arylsulfatase B

Mediators generated as a consequence of mast cell activation/secretion
- Slow-reacting substance (leukotrienes)
- Prostaglandins
- Thromboxanes
- Platelet activating factor

[x]From Reference 1.

It should be kept in mind that degranulation does not necessarily lead to pathological manifestations. Many parasitic infections cause increased IgE synthesis and it is believed that the resulting release of mediators plays a role in eliminating the infection.[18-20] Furthermore, it has been postulated that mediators such as the leukotrienes play an important physiological role in the regulation of the microenvironment.[21]

Degranulation - The role of a serum factor in the activation of granulocytes and the release of granules was recognized early, and subsequent work established this factor to be IgE.[22,23] As will be discussed below in more detail, IgE binds via its Fc fragment to the receptor molecule on the cell surface. Polyvalent antigens bind to this cell-bound IgE and this results in degranulation. Recently, it has been recognized that degranulation in rodents, rabbits and guinea pigs can also be mediated by subclasses of IgG that bind to receptors that are different from those for IgE.[24-28] The evidence for the possible involvement of human IgG in mast cell degranulation is inconclusive.[29,30]

A number of non-immunological compounds also induce degranulation. Many of these such as 48/80[31] and the calcium ionophore A23187[32] are non-physiological. On the other hand, degranulation by anaphylatoxins that are generated during complement activation[33] and low molecular weight cationic polypeptides that are released by polymorphonuclear leukocytes[34] may be physiologically important.

Earlier studies on mast cell degranulation were carried out on mixtures of cells or on whole tissues. In recent experiments using IgE affinity chromatography, it was established that human basophils and lung mast cells can be obtained as highly purified populations.[35,36] However, purification procedures are laborious and the yields are relatively low so that biochemical studies are difficult to pursue. Therefore, most biochemical analyses have been done with pure populations of rodent peritoneal mast cells that can be purified readily or with rat basophil leukemia (RBL) cells. The latter cells grow in tissue culture to high yields, they contain histamine and other mediators and they can be stimulated with IgE and antigen.[37,38] However, it should be kept in mind that RBL cells may represent an artifact and experimental results should be verified against freshly isolated human cells.

Regulation of IgE Biosynthesis - One obvious target for the development of antiallergic drugs would be the control of IgE biosynthesis. Evidence for the existence of T cells that regulate IgE formation has been presented,[46,47] suggesting the potential for anti-allergic immunoregulatory drugs. Indeed it has been reported that n-pentyl-β-D-fructopyranoside selectively inhibits IgE antibody formation.[48] However, this area is outside the scope of this review.

Binding of IgE to Mast Cells - Binding of IgE to mast cells or basophils is the initial event that leads to degranulation. Receptor molecules (10^5-10^6 per cell) are present at the cell surface that specifically interacts with the Fc fragment of the IgE molecule.[49,50] IgE is structurally very similar to IgG but the heavy chain contains one extra domain, called $C_\varepsilon 4$.[51-54] This domain as well as $C_\varepsilon 3$ seems to be involved in receptor binding. IgE contains large amounts of carbohydrate but this is not necessary for receptor binding.[55] Early studies on the biochemical properties of IgE and its interaction with mast cells were made possible by the availability of myeloma proteins.[23,40] More recently, hybridomas have been developed that secrete monoclonal IgE so that milligram quantities of pure IgE are now available.[41-43] The availability of large amounts of IgE will greatly facilitate studies on the molecular basis of IgE binding. It will also make it possible to investigate a controversial observation that a specific pentapeptide in the Fc fragment is responsible for binding to the receptors.[56,57]

IgE-Receptors - Receptor molecules on the surface of RBL cells that specifically bind IgE were characterized as follows. Surface proteins were labeled with 125-iodine, solubilized with non-ionic detergents, and receptor molecules were purified by affinity chromatography using IgE bound to Sepharose.[49,58-60] From these studies, it was established that the IgE receptor consists of at least one membrane glycoprotein with a molecular weight of 45,000-55,000 as judged by SDS-polyacrylamide gel electrophoresis. Chemical cross-linking studies provided evidence that another polypeptide with a molecular weight of 30,000-35,000 is part of the receptor complex.[61] This polypeptide can be cross-linked chemically to the 50,000 D IgE binding protein but not to receptor bound IgE. Other investigators have reported two IgE binding polypeptides with molecular weights of 45,000 and 55,000, respectively.[58,62] The 45,000 D to 55,000 D receptor polypeptide could be a mixture of two polypeptides; alternatively, in the 45,000 D and 55,000 D polypeptides could represent the same polypeptide with, for example, different degrees of glycosylation. Functional heterogeneity in IgE receptor complexes was observed by Sterk and Ishizaki[63] who demonstrated that mouse mast cells have two distinct receptors; one that binds rat and mouse IgE and the other that binds mouse IgE only. Using affinity chromatography, a presumed human IgE receptor

(molecular weight 58,000 to 68,000) was demonstrated at the surface of basophils from patients with basophilic leukomia and chronic myelogenous leukemia.[64]

Receptor Cross-Linking - Binding of IgE to the receptor is not sufficient to induce degranulation. Adjacent IgE molecules need to be cross-linked and this is accomplished physiologically by the reaction of multivalent antigens with the antibody binding sites on the IgE molecules. Cross-linking of IgE molecules brings the receptor molecules in closer proximity and this triggers a number of biochemical events that ultimately lead to mast cell degranulation. The evidence for this comes from the observations that chemically cross-linked IgE will induce degranulation without the addition of antigen;[40] that the addition of the small monovalent hapten dinitrophenol (DNP) to anti-DNP monoclonal IgE-mast cell complexes will not result in degranulation, whereas the addition of multivalent DNP (as DNP-bovine serum albumin complexes) will lead to degranulation; that bivalent $F(ab^1)_2$ fragments of antibodies against IgE will induce degranulation, whereas monovalent Fab fragments will not;[65] and that $F(ab^1)_2$ fragments of antibodies against the receptor molecule will degranulate mast cells without addition of IgE, whereas Fab fragments will not.[66,67]

Changes in Phospholipid Metabolism - Cross-linking of receptor molecules triggers changes in lipid metabolism; phosphatidic acid, phosphatidylinositol and phosphatidylcholine are phosphorylated and there is a rapid release of arachidonic acid[69] which is then converted to products of the lipoxygenase and cyclooxygenase pathways.[70,71] Hirata et al.[72] demonstrated that receptor bridging of rat mast cells with concanavalin A resulted in the methylation of phosphatidylethanolamine to form phosphatidylcholine. This conversion is mediated through S-adenosylmethionine and two methyltransferases that are present in the plasma membrane. A similar methylation pattern was observed when receptors were cross-linked with receptor-specific antibodies or with IgE and anti-IgE antibodies.[73] Kinetic studies showed that methylation occurred within 30 seconds after receptor cross-linking and this was followed by an influx of Ca^{++} and release of histamine. It has been proposed[69,74] that Ca^{++} uptake and arachidonic acid release are closely coupled events.

Cyclic AMP - Rapid changes in cyclic AMP (cAMP) levels occur prior to mast cell degranulation.[75-78] Within 15 seconds after receptor bridging cAMP levels increase 2-3 fold; and within 60 seconds cAMP levels have returned to normal. This rapid increase and decrease in cAMP parallels the kinetics of phospholipid methylation. A second and much less significant increase is observed approximately 3 minutes later. This second cAMP increase does not seem to be a significant factor in promoting degranulation since indomethacin inhibits this rise without inhibiting degranulation.[75]

The mechanism by which cAMP is synthesized after bridging of the IgE receptor has been reviewed recently by Winslow and Austen[79] (see also References 80 and 81). A membrane associated regulatory multi-subunit coupling protein (the G/F protein) responds to receptor cross-linking and activates adenylate cyclase which converts ATP to cAMP. Levels of cAMP are also controlled by phosphodiesterase which converts cAMP to 5'-AMP.

cAMP activates cAMP-dependent protein kinases. The inactive form of the kinase is a tetramer with a regulatory dimer bound to two catalytic subunits. Binding of cAMP to the regulatory polypeptide releases active subunits that catalyze the transfer of the terminal phosphorus from ATP to serine or threonine on receptor proteins. These are involved in cellular functions such as growth, differentiation, and

metabolic regulation.[83] Two isoenzymatic forms of cAMP-dependent protein kinase are present in mast cells and the increase in their activity parallels that of histamine release.[79,84]

Exposure of mast cells to A23187 leads to the phosphorylation of a number of polypeptides including the IgE binding polypeptide.[85-87] However, so far, phosphorylation of mast cell proteins under more physiological conditions has not been reported.

A number of experiments (mostly with rat mast cells) have demonstrated the importance of changes in cAMP levels for the release of mediators from mast cells. Compounds that increase adenylate cyclase activity through their action on the G/F protein [e.g. adenosine or N^6-phenylisopropyl adenosine (PIA)] increase cAMP levels and potentiate IgE stimulated mediator release; whereas compounds that inhibit adenylate cyclase activity [e.g. 2',5'-dideoxyadenosine (DDA)] inhibit the initial rise in cAMP and suppress degranulation.[82] The addition of adenosine or PIA to mast cells without IgE receptor cross-linking increases cAMP levels but it does not result in mediator release demonstrating that increased cAMP alone is not sufficient for degranulation.

It is not clear how these changes relate to phospholipid methylation. This reflects, in part, the complexity and multitude of stimuli that change cAMP levels, and of the biochemical events that are mediated by cAMP. Dibutyryl cAMP raises cAMP levels and inhibits the methylation of phosphatidylethanolamine to phosphatidylcholine;[79,91] and theophylline, a phosphodiesterase inhibitor prevents methylation, Ca^{++} influx and histamine release in a dose-dependent fashion.[82,91] It was speculated that the inhibitory effect of cAMP is mediated through the generation of an inhibitory phosphorylated protein[79] or through an inhibitor of protein kinase.[82] On the other hand, 2',5'-dideoxyadenosine, which inhibits the initial rise in cAMP and suppresses mediator release, does not inhibit phospholipid methylation;[88] and inhibition of phospholipid methylation by 3-deaza-SIBA only partially suppresses the initial rise in cAMP.[82] Thus, it was suggested by Winslow and Austen[79] that phospholipid methylation and activation of adenylate cyclase are parallel responses to IgE receptor binding. Possibly each of these events will promote Ca^{++} uptake.[100]

Proteases - An involvement of proteases in mast cell degranulation has been suggested by Austen and Brocklehurst[88] and this has been supported by the observation of Ishazaki[78] that inhibitors of serine esterases prevent phospholipid methylation and changes in cAMP levels in RBL cells when challenged with anti-receptor antibodies. Similar results are obtained when RBL cell plasma membrane preparations are used in such studies.[77]

Ca^{++} Influx - As was discussed above, changes in phospholipid methylation and cAMP levels occur within 30 seconds after IgE receptor bridging. These changes are followed by an influx of Ca^{++} and the release of histamine.[74,75,77,91] Maximum levels of Ca^{++} influx and histamine release were observed 2 and 3 minutes after stimulation, respectively. Inhibitors of glycolytic or oxidative phosphorylation do not affect Ca^{++} uptake significantly but they do abolish histamine release,[91] demonstrating that Ca^{++} uptake is not a consequence of degranulation. Membrane permeability to Ca^{++} after receptor cross-linking is a transient phenomenon and its decay parallels that of histamine release.[91] As will be discussed in more detail below, changes in membrane phospholipid composition lead to Ca^{++} uptake. In regard to its mechanism, the involvement of Ca^{++}-Mg^{++}

activated adenosine triphosphatase in Ca^{++} transport has been demonstrated for the sarcoplasmic reticulum,[92,93] and has been strongly suggested for mast cells. The enzyme is present in mast cell plasma and granule membranes,[94,95] and inhibitors of Ca^{++}-Mg^{++} ATPase also inhibit histamine release.[96] A role of Na^{+}-Ca^{++} channels has been implicated by the recent observation that amiloride (a Na^{+} channel blocker) induces histamine release[97], presumably through the accumulation of cytoplasmic Ca^{++}.

Ennis et al. reported that histamine will be released from rat peritoneal mast cells in the absence of extracellular Ca^{++}. At least three pools may be present: extracellular Ca^{++} that is loosely bound to the plasma membrane, Ca^{++} bound to regulatory sites in the membrane, and intracellular Ca^{++}. Extracellular membrane-bound Ca^{++} is probably utilized for optimal IgE-mediated degranulation.

Mechanism of Degranulation - Increases in cytoplasmic Ca^{++} concentration change the integrity of intracellular cytoskeleton and this may induce degranulation. The role of the cytoskeleton in the fusion of granules to the plasma membrane and mediator release is illustrated by the fact that heavy water, which stabilizes microtubules, inhibits degranulation, and that choleratoxin which dissociates microtubules, facilitates mast cell degranulation.[38,89] Altered phospholipid metabolism after receptor cross-linking results in the accumulaton of a number of potent fusogens that include 1,2-diacylglycerol.[89] It seems likely that these fusogens facilitate the fusion of granules with the cell membrane and the subsequent release of the contents of granules.

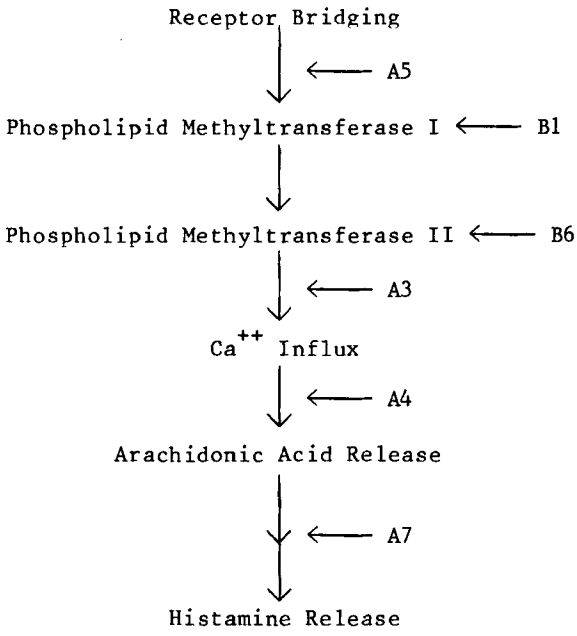
Pathway of Biochemical Events Leading to Mast Cell Degranulation - It has not been established whether all biochemical reactions described above are directly responsible for mast cell degranulation or are a consequence of degranulation. Neither is it known whether these reactions can be arranged into a pathway between IgE binding and degranulation. These questions can be approached by studying the effect of specific inhibitors of these biochemical reactions on degranulation, or by analyzing cell mutants that are defective in histamine release for their biochemical defects.

Cell Mutants - RBL cells that release histamine when stimulated with A23187 or with IgE and antigen were mutagenized[38,90] and two classes of cells with normal IgE receptor activity were selected: the first class fails to release histamine when stimulated with IgE or ionophore and the second class releases histamine after A23187 stimulation but not after stimulation with IgE. The second class of mutants are presumably defective in one (or more) of the early biochemical events but normal in the late events. Mutants of the second class were stimulated with IgE and antigen and analyzed for phospholipid methyltransferase I and II activities, percent change in phospholipid methylation, Ca^{++} influx, and Ca^{++}-dependent phospholipase activity (as measured by arachidonic acid release). In wild type cells, IgE stimulation resulted in an increase of all activities, and A23187 stimulation increased all activities except phospholipid methylation. One variant (called A5) failed to increase phospholipid methylation (although phospholipid methyltransferase activity was within the normal range), Ca^{++} influx and Ca^{++}-dependent phospholipase activity. Two other variants (B1 and B6) lacked either phospholipid methyltransferase I or II and showed no increase in methylation, Ca^{++} influx and phospholipase activity. Variant A3 showed a normal pattern of phospholipid methylation but no increase in Ca^{++} influx and phospholipase

activity and the two variants A4 and A7 failed to increase phospholipid methylation but did show an increase in Ca^{++} influx. Of these, variants, A7 showed increased phospholipase activity, whereas variant A4 did not.

From these studies, a sequence of events was proposed by Siraganian et al.[38] that is initiated by IgE binding and receptor bridging and that leads to the release of histamine (Figure 1).

FIGURE 1



Biochemical Steps Leading to Mast Cell Degranulation (From Reference 38)

(A5, B1, B6, A3, A4, A7 are variants of RBL cells discussed in the text.)

The results obtained with variants A4 and A7 could suggest that phospholipid methylation is not essential for Ca^{++} influx. However, these variants failed to release histamine which indicates that the increased Ca^{++} levels did not contribute to degranulation. Furthermore, at this time it is not known whether these variants represent single or multiple site mutational events. The importance of phospholipid methylation was demonstrated in an experiment where variant B1 (phospholipid methyltransferase I and histamine release deficient) was fused with variant B6 (phospholipid methyltransferase II and histamine release deficient). Hybrid cells were isolated with activities of both methyltransferases within the normal range, and these cells were capable of releasing histamine upon IgE stimulation.

Inhibitors - S-isobutyryl-3-deazaadenosine (3-deaza-SIBA), a specific inhibitor of S-adenosyl-L-methionine-mediated methylation, was used to verify the scheme of events proposed in Figure 1. Preincubation of mast cells with 3-deaza-SIBA followed by receptor cross-linking inhibited

phospholipid methylation, Ca^{++} uptake, and histamine release in a dose-dependent manner,[82] again suggesting the importance of phospholipid methylation in degranulation. Other evidence for the dependence of histamine release of Ca^{++} uptake has been presented in the sections dealing with Ca^{++} uptake and cAMP.

Conclusion - The purpose of this paper was to demonstrate that a number of unique biochemical reactions are responsible for IgE-mediated mast cell degranulation; and that the reagents and suitable assays and screens are now available for the development of specific and novel anti-allergic drugs. Some reactions (IgE binding, receptor bridging and possibly the fusion of granules with the mast cell or basophil plasma membrane) are unique to mast cells and basophils and are the most likely ones to yield drugs with the highest degree of specificity. Others (phospholipid methylation, Ca^{++} uptake, cAMP changes) occur in many other cell types after activation. Thus, drugs interfering at this level are less likely to be specific. However purified mast cells and basophils can be used for comparative studies with other cell types thus increasing the likelihood of developing selective inhibitors.

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Chapter 26. Plasminogen Activators

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Introduction – Proteases function through limited, selective, enzymatic cleavage of peptide bonds and are well-recognized for their regulatory capacity in diverse physiological and pathological processes, including: blood coagulation, fibrinolysis and hormone activation.[1] Limited proteolysis may be limited to a single event or may trigger sequential, cascading steps by specific, rapid and irreversible zymogen activation(s).[1] Proteolytic action can take place extracellularly, intracellularly or intercellularly. Proteolytic enzymes therefore have the capacity to regulate biological functions within these microenvironments. The specificity of proteolytic peptide bond cleavage, coupled with irreversible consequences of limited proteolysis, are key factors involved in the regulatory action of proteases in multiple control mechanisms.[2]

This chapter will review recent developments with an intriguing group of specific, neutral, serine proteases that proteolytically convert plasminogen, a serum zymogen, to the active enzyme plasmin: the plasminogen activators (PAs). For extensive and historical accounts of PAs beyond the scope of this chapter, the reader is directed to a number of reviews dealing with their biochemistry, cell biology and role in normal and pathological degradative function.[3-9] This review will summarize PA biochemical characterization to highlight the relationship between the molecular properties of PAs and their biological function. The association of PA and malignant cells will also be emphasized as an example of the role of PAs in a variety of invasive and degradative biological processes.

PA Characterization – PAs may be broadly classified as being urokinase-like, non urokinase-like or as bacterial activators.[9-11] Urokinase (UK) is a PA produced in the kidney and excreted in urine. UK-like PAs have also been found in kidney cell cultures, in malignantly transformed cells in culture and in many tumor-derived tissues. Non urokinase-like PAs have been isolated from many organs and tissues, secretions, vascular endothelium, plasma, malignantly transformed cells in culture and from some tumors. Streptokinase, which upon binding to plasminogen yields an efficient plasminogen-activating complex, is an example of a bacterial activator. UK-like and non UK-like PAs have distinct biochemical and immunochemical differences that appear to be related to particular biological properties, as described below.

Urokinase – Human UK is an extensively studied enzyme and is widely considered as a prototypical PA. Human UK exists in several molecular weight forms. Predominant forms are termed "high molecular weight" (HMW) and "low molecular weight" (LMW); HMW UK is of approximately 54,000 Mr, and LWM UK is approximately 32,000 Mr.[10-11] HMW UK is a two-chain molecule with polypeptide chains of 20,000 Mr and 34,000 Mr connected by a single disulfide bridge.[11-12] Recently, a single chain UK with a Mr of 50,000 has also been described.[13] LMW UK has generally been considered to be a single chain molecule, probably identical to the heavy chain of HMW UK.[14] Recent studies, however, have reported that the LMW form of UK has either a two-chain minor component[15-16] or a two-chain composition.[17-19] It has been suggested that LMW UK is a proteolytic degradation product of HMW UK.[18] It remains unclear whether proteolytic degradation of HMW to LMW UK is mediated by autocatalysis[17] or by plasmin.[20] Although both UK forms cleave plasminogen to plasmin in the same manner, it has been suggested that they display enzymatic differences.[10,21] HMW and LMW UK differ in their activation of glu-plasminogen and lys-plasminogen substrates,[17,21] in their response to fluorescent active-site probes[22] and in their rate of inhibition with plasma inhibitors.[23] Immunochemical studies have also probed the relationship between HMW and LMW UK.[14,24] It appears that LWM UK does not show

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immunochemical relatedness to the light chain of HMW UK.[24] PA characterization by radioimmunoassay has shown that LMW UK appears to be a component of HMW UK, which in turn contains determinants lacking from LMW UK.[25]

Tissue PA – Tissue PA (TPA) and UK are immunologically distinct and unrelated PAs.[26] TPA, however, does appear to be related or identical to the PA in the vascular wall which is released into circulating blood. TPA has been purified from a human melanoma cell culture and is identical to human uterine PA.[27] The Mr of TPAs range between 60,000 and 75,000 and can exist as two polypeptide chains linked by disulfide bridges or as a single chain.[28,29] The one-chain form of TPA has a Mr of approximately 72,000, and the two-chain form has a Mr of approximately 33,000 and 39,000.[28] A key biochemical difference between TPA and UK is that the former PA type[27] efficiently adsorbs to fibrin and fibrin clots, whereas with the exception of a single chain form of UK,[13] the latter PA type does not. Kinetic studies suggest that TPA yields efficient activation of plasminogen on a fibrin clot but prevents efficient activation in the absence of fibrin.[30] The potential of TPA, relative to UK, for thrombolytic therapy is discussed later in this chapter.

Although UK is widely considered to be the kidney PA and TPA is considered to be the PA in blood, recent reports have added new complexity to the issue of PA classification: in addition to UK, TPA has been found in urine[26] and kidney;[31] in contrast, it has recently been shown that human plasma contains a PA identical to UK.[32-34] Several studies have shown that several PAs of UK-like and TPA-like antigenic characteristics can be simultaneously produced by cultured human cells[35,36] and hamster cells.[37] It is of interest that all classes of PAs appear to exist in multiple molecular weight forms[37,38] and appear to be glycosylated.[19,39]

Zymogen Forms of PA – Zymogen forms exist for neutral serine proteases,[1,2] and recent reports indicate that PAs are not exceptions to this general rule. Although a trypsin-activatable PA produced by kidney cells and the conversion of a plasma plasminogen proactivator has been described,[40,41] more recent studies with cells in culture have further elucidated the existence of PA precursor forms. For example, activatable proenzyme and active forms of PA can be found in the conditioned medium of human embryonic kidney cells.[42] Recently, the proenzyme form of a PA produced by a human epidermoid carcinoma (HEp3), indistinguishable from UK, has been described.[43] The enzyme exists as a single chain form of 53,000 Mr, has only barely detectable catalytic activity and cannot be easily labeled with diisopropyl fluorophosphate (DFP).[43] Upon activation with plasmin, the HEp3 PA zymogen is converted to an active enzyme that is identical to urinary UK in specific catalytic reactivity, peptide chain composition and DFP labeling. The potential relationship of the single chain proenzyme to the recently described single chain, fibrin-binding, UK remains unknown.[13] An inactive form of PA from human glioblastoma cells in culture has also been isolated by affinity chromatography on a sepharose-bound anti-UK monoclonal antibody.[44] Studies with sarcoma-virus transformed murine cells have also revealed that PA in culture is released as an inactive proenzyme;[45] the inactive form consists of a single polypeptide chain of 48,000 Mr, as compared to the two-chain active forms with Mr of approximately 29,000 and 18,000. As for the human zymogens, DFP does not label the murine proenzyme but is incorporated into the larger chain of the active enzyme.[45]

Subcellular Distribution of PA – PA exists in a cell-associated form in addition to a soluble, extracellular form.[6] Early studies indicated that the subcellular location of PA was in two particulate subcellular fractions: the lysosome-rich fraction and the microsome fraction.[46] More recent studies have shown that PA is associated with plasma membrane-like elements of the cell; in studies employing differential centrifugation and sucrose gradient centrifugation, PA was isolated in a membrane fraction (that contains less than 10% of the total cellular protein), which contained plasma membrane markers but little or no nuclear or cytoplasmic contaminants.[47] The electron-microscopic appearance of this fraction, the high specific activities of Na^+K^+ ATPase, ^3H fucose and 5'nucleotidase and its buoyant density indicated that this fraction was enriched in surface membrane.[47] Additional biochemical[48-53] and immunocytochemical[54] studies also indicated a plasma-membrane association for PA in a

wide variety of cell types. However, an immunofluorescent study suggests that PA might be found in intracellular granules contaminating membrane fractions.[55] PA of cultured human fibrosarcoma cells is reportedly localized to light subcellular structures likely associated with the membrane of Golgi-derived vesicles.[56] Thus, most studies suggest that PA is closely associated with plasma membrane-like or Golgi membrane-like cellular entities which are not enclosed within granules but are rather firmly associated with membrane components.[47]

Release of PA – Recent studies have probed the mechanism whereby the cell-associated, membrane-bound PA is processed to an extracellular soluble form.[57] It has been found that chymostatin, an inhibitor of chymotryptic proteases, inhibits the release of PA.[57] A chymostatin-sensitive enzyme associated with a membrane fraction, which may play a direct role in the proteolytic release of PA from its membrane association, has recently been described.[58] The issue of cell density-dependent secretion of PA has been examined by a number of investigators with conflicting results.[59] The possibility that PA release may be due to membrane shedding, rather than due to secretion, has also been discussed.[60]

Plasminogen Activation – All PAs studied to date function as zymogen activators through proteolytic hydrolysis of the Arg 560-Val 561 peptide bond in plasminogen to produce the two-chain plasmin molecule.[10] Human plasminogen exists as a single-chain glycoprotein with a Mr value of approximately 93,000; native glu-plasminogen has amino-terminal glutamic acid and is readily converted to modified amino-terminal forms: lys-plasminogen, val-plasminogen and methionine-plasminogen.[7,9,10] Plasminogen contains lysine binding sites which interact with lysine, 6-aminohexanoic acid and trans-4-aminomethylcyclohexane-1-carboxylic acid; one high-affinity binding site and four low-affinity binding sites are located in the plasmin A chain.[9] The two-chain plasmin molecule is comprised of a heavy A chain originating from the amino terminus of plasminogen and a light B chain derived from the carboxyl terminus of plasminogen; the B chain contains the trypsin-like, DFP-sensitive, active site.[9] Activation of glu-plasminogen to plasmin by UK appears to be 20 times slower than activation of lys-plasminogen, and in both cases lys-plasmin is formed.[9] Studies with monoclonal antibodies exploring the domains of plasminogen have recently been described.[61]

PA and Degradative Processes: Overview – Previous reviews have described the association of PA in embryonic development, ovulation, blastocyst implantation, inflammation and mammary gland involution.[4,62] PA has been well-correlated with cellular migration of malignant cells,[63] Schwann cells,[64] neuronal migration[65] and embryonic cell migration.[66] Recent reports have demonstrated that PA is also associated with morphogenetic remodeling in the Bursa of Fabricius during embryonic development,[66] disease activity in psoriasis,[67] granulomatous tissue reaction in murine leprosy[68] and, as a consequence of secretion by skeletal muscle cells, may play a role in degeneration of neuromuscular contacts in pathologic denervation.[69] The general role of PA in cellular migration, tissue modeling and invasive processes, is abundantly illustrated in the relationship between PA and the malignant phenotype, as described below.

PA and the Malignant Phenotype – PA activity associated with malignant transformation is dependent upon the interaction of PA, produced by transformed cells and the serum zymogen, plasminogen.[4,6] PA activity is barely detectable in many normal cells, such as chick embryo fibroblasts, but is elevated ten to one-hundred fold in primary cultures of cells malignantly transformed by RNA or DNA tumor viruses, primary cultures of tumor cells, cells transformed by carcinogens, chemically induced tumors and in numerous tumor cell lines, including human tumor cell lines.[3,4,6,8] Cytocidal DNA or RNA viruses and non-transforming avian leukosis viruses, in contrast, have no PA enhancing effect.[6] The production of PA in cultures of malignant cells is an early event and precedes morphological evidence of transformation by temperature-sensitive RNA tumor viruses.[4,6] The generation of plasmin, mediated by PA activation of plasminogen, further enhances proteolytic activity in the microenvironment of the malignantly transformed cell; plasmin can modify cytoskeletal components, induce cellular division and alter proteins at the cell surface and within the basement membrane.[6,8,70] Both the production of PA and catalytic activation of

plasminogen to plasmin mediated by PA has been strongly correlated with a variety of processes that characterize the malignant phenotype, including: anchorage independent growth in agar,[71] tumorigenesis of DNA tumor virus transformants in nude mice,[72] tumorigenicity of bromodeoxyuridine-regulated, murine melanoma cells,[73] tumorigenesis of dihydrotestosterone-dependent murine, mammary carcinoma cells,[74] temperature-sensitive expression of the Rous sarcoma virus src gene product[6] and tumor promoter treatment of normal and malignant cells.[8] In addition, transformed cell morphology, adhesion and migration have also been linked, to a substantial degree, to PA production or conversion of plasminogen to plasmin.[6,8] The PA-plasmin system has been correlated with the induction of celllular proliferation,[10] with the disappearance of intracellular actin cables, anchorage independent growth and malignant potential.[74] A direct relationship among PA production, growth in agar and the ability to form tumors in nude mice has been shown for independently isolated clones of simian virus 40 transformed rat embryo cells.[72] It has recently been reported that PA production, acquisition of anchorage independence and phosphorylation of a 36,000 Mr protein of chick cells infected with partial Rous sarcoma virus mutants show good correlation with their tumor-forming ability in nude mice and chickens.[75] PA and anchorage-independent growth in agar have also been considered as markers of neoplastic cells transformed in culture following exposure to a chemical carcinogen.[76] Recent studies with chemical carcinogen-induced transformed guinea pig fibroblast lines possessing thousand-fold differences in tumorigenicity showed a quantitative correlation between PA production, lymphotoxin sensitivity, induction of delayed-type skin reactivity and the capacity to give rise to tumors;[77] tumorigenic cells produced PA at a level which directly correlated with tumorigenic potential, whereas non-tumorigenic cells failed to produce PA. In summary, a large body of experimental evidence suggests an association between PA activity and the malignant phenotype. It should be re-emphasized, however, that cells other than malignant cells also produce PA; for many normal cells it appears that PA synthesis is under developmental, temporal, physiologic or hormonal regulation.[6,8,62] In normal cells a relationship between PA production and cell-remodeling has been observed.[4,62] For example, extensive degradation of the graafian follicle at the time of ovulation has been described, as has transient production of PA by trophoblasts during the time period in which invasive embryonic implantation takes place.[4,8,62] It should also be noted that some exceptions to the correlation between PA production and malignant transformation have been noted. For a number of these studies, however, issues of accurate quantitative methods, cells with variable karyotypes, failure to examine extracellular as well as cell associated PA levels and culture media with inappropriate levels of hormones have not been adequately addressed.[6,62]

PA Substrate in Malignant Cells – It has been suggested that PA can function catalytically on a cellular substrate other than plasminogen to directly modify the morphology and behavior of malignant cells.[78] Evidence to support this claim is based on studies in which protease inhibitors were employed to block alterations in the colonial morphology resulting from the high PA levels observed during super-transformation mediated by tumor virus-tumor promoter interactions.[78,79] Compounds that inhibited the colonial aggregates that characterize the super-transformed phenotype were shown to be inhibitors of PA by a direct fluorometric assay that employed the fluorogenic peptide substrate Cbz-gly-gly-arg-amino-4-methyl-coumarin.[78,80] This substrate allows for direct determination of PA independently of the interfering potential of plasmin.[80] Experimental results demonstrated that a DFP sensitive protease with arginine specificity is involved in producing the morphological alterations that accompany tumor promoter treated, virally transformed cell cultures.[78,79] It appears that PA itself, and not plasmin, is involved in mediating these morphological alterations.[78] A recent report has shown that UK can cleave a 66,000 Mr human lung, pericellular-matrix protein to a 62,000 Mr product;[81] this protein, which is also cleaved by alpha thrombin, is the first described cellular target for PA.

PA in Tumor Invasion and Metastasis – The PA-plasmin system has been examined for its potential role in tumor invasion and metastasis.[8] Several studies have suggested that PA, or the enzymatic activity it generates, may play a role in the local and rapid dissolution of basement membrane required for metastasis by invasive malignant cells.[70,82,83a,83b] It

appears that PA, through generation of plasmin, plays a role in destruction of the basement membrane, since the latter enzyme can degrade glycoproteins associated with the basement membrane: laminin and fibronectin.[8,70,84] In addition, plasmin also activates latent forms of type IV and type V collagenase that play a role in degradation of the collagenous components of the basement membrane.[8,70] Although PA (UK) does not appear to directly cleave fibronectin, laminin, type IV collagen or type V collagen, the potential direct role of PA in activation of latent collagenase, or cathepsin B, is still an open question;[8,70] the potential role of TPA and the possible existence of additional basement membrane substrates for PA also remain open questions. The potential role of PA in invasive cell migration and/or in chemotaxis across the basement membrane are also unanswered questions. One report, however, has suggested that the generation of plasmin from plasminogen by metastatic cells is not necessary for the degradation of subendothelial matrix by metastatic cells.[85] This report demonstrated that in the absence of serum protease inhibitors and/or plasminogen, *in vitro* matrix solubilization is enhanced; this suggests that a cell surface-associated or rapidly inactivated, released protease, perhaps PA, plays a role in matrix solubilization upon tumor cell surface-matrix interaction.

It has been suggested that PA may play a role in tumor neovascularization, a process prerequisite to the metastatic spread of cancer.[86] It has recently been demonstrated that homogeneously pure PA (UK) but not active-site inhibited PA is angiogenic in the rabbit cornea.[8,87] These results suggest that PA may function, in part, by contributing to the process of tumor angiogenesis and thereby play some role in this aspect of tumor invasion and metastasis.

A number of studies have examined the issue of whether a quantitative relationship exists between PA production by malignant cells and their capacity to metastasize. Studies employing melanoma variant sublines of differing metastatic potential have given conflicting results. One report has indicated that low metastatic B16F1 and variants of increasing potential (B16F5, B16F10 and B16F13) showed similar levels of high PA activity.[88] In contrast, a quantitative difference in PA production between B16F1 and B16F10 sublines has also been described.[89] It has been suggested that this quantitative difference in PA production may contribute to the varying metastatic capability of the two melanoma variants. Studies with UV 2237 fibrosarcoma cells have shown that some cloned tumor cell lines (e.g., clone 12) with high *in vivo* metastatic potential produce low levels of PA relative to cells with lower metastatic capacity (e.g., clone 46).[90] However, this study is not conclusive relative to the role of PA in metastasis, since it is not known what level of PA is required for contribution to the metastatic process. For example, clones of relatively "low PA levels" may provide adequate enzyme for contributing to metastatic spread.[90] It is also worth noting that in immuno-suppressed hosts clone 46 is highly metastatic.[90] In the Lewis lung carcinoma system no consistent difference was noted for PA levels in metastatic versus primary tumor cell cultures;[91] however, the cultures studied were atypical upon comparison to parental tumors with respect to slow cell growth upon reinjection at the primary site and with respect to their decreased metastatic potential. The question has been raised that PA levels in cultured cells may not reflect PA levels of the original tumor cells; it has been noted that very high-PA producing clones can be selected by anchorage-independent growth from Lewis lung carcinomas.[91] Studies with metastatic variants from a cloned cell line, derived from an epitheloid cell line of hepatic origin, have shown no correlation between metastatic ability and fibrinolytic activity.[92] In contrast, quantitative models of metastasis employing HEp-3 human epidermoid carcinoma cells in the chick embryo have utilized PA as a quantitative marker for HEp-3 metastasis in both chick embryos and in newly hatched chicks.[93] In addition, recent studies dealing with the secretion of basement membrane degrading collagenase and PA by transformed cells suggest that the concommitant secretion of both enzymes is a prerequisite for metastasis.[94a] PA levels have also been associated with the metastatic spread of rat prostate adenocarcinoma cells.[94b] It is interesting to note that in studies with human colon tumors, tumor samples produced high PA levels in comparison to normal mucosael samples and that a correlation was found for high PA levels in tumors with a phenotype of invasiveness and metastasis.[95] In contrast, primary and metastatic tumors of human breast cancer did not show significant differences in their PA content.[96] For further

discussion of the role of PA in metastasis, the reader is directed to several reviews.[8,97,98,99] It is clear that further experimentation will be required to delineate the exact scope and role of PA in metastasis. Critical analysis of this issue must await, as previously suggested, thorough testing in models of metastasis *in vivo*, and the development of as yet to be described specific, efficient, potent, non-toxic, non-antigenic, bioavailable inhibitors.[100]

PAs and Thrombolytic Therapy – An important therapeutic application of the degradative capacity of PAs involves the lysis of fibrin clots; differences in the biological role of distinct molecular classes of PA have been clearly noted in this regard.[27] Although streptokinase and UK have received widespread scientific and clinical attention for their capacity to mediate thrombolytic therapy,[101-105] recent advances with TPA has further heightened interest in this area.[27,28,105] It has been suggested that TPA is a more effective and specific thrombolytic agent than UK;[106] it appears that TPA is more efficient than UK due to its high affinity for fibrin under conditions that do not lead to degradation of fibrinogen. It has been suggested that TPA has a lower threshold activity than UK and also displays a more progressive effect than UK with time.[106] It therefore appears that TPA in thrombolytic therapy might eliminate bleeding tendency noted for thrombolytic agents (UK and streptokinase), which activate both fibrin-bound as well as circulating plasminogen.[107] TPA has recently been used therapeutically in man, and rapid lysis of an old intravascular, iliofemoral thrombus was noted, in the absence of bleeding, as a result of systemic activation of the fibrinolytic system. Direct comparison of thrombolysis by TPA and UK in rabbits with experimental pulmonary embolus has shown that the former enzyme is thrombolytic at lower doses than UK without widespread plasminogen activation in the blood.[108] Similar findings have been noted for TPA in dogs with femoral vein thrombosis and in rabbits with experimental jugular vein thrombosis.[109,110] An interesting preliminary report has indicated that TPA can yield coronary thrombolysis upon intravenous administration to dogs bearing coronary clots.[111] Interest in the role of PA in thrombolytic therapy has led to exciting, recent advances in achieving mass production of PAs by methods including recombinant DNA technology. The construction of hybrid bacterial plasmids containing DNA sequences for UK that produce UK in *Escherichia coli* has been described.[112] It has also been reported that UK-like material is synthesized from poly A (+) RNA isolated from cultured human embryonic kidney cells.[113] In addition, the messenger RNA for human TPA has been described,[114] and the cloning and expression of TPA cDNA in *Escherichia coli* has been reported.[115] The isolation of a bacterial clone bearing a cDNA sequence for a portion of human TPA has also been described.[116] The purification of TPA in centigram quantities from human melanoma cells in cultures has also been reported.[117]

Conclusions – As described above, PAs have many regulatory functions and contribute to a number of biological degradative processes. In the case of malignant cells, the expression of PA may be related to pleiotropic aspects of the malignant phenotype: invasive degradation, cell migration, cell proliferation, angiogenesis and modification of cell surface and cytoskeletal composition. In the case of thrombolytic reactivity, the degradative properties of PAs can be of great importance in therapeutic lysis of clots. Recent observations concerning the production of PA by leukemic cells and natural killer cells[118-120] and the regulation of PA by protease nexin[121] indicate that future studies may elucidate additional biological roles for the PAs.

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Chapter 27. Natural Killer Cells: Role in Cell-Mediated Immunity

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Introduction - The role of the immune response in host resistance against tumor development and infection by microbial organisms has been an area of intense experimental and clinical interest. In the past, T-lymphocytes have received major attention as the key effector cell population in immune surveillance against cancer.[1-3] Although mature, immune T-cells play some role in *in vivo* resistance to tumor challenge, substantial experimental evidence has accumulated to indicate that this effector cell population fails to account for all facets of host immune resistance to either tumor development or microbial infection.[2] During the last few years, considerable interest has been focused on the role of natural cell-mediated immunity in host defense against primary and metastatic tumors, as well as microbial infection. This chapter will summarize recent findings with the most extensively studied and characterized effector cell that mediates natural immunity: natural killer (NK) cells. This review will discuss the characteristics of NK cells, the regulation of their cytotoxic potential, the role of NK cells in immune reactivity, and the potential widespread clinical relevance of the modulation of NK activity. Emphasis will be placed on the role of NK cells as antitumor effector cells in resistance against malignant disease. The reader is directed to a number of recent and comprehensive reviews and texts beyond the scope of the present discussion.[4-12]

Characteristics of NK Cells - NK cells were discovered less than ten years ago during studies dealing with the specific cytotoxic activity of lymphocytes of tumor-bearing individuals against either their own tumors, or against tumors of the same etiologic or histologic type. Although specific cytotoxic activity was observed in several studies dealing with cancer patients,[3] or with virally induced tumors in animals,[1] it was also found that lymphocytes of some normal, control individuals were indeed more cytotoxic against some tumor target cells, than were those from the tumor-bearing individuals.[9] The term "natural killer" was employed since the cytotoxic reactivity of the lymphoid effector cell population was observed spontaneously, without the requirement for prior immune sensitization, i.e., naturally. Since NK cells are present in unimmunized individuals, and unlike other lymphoid effector cell populations do not require long time periods to be activated or primed for cytotoxic capability, they have been considered as a first line of immune defense against cancer.

NK cells are nonadherent and nonphagocytic, and have therefore been considered to be a subpopulation of lymphocytes rather than macrophages or monocytes.[10-12] NK cells are distinguishable from mature T cells since high levels of NK activity are found in neonatally thymectomized, or athymic nude mice. Although NK cells are thymic-independent, they do possess a number of T cell-associated markers and it appears that NK cells may be of the T cell lineage.[11] Approximately 50% of human NK cells have receptors for sheep erythrocytes and most can react with monoclonal antibodies to T cell-associated antigens.[10] At least 50% of murine NK cells express THY 1 and approximately 20% react with a monoclonal anti-Lyt1 antibody.[10] In addition, NK cells both produce and grow in response to stimulation by IL2 (T cell growth factor, TCGF) and additional T cell mitogens. It should be noted, however, that NK cells also possess some cell surface markers that are in common with PMN's and macrophages. Human NK cells, for example, react with OKM1 and antibodies to asialo GM1 and MAC 1 (see below). The receptor for the Fc portion of IgG is also found on the cell surface of NK cells[11] and has raised the question of the relationship between NK cells and K cells that mediate antibody-dependent cell-mediated cytotoxicity (ADCC). It appears that NK and K cells are in the same subpopulation of lymphocytes and that NK and ADCC functional activities may be mediated by the same cells. Cytotoxic effects might therefore be mediated

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by either interaction with antibody-coated target cells through the Fc receptor, or by distinct NK surface antigen-mediated binding to target cells (see below).

NK Cells are Large Granular Lymphocytes - It has recently been noted[12] that NK cells are large granular lymphocytes (LGL), which are large lymphocytes with prominent azurophilic granules in the cytoplasm and which contain an indented, kidney-shaped nucleus. LGL have a high cytoplasmic:nuclear ratio, and therefore have a relatively low density when compared to other lymphoid populations.[12] It has been possible to enrich human LGL from their frequency in the peripheral blood of 2-6% to a purity of 90-95% by employing a number of purification steps[13,14] including discontinuous Percoll gradient centrifugation and elimination of cells that form high affinity rosettes with sheep erythrocytes at 29 degrees Centrigrade. The majority of NK cell activity, as well as ADCC activity, has been found in Percoll fractions that contain 75-85% LGL, whereas these fractions contained only 10-20% of the input peripheral blood lymphocytes.[13,14] The Percoll fractions containing the majority of the small to medium lymphocytes, however, have been devoid of NK or ADCC activity. That LGL are responsible for NK cytotoxic reactivity is supported by observations that approximately 50% of LGL form conjugates with, and lyse, highly NK-cell sensitive target cells. It appears, that LGL also account for a large percent of human T gamma (T_G) cells. Only T_G cells with LGL morphology have NK cell and ADCC activities.[11,12]

Rat NK cells in the peripheral blood and spleen also appear to be LGL, and are enriched by a procedure similar to that used for the isolation of human LGL.[14,15] LGL have also been detected in mouse spleen, peripheral blood, lung, bone marrow, and lymph nodes.[11,16-18] Recently, a naturally occurring LGL rat leukemia has been identified, which may be a useful means for obtaining large numbers of LGL.[19]

Studies with monoclonal antibodies, reported to be selective for T cells or other lymphoid populations, and fluoresence activated cell sorting, have demonstrated that LGL show a pattern of reactivity distinct from T cells and from monocytes or granulocytes.[20,21] A portion of LGL express several T-cell associated markers (OKT8,OKT10,LyT3) but fail to express other markers (OKT3,OKT4,OKT6,OKT11).[20] In contrast, some human LGL are reactive for antigens which are undetectable on typical T cells, but present on granulocytes and/or monocytes (OKM1, asialo-GM1, and Ia).[10] It therefore appears that the LGL phenotype is distinct from that of T cells or macrophages. It is not clear whether LGL are in the T-cell lineage, the monocyte lineage, or represent a separate lineage of lymphoid cells.[5]

Interferon(IFN)-augmented, as well as spontaneous NK and ADCC activities, are exclusively associated with LGL-enriched fractions.[14] It has been suggested that LGL are also the main effector cells exerting spontaneous and IFN-boosted activity against adherent as well as nonadherent tumor cell lines.[22] A single cell cytotoxicity assay has been employed in the examination of the frequency of NK cells among LGL, the effects of IFN on their tumor cell-binding, and lytic reactivity.[23] It has been found that up to 80% of human LGL can function as NK cells. It was also found that IFN affects human NK activity in a number of distinct ways: IFN can convert some nonbinding LGL to tumor cell-binding LGL; IFN can activate non-lytic, target-cell binding LGL; IFN can increase the rate of lysis of lytically-active, target-cell binding LGL; and IFN can facilitate the recycling of NK cell binding to additional target cells by eliminating refractoriness to rebinding by LGL.[23] It has recently been noted that IFN influences restricted subpopulations of NK cells, primarily HNK-1^+ cells, by influencing cytotoxic events rather than by triggering cellular maturation.[24-26]

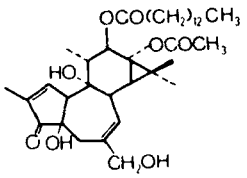
A number of studies have recently employed LGL in a variety of experimental settings. For example, LGL treatment with Sr^{2+}, an alkaline ion known to cause granulocyte degranulation, has led to LGL degranulation and loss of NK lytic activity.27 Upon *in vitro* cultivation, Sr^{2+}-treated LGL recovered NK lytic function as cytoplasmic granules reappeared. It therefore appears that LGL intracytoplasmic granules may play a role in the mechanism of LGL-mediated tumor cell lysis. The effect of a monoclonal anti-LGL antibody on human NK reactivity has also been investigated.[28] The NK-8 hybridoma was found to react with 33% of peripheral blood LGL and 70% of LGL forming conjugates with NK-susceptible K562 target

cells.[28] NK-8 inhibited LGL cytotoxicity against K562 cells by 60%, in the absence of complement, and inhibited tumor cell induction of IFN by LGL.[28] In contrast, monocytes, granulocytes, and other lymphocytes were nonreactive with NK-8. Studies with the carboxylic ionophore, monensin, has led to irreversible inhibition of the NK lytic mechanism and has shown that LGL require an intact secretory process to mediate cytotoxicity.[29] Studies have indicated that IL 2 plays a role in the activation of human LGL activity.[30] The characteristics of purified LGL cultured in the presence of IL 2 have also been examined.[31] Upon culturing, LGL lost reactivity with monoclonal antibodies OKM1 and OKT10, expressed increased reactivity with OKT3 monoclonal antibodies and with anti-Ia, and maintained their IFN-augmentable cytolytic profile.[31] The rationale behind attempted long-term culturing of LGL is to obtain large amounts of cells for detailed biochemical studies. Another approach to this goal has been directed towards the cloning of NK cells.[32-34]

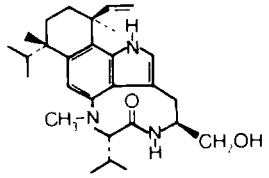
Regulation of NK Cytotoxic Activity - To date, IFN and IFN-inducers appear to be the major positive regulators of NK cytotoxic activity.[35] Experiments with pure human leukocyte and fibroblast IFNs have led to substantial augmentation of NK activity,[35] thereby confirming that IFNs have direct NK-boosting capacity. The issue of whether IFN is the only positive regulator of NK activity has also been examined. It has been suggested that some of the NK-augmenting activity mediated by *C. parvum* may be caused by a factor other than IFN.[36,37] A more pronounced example of an IFN-independent augmentation of NK activity has been noted in studies dealing with alloantisera and monoclonal antibodies, and lectins.[38,39] Retinoic acid has also been found to augment NK activity and may do so in an IFN-independent manner since retinoids often inhibit IFN production in a number of cell types.[40,41]

Inhibition of NK activity may be classified into several categories and a number of agents that inhibit NK activity have been described.[35] NK cells can be directly inhibited, accessory cells can be inhibited, and suppressor cells can inhibit NK reactivity.[1,2,35] Prostaglandins (PG) have been reported as inhibitors of both spontaneous and IFN-augmented murine and human NK activity *in vitro*.[35,42] Aspirin or indomethacin, inhibitors of PG synthesis, were reported to partially restore NK activity in tumor bearing animals.[43] Pretreatment of NK cells by PGE_2 can activate NK activity by increasing the frequency of NK cells in the absence of alterations to the number of target-binding lymphocytes.[44] Exposure of NK cell-target cell conjugates to PGE_2 during the cytotoxic assay led to inhibition of NK lytic activity. It was subsequently noted that activation of NK lysis can be either enhanced or blocked by the interaction of PGE_2 and IFN.[45] If PGE_2 was present during cellular processing needed for NK activation induced by IFN, pre-NK lytic potential was blocked. If PGE_2 was added following complete IFN-induced activation of NK cells, there was an enhanced recycling capability of the same number of NK cells.[45] It has also been reported that IFN-stimulated NK cells are protected from suppression by PGE_2.[46]

Tumor promoting phorbol esters and teleocidin have been found to inhibit both murine and human NK cell activity.[40,47] Phorbol-12- myristate-13-acetate (PMA), but not non-tumor promoting phorbol esters, was found to inhibit both spontaneous as well as IFN-boosted murine NK activity.[40] In studies with human LGL, it appears that PMA can inhibit either both spontaneous and IFN-augmented NK activity, or only IFN- boosted activity.[40]



Phorbol-12-myristate-13-acetate (PMA)



Teleocidin

Intact cholera toxin, but neither subunit A or subunit B of the toxin, blocks both spontaneous, as well as IFN-augmented, or retinoic acid augmented, NK activity of LGL.[40,47] It therefore appears that the inhibitory effect of cholera toxin requries both binding through the B subunit, as well as activation of adenylate cyclase through the A subunit.[40,48,49] It has been reported that agents that increase cAMP inhibit human NK activity.[50] Studies with LGL have demonstrated that increased levels of cellular cyclic AMP mediates the action of PGE_2 on the suppression of NK lytic activity.[51]

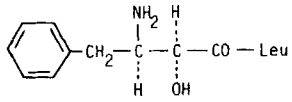
Suppressor cells also appear to be involved in aspects of NK cell inhibition.[35] Suppressor cells induced by pyran copolymer were presumed to be macrophages since they were adherent and phagocytic.[52] A soluble factor appears to play some role in the mechanism of inhibition. It has been suggested that macrophages may contribute to low NK activity in various murine strains.[53] Adherent peritoneal cells from *C. parvum*- injected mice had reduced capacity to inhibit NK activity.[54] A naturally occurring cell type with inhibitory activity for NK cells, which is non- adherent, has been isolated from lymphoid organs of both mice and rats.[55] The cell, which lacks surface Ig and Fc receptors for IgG, expresses receptors for Helix pomatia A agglutinin and bears Thy-1 antigen, suggesting that the cell is within the T lymphocyte lineage, and is not a B lymphocyte or a monocyte/macrophage.[55] Studies of cellular regulation of human NK cells have demonstrated that monocytes or polymorphonuclear leukocytes can suppress NK activity, apparently through the generation of reactive forms of molecular oxygen.[56] Of mononuclear phagocytes isolated by adherence from various sources, only bronchoalveolar macrophages appeared to inhibit the expression of human NK cell activity.[57] A recent study has indicated that peripheral blood granulocytes can inhibit human NK cell activity *in vitro*.[58]

Mechanism of NK Cytotoxic Activity - The biochemical and molecular basis by which NK cells recognize, interact with, and lyse tumor target cells has not been fully elucidated.[11,59-62] It has been demonstrated, however, that the binding of NK cells to tumor cells usually precedes target cell cytolysis.[11,59-62] Steps involved in the NK lytic mechanism have been resolved into binding, programming for lysis, and killer cell- independent steps.[63] Isolation of target structures from NK-susceptible tumor cells have been reported.[64] The NK target structures of YAC lymphoma cells appear to be cell surface glycoproteins of 130,000, 160,000 and 240,000 Mr.[64] Although the target antigens prevented binding of intact target cells to NK-containing cells, they failed to inhibit cytolysis. Target molecules were not observed in NK insensitive target cells, and their expression varied directly among target cells according to their sensitivity to NK cell-mediated lysis.[64] A correlation between the expression of a glycolipid, asialo GM_2, and the susceptibility of target cells to NK lysis has also been observed.[65] The ratio of expression of asialo GM_2 to GM_2, has been shown to have a good correlation with NK cell-mediated lysis of target cells.[66] It has been suggested that asialo GM_2 and some gangliosides could play a role in the binding or lytic events involved in NK cell-target cell interaction. High levels of sialic acid and sialylation at the cell surface could modify or inhibit such interactions.[66] It has recently been suggested that receptors for transferrin may function as a target structure for recognition by human NK cells.[67] A number of studies have examined the morphological aspects of NK cell-target cell interaction.[27,68-70] Studies have implicated that LGL granules play a role in the mechanism of cytotoxicity.[27,68,69] The role of uropods of LGL in NK effector cell function has also been discussed.[69,70]

A number of biochemical processes have been implicated in the lytic mechanism of NK cells.[11,59-62] It has been demonstrated that human LGL produce neutral serine proteolytic activity, and that tryptic and chymotryptic inhibitors block cytolysis *in vitro*.[11,59,71-72] The use of protease inhibitors for NK cells of a variety of species has also suggested some role in the lytic process by neutral serine proteases.[73,74] A role for phospholipid methylation and phospholipase A_2 has also been suggested.[59,75] A role for oxygen intermediates in NK cell-mediated cytotoxicity has been reported.[76] Since highly purified human LGL fail to produce an oxidative burst in response to a number of stimuli, including PMA, some skepticism has been directed towards the involvement of oxygen intermediates in NK killling.[11,59,77] Much attention has recently been focused on the production by NK cells of soluble cytotoxins that

appear to be specific for NK-sensitive target cells.[78,79] Murine target cells, resistant to NK cytotoxic factors, are also resistant to NK cell-mediated cytotoxicity.[80] It has been suggested that, for lysis to take place, a NK sensitive target cell must be recognized by the NK cell, NK cytotoxic factors must be released by the effector cells, and the factor must bind to the target cells.[80] It appears that NK resistant murine target cell clones lack, or display decreased levels of binding sites for NK cytotoxic factors.[80] The specificity of cytotoxic factors produced by human LGL has also been described.[81] It appears that the specificity of the human factor depends on target cell recognition. Several reports have also suggested that ultrastructural membrane lesions take place during cytolysis, and that target cell membrane lesions may result from the insertion of tubular complexes inserted into the membrane during cytolysis.[82,83]

Biological Response Modifiers and NK Cells - The use of biological response modifiers to augment the *in vitro* and *in vivo* antitumor reactivity of human and rodent NK cells has received considerable investigative attention over the past few years.[4,5] It has been hypothesized that the manipulation of NK cell function above spontaneous background levels by adjuvant immunotherapy in cancer patients may be of therapeutic benefit.[84] Interferon, as discussed above, appears to be the major regulator of NK cytolytic function.[4,5] Many additional biological response modifiers of both microbial and chemical origin can increase resistance to tumor growth. A partial list of agents which have been shown to induce higher levels of NK cell cytotoxicity and increase antitumor resistance follows: *C. Parvum*;[85] BCG;[86] OK432;[87] bestatin;[88] lipopolysaccharide;[89] poly I:C;[89] pyran copolymer;[90] lentinan;[91] retinoic acid;[40] Tuftsin;[92] MDP;[93] and Cis-platinum.[94] Many of these agents stimulate macrophage reactivity as well as NK activity, and further study is required to evaluate the selective role of a given effector mechanism.



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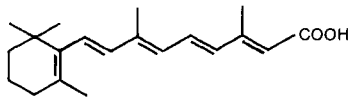

Retinoic Acid

NK Cell-Mediated Immunity Against Tumors - The overall *in vitro* profile of NK cell activity of lymphocytes from normal donors against established tumor cell lines has been well documented in a number of species including human, rat, and mouse.[4,5] More recently, a profile of reactivity for NK cells *in vitro* against spontaneous rodent and human tumors has been steadily emerging. Studies with murine splenic cells against a variety of mouse spontaneous mammary tumors has shown that a considerable number of tumors were sensitive to natural cell-mediated cytotoxicity.[95] The cytotoxic activity was associated with LGL fractions and could be augmented with IFN. Recent studies have shown that the lytic capacity of human LGL are not limited to *in vitro* tumor cell lines since allogenic, freshly isolated tumor biopsy cells from carcinomas and sarcomas were also killed with a high level of efficiency.[96] In studies dealing with tumor cell suspensions derived from human carcinomas of the lung, colon, breast, or neck, and melanomas, the cytotoxic reactivity of different subpopulations of lymphoid cells from the peripheral blood of normal donors was examined.[97] It was found that the majority of most primary tumors can be lysed by normal lymphoid cells and that the activity is strongly associated with LGL. Additional work is yet to be done in comparing the sensitivity of human, and rodent, tumor cells taken from primary autochthonous tumors and their subsequent metastases.

Considerable evidence has also been steadily accumulating which shows that NK cells function *in vivo* to eliminate tumor cells. NK cells prevent *in vivo* growth of lymphomas[98] and, in addition, play a major role in the elimination of tumor cells *in vivo*.[99] In contrast, it

has been reported that NK activity is inhibited by urethan carcinogenesis.[100] An important role for NK cells in radiation-induced leukemogenesis has also been reported.[101] Selective depletion of NK cell activity with anti- asialo GM1 or anti-NK 1 antisera has established an important role for NK cells in controlling primary tumor growth of experimental tumors, and has provided direct evidence that NK cells are involved with tumor cell elimination *in vivo*.[102-104] A number of studies have also examined the relationship between NK cells and metastatic spread of cancer.[105-115] It has been shown that metastatic cells are more resistant to NK activity than cells derived from local tumors, and it has been suggested that this increased resistance may play a role in metastatic spread.[107] Low levels of NK cell activity have been found to be associated with enhanced incidence of experimental metastasis.[108] A close correlation between host NK cell activity and the capacity to eliminate blood-borne tumor cells suggests that NK cells play an important role in host defense against circulating tumor cells and can therefore prevent the development of tumor metastasis.[109] Additional studies in NK-deficient beige mice have also indicated that NK cells play an important role in host control over tumor growth and metastasis.[110] Direct evidence for inhibition of experimental tumor metastasis *in vivo* by NK cells has recently been demonstrated.[111-115] A cloned cell line with NK activity, upon transfer into NK-deficient hosts, has been shown to have anti-metastatic activity against melanoma tumor cells.[112] Studies with anti-asialo GM1 serum have demonstrated that NK cells play an important role in resistance to tumor cell dissemination, and thereby contribute to control over tumor cell growth and metastatic spread in mice.[114] Recently, it has been shown that the adoptive transfer of an enriched rat LGL population to asialo GM1 treated rats led to partial restoration of their cytotoxic activity against established cell lines.[116,117] Conclusive experiments concerning the role of NK cells await the critical testing of adoptive transfer of LGL in models of experimental and spontaneous metastasis *in vivo*. A number of intriguing observations have recently been made concerning NK cell activity in patients with advanced cancer. NK activity measured in peripheral blood mononuclear cells of patients with disseminated disease was significantly lower than NK activity of either patients with localized disease, or normal donors.[118] Defective NK activity in cancer patients has been correlated with LGL recycling capacity[119] or low LGL presence *in situ*.[120] It has also recently been found that NK activity of patients with advanced carcinoma of the lung is a useful prognostic indication of the effect of chemotherapy on patient prognosis and may be useful for the establishment of improved treatment regimens for advanced carcinoma of the lung.[121] Studies of patients with acute leukemia in remission have suggested that during remission there is a gradual recovery from low to normal NK activity when compared to the NK activity of healthy donors. In contrast, patients who relapsed were found to have lower activity than patients who remained in remission.[122] The exact role and scope of NK activation in evaluation of the effects of biological response modifiers, and the extent of NK activity in human anti-tumor reactivity, remains unknown.[5] Clinical studies with IFN, for example, have indicated a variety of results ranging from a highly significant and consistent increase in NK cell cytotoxicity of some patients, to a diminished NK cell response, to a transient increase in NK activity that then declines.[123-125] It is clear that issues of route and timing of biological response modifiers, as well as dosage will be critical in determining biological activity of any endpoint, including NK cell activity.[5,84,94]

Conclusions - Natural killer cells, a specific lymphoid subpopulation comprised of large granular lymphocytes, appears to play an important role as a natural effector cell against primary and metastatic tumor cells. Rapid advances and widespread interest in this area of cell- mediated immunity promises to fully define the role of NK cells in anti- tumor immune reactivity in the near future. Recent studies exploring the role of NK cells in a number of disease states, such as X-linked lymphoproliferative syndrome, multiple sclerosis, atopic dermatitis, as well as studies examining the *in vivo* reactivity of NK cells against a number of microbial agents, including herpesviruses, cytomegalovirus, hepatitis virus, babesia microti, malaria, cryptococcus, and various bacterial infections, suggests that NK cells, and natural immunity, will continue to receive substantial experimental and clinical attention during the next few years. Studies dealing with the role of NK cells in bone marrow transplantation reinforce this view.[5]

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Section VI - Topics in Chemistry and Drug Design

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Chapter 28. Recent Advances in Drug Delivery System Technology

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Introduction - During recent years, much attention has been focused on new systems of drug delivery. The goal of this new technology is to optimize drug therapy by enhancing the specificity and efficacy of drug action and by decreasing the incidence of side effects and undesired responses in non-target tissues. The ideal drug delivery system would deliver drug only to the target tissue where it would maintain a therapeutic concentration for a specified period of time. These new systems would also improve drug therapy by promoting patient compliance. No drug can be effective if the patient does not follow a prescribed dosage regimen because of drug side effects or because an excessive frequency of dosing is required.

A review of the literature since the previous reviews in this series in 1979 on liposomes,[1] and in 1980 on drug delivery systems[2] and antibodies as drug carriers,[3] indicates that this new technology has become a burgeoning multi-disciplinary science in which a variety of approaches have been employed in the search for new systems. Recent publications, which provide general descriptions of the new approaches, as well as more detailed discussions on the rationale for drug delivery systems, refer to the systems as controlled,[4] intelligent,[5] novel,[6] rate controlled,[7] smart,[8] targeted,[9,10] therapeutic,[11] and even as a "magic gun" to deliver "magic bullets."[12]

Reports of new developments are scattered across the literature of many disciplines under a variety of keywords such as liposomes, monoclonal antibodies, polymeric carriers, prodrugs, transdermal devices, etc. In addition, one can now find reports of transdermal delivery systems containing liposomes,[13] or of liposomes containing monoclonal antibodies.[14] Clearly, the topic of drug delivery systems is rapidly becoming very complex. When discussing drug delivery systems, therefore, it will be helpful to realize that:

1) They are not single entities such as prodrugs or liposomes. They are systems composed of several interdependent components. The design of a drug delivery system must take into consideration the drug component, the carrier or vehicle component, and the intended route of administration to the body.

2) These systems are not universal but are rather specific; that is, a system developed for one drug may not be usable per se for another drug with different physicochemical and pharmacokinetic properties. Nor can a transdermal system developed for a drug be used per se as an ocular system with the same drug.

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A review article on this complex topic cannot provide a textbook review of the subject, but rather only a global impression or overview. Therefore, recent developments in drug delivery system technology will be discussed in progression from chemical approaches, through polymeric and biological approaches to pharmaceutical approaches. They will be highlighted according to keywords for the benefit of the reader who has an interest in a particular aspect of this new technology.

I. Drug Components

Soft Drugs and Antedrugs - "Soft Drug" is a term proposed by Bodor for active compounds that undergo in vivo metabolism to inactive and non-toxic moieties after achieving their therapeutic roles.[15] Soft drugs have been proposed for both systemic and topical use. "Antedrug" is a term proposed by Lee and Soliman for derivatives of parent compounds that are active but which are metabolized to an inactive form upon entry into the general circulation.[16] Antedrugs are intended only for local activity. Both concepts have been applied to topical steroids as a means of providing local activity without systemic side effects. These concepts should find application for drugs used in other tissues of the body.

Prodrugs - The topic of prodrugs is vast enough to require a separate comprehensive review article. Therefore, the following discussion will attempt only to provide a brief summary of prodrugs with respect to their general application to drug delivery systems.

Prodrugs have been defined by Stella et al. as agents "which must undergo chemical or enzymatic transformation to the active or parent drug after administration."[17] The general subject of prodrugs has also been reviewed recently by Bodor,[18] Bundgaard and Hansen,[19] and Notari.[20]

The concept of prodrugs finds application in two areas. The first is to improve the overall properties of a parent drug with respect to its absorption, bioavailability, duration of action, safety, solubility, stability or taste. The second is to provide targeting or site-specific drug delivery by enhancing selective concentration in the target tissue and/or by selective conversion to its active moiety by an enzyme which is present only in the target tissue.[19,20] The design of prodrugs for use in drug delivery systems must take into consideration the enzyme systems available at the intended site of application.

Readers with interest in specific prodrugs are directed to the most recent publications in the comprehensive series by: Bodor et al.[21,22] on "Improved Delivery Through Biological Membranes"; Bundgaard et al.[23,24] on "Prodrugs as Drug Delivery Systems"; and Repta et al.[25] on "Enol Esters as Potential Prodrugs." The preparation of prodrugs of amides, imides and amines through N-alkylation, N-acylation, reduction or ring-opened derivatization of cyclic compounds has been reviewed by Pitman.[26] The application of the prodrug principle to antibiotics has been reviewed by Ekström.[27]

Recent developments in prodrug design which should be considered significant include a renewed interest in the concept of mutual prodrugs, the concept of tripartate prodrugs, and polymeric prodrugs.

Mutual prodrugs may be defined as the coupling of two active moieties so that each acts as the prodrug of the other.[28] The concept of mutual prodrugs is not new, since such compounds were in use even before the concept of prodrugs was developed. For example, sulfasalazine was developed in the 1940's for use in treating ulcerative colitus by releasing sulfapyridine and 5-aminosalicylic acid in the intestine. However, the concept does offer interesting possibilities and is finding

new applications. Baltzer and Godtfredsen reported the development of mutual prodrugs of β-lactam antibiotics and β-lactamase inhibitors.[28] In principle, the objective is to deliver an antibiotic and its potentiating agent (enzyme inhibitor) to the body in a 1:1 ratio. A recent example is the development of sultamicillin a mutual prodrug of ampicillin and the β-lactamase inhibitor sulbactam.[29] The ideal mutual prodrug would be well absorbed and its two components released, distributed and eliminated in a similar fashion.

Tripartate prodrugs were recently suggested by Carl et al.[30] To date, prodrugs have been essentially bipartate in nature, with the drug or active moiety attached directly to the carrier or specifier moiety. A tripartate prodrug utilizes a spacer or connector group between the active and specifier moieties to provide better selection and control over the enzymatic system or systems required to release the drug.

Polymeric prodrugs are formed when drugs are attached to a polymer through chemical bonds. A polymeric prodrug may also be considered to be a type of macromolecular drug carrier. Langer refers to these compounds as pendant chain polymeric controlled release systems.[31] Polymeric prodrugs will be considered under drug carrier systems, along with other types of macromolecular drug carriers in which drugs are weakly bonded to and/or encapsulated in macromolecules.

II. Drug Carriers or Vehicles

The majority of recent developments in drug delivery system technology have been in the area of drug carrier systems. These carrier systems have utilized a variety of materials to provide controlled release rates with implants and injections, or to provide better target tissue selectivity.

Classification of these various carrier systems depends upon the viewpoint of the discipline involved. For example, in his book, Drug Carriers in Biology and Medicine, Gregoriadis[32] provides a biological viewpoint and classifies carriers as: macromolecular (albumin, antibodies, glycoproteins, etc.); cellular (erythrocytes, fibroblasts); and synthetic (synthetic polymers, liposomes). On the other hand, in their paper "Present and Future Applications of Biomaterials in Controlled Drug Delivery Systems," Langer and Peppas[31] provide a physical polymeric chemistry viewpoint and classify drug carriers as: diffusion controlled (liposomes, membrane systems, non-bioerodible matrices); chemically controlled (bioerodible matrices and pendant chain systems); swelling controlled and magnetically controlled. They also classify polymers as being either hydrophilic, hydrophobic or biodegradable. The aforementioned references[31,32] and an article by Wood[33] on "Bioerodible Drug Delivery Systems" will provide the reader with detailed discussions on carrier systems.

Polymeric Prodrugs - The design of polymeric prodrugs using biodegradable polymers has been reviewed by Kim et al.[34] They define three different models of chemically bound drug/polymer systems: monomeric drugs attached to a polymeric chain; drugs incorporated into the polymeric chain; and drugs in oligomeric or polymeric form attached to a polymer chain.

Recent developments of interest in this area include:

- small synthetic glycolipids or glycopeptides, such as trimannosyldilysine as cell specific ligands for the anti-inflammatory steroid dexamethasone.[35] The ligands are reported to be attacked by microsomal enzymes in the macrophages present in inflamed tissue. This type of prodrug is expected to provide a targeted delivery of an anti-inflammatory drug to the site of inflammation.

- glycosaminoglycans, which are polydisaccharides derived from connective tissue, as inert biocompatible carriers.[36] Drugs can be attached to the polymer backbone through derivatization with a variety of functional groups. This approach provides control over the rate of drug release depending upon whether the derivative bond is attacked rapidly by hydrolysis or over a longer period of time by enzymatic action. Since the glycosaminoglycans are not species-specific, these types of prodrugs would be free of the immune reactions possible with some other polymeric carriers.

- oxidized cellulose as a biodegradable carrier for α-chymotrypsin to provide prolonged enzyme activity and increased enzyme stability.[37] The enzyme is immobilized through an imine linkage and is protected from proteolysis until the matrix decreases by hydrolysis. This system can be utilized for other compounds containing amino groups.

- an alternating copolymer of vinyl pyrrolidine and maleic anhydride to form polymeric prodrugs of a number of drugs, including quinidine and the antineoplastic agent melphalan.[38]

Polymers of organometallic compounds have been proposed as drug delivery carriers for antitumor agents, such as platinum diamminodichloride.[39] Dextrans have been reviewed by Molteni with respect to their use as carriers for antibiotics, enzymes, peptides and metals through the formation of various linkages.[40]

Biomaterial Vehicles - In addition to the aforementioned polymer backbones, a host of other materials has been employed as biocompatible carriers for drugs in an attempt to provide better control over the rate and site of drug action. The list of these biomaterials is long and growing. In the book by Gregoriadis, albumin, antibodies, artificial cells, DNA, glycoproteins, erythrocytes, synthetic polymers and liposomes, as well as many others, are discussed.[32] The nature of the drug/carrier interactions range from true covalent bonds, to weak or ill-defined bonds, to simple entrapment or encapsulation of the drug by the macro-molecular material.

The development of tumor-specific monoclonal antibodies[41] has renewed interest in their use for targeting drugs to tumor cells.[42] Cell-specific delivery of ricin to leukemia cells[43] and of methotrexate to tumor cells[14] with monoclonal antibodies have recently been reported. Monoclonal antibodies containing radioactive metal chelates have now been used to permit tumor imaging by γ-ray scintigraphy.[44]

Insofar as other biomaterials are concerned, it should be noted that chitin,[45] fibrin film,[46] and lipoproteins[47] have recently been suggested for use as drug carriers.

Liposomes - Liposomes were previously reviewed in this series in 1979 by Papahadjopoulos.[1] He defined liposomes as being "vesicles composed of one or more lipid bilayers completely surrounding an internal aqueous space . . . usually composed of phospholipids . . . ranging in size from 0.5-5 μm." The phospholipid normally used in liposomes is lecithin. These phospholipid vesicles closely resemble biomembranes and liposomes were developed originally as model systems for biomembranes. The ability of liposomes to carry both hydrophilic and lipophilic substances led to their evaluation as drug carriers. Detailed discussions on the use of various types of liposomes have been presented recently by Weinstein,[9] Gregoriadis,[32] Juliano,[48] and Kaye.[49]

Liposomes have great potential as drug delivery vehicles, but they also have significant problems with respect to their manufacture as sterile and stable preparations on a large scale.[50] While many drugs have been incorporated into liposomes, not one has yet advanced past the clinical stage of development. Today, however, there is a greater awareness that the characteristics of liposomes can be adjusted by varying their size, surface charge and lipid composition. It is also safe to assume that problems relating to the manufacture and stability of liposomes can be resolved with time.

The recent literature contains reports on the use of liposomes by various routes of administration:

Parenteral Use - Liposomes containing methotrexate in monoclonal antibodies have been used to deliver drug to specific cells in vitro.[14] Liposomes have also been used to deliver methotrexate to tumors in vivo. The tumor was preheated by ultrasound to 42°C, the phase transition temperature of the liposomal bilayers, to promote release of drug at the intended site of action.[51]

Topical Use - Liposomes of triamcinolone incorporated into a gel resulted in improved local drug activity. Significantly higher concentrations of drug were found in the skin and significantly lower concentrations were determined in the blood than with the non-liposome control preparation.[13]

Ophthalmic Use - The uptake of penicillin G unilamellar liposomes into rabbit corneas was found to be highest from positively charged liposomes, less from negatively charged liposomes, and least from neutral liposomes.[52]

Oral Use - Liposomes of heparin administered by mouth significantly increased prothrombin times in dogs.[53] Liposomes of insulin administered by mouth to diabetic rats and rabbits provided unpredictable results in that they were effective in some but not all cases.[54]

Nanoparticles and Microspheres - Many of the aforementioned drug delivery vehicles such as macromolecular complexes and liposomes are in fact colloidal drug delivery systems. Other colloidal drug delivery systems which are intended primarily for injectable use include: emulsions, nanoparticles and microspheres.

Multiple emulsion systems (w/o/w and o/w/o) have been proposed as drug delivery system vehicles for parenteral use. Like liposomes, multiple emulsion systems hold much promise but as yet have little application, due to their inherent instability.[55] Attempts to resolve the problems associated with the preparation and stability of liposomes and multiple emulsions as drug delivery carriers probably contributed to the development of the solid colloidal sized systems, namely, nanoparticles and microspheres. In theory, these microparticulate carriers are small enough to permit parenteral administration and transport via the general circulation. Selective uptake of the drug carrier by various tissues would be obtained by controlling the size and shape of the particles, their surface charge and their hydrophobicity. In their review of the use of microspheres to target drugs, Illum and Davis stated that particles larger than 7 μm would be entrapped in the lungs, particles larger than 100 nm would normally be entrapped by the liver and spleen, and particles less than 100 nm would be taken up by bone marrow and tumors.[56]

Nanoparticles have been defined by Oppenheim et al. as "cross-linked, non-porous aggregates of naturally occuring macromolecules. They are submicron in size and can be stored as a freeze dried powder."[57] Nanoparticles normally range in particle size between 50 to 500 nm. They can be dispersed easily in water to form relatively stable dispersions that can pass through hypodermic needles. For

discussions on the properties and preparation of nanoparticles the reader is referred to Marty et al.[58] and Oppenheim.[59] Recent reports on the successful use of nanoparticles include the development of triamcinolone diacetate nanoparticles as a parenteral sustained release system.[60] The use of insulin nanoparticles to achieve a limited degree of gastrointestinal absorption has been demonstrated.[57]

Microspheres are discrete micron sized particles containing a drug and a selected carrier. Thus far, serum albumin has been utilized most commonly as the carrier material to form microspheres or beads. Sokolski and Royer reported the preparation of microspheres (100-200 μm) of progesterone in glutaraldehyde cross-linked bovine serum albumin.[61] Longo et al. reported the preparation of glutaraldehyde cross-linked human serum albumin (3-150 μm).[62] Other materials used to prepare microspheres include: modified cellulose[63] beads (40-160 μm) containing ^{131}I; polyacrylamide[64] beads of tetracycline (ca. 150-350 μm) and theophylline (ca. 350-600 μm); and polylactic acid[65] beads of local anesthetics (ca. 50 μm). Morris and Warburton reported the preparation of dry, free-flowing spherical microcapsules (3-50 μm) from w/o/w multiple emulsions.[66] Yoshioka et al. reported the incorporation of gelatin microspheres of bleomycin into a water-in-oil emulsion to form a sphere in oil-in-water emulsion (s/o/w) which provided prolonged release of bleomycin after injection.[67]

Widder et al. proposed the use of human albumin microspheres (ca. 1 μm) containing drug and a magnetizable material such as magnetite as a means of achieving localization of drug action through the use of externally applied magnets.[68] Since then, Morimoto et al. have been able to localize microspheres (ca. 1 μm) containing ^{125}I in either the lungs or the kidneys of mice using external magnets.[69] When external magnets were not used, the microspheres concentrated in the liver. Ibrahim et al. reported the development of magnetic polyalkylcyanoacrylate nanoparticles (ca. 300 nm).[70]

Devices, Implants, Infusion Pumps and Other Controlled Release Systems - Devices developed for use in drug delivery systems were reviewed in this series by Shaw in 1980.[2] These devices include the Ocusert® ophthalmic system, the Oros® oral system, transdermal systems, and infusion pump systems. A more detailed review of this topic was published by Theeuwes.[71] These systems find their most promising applications with drugs of low molecular weight or low daily doses. Thus far, the ophthalmic system for pilocarpine and transdermal systems for scopolamine and nitroglycerin have been marketed in the United States. The oral system which provides a once-daily dosage for indomethacin has recently been approved in the United Kingdom.[72]

Implantable drug delivery devices, including contraceptive implants, narcotic antagonist implants, magnetically controlled implants, devices to provide zero-order release of macromolecules, and implantable infusion pumps have been reviewed by Langer.[31,73]

The magnetically controlled devices use biocompatible polymers such as ethylene-vinyl acetate copolymers to form matrices containing drug and magnetizable materials. After implantation the rate of drug release can be adjusted by applying an external magnetic flux. Release rates have been increased as much as 200% by this method, probably as the result of alternating compression and expansion of channels in the matrix during periods of magnetic flux. This device offers the possibility of increasing or decreasing the release rates of a drug like insulin as required during the day by means of a programmed external magnet. Hsieh et al. have developed a laminated hemispherical device which is claimed to provide zero-order release for compounds with molecular weights up to 68,000 for up to 60 days.[74]

Infusion pumps, whether implanted or worn externally, are finding more frequent application in the treatment of diabetes, cancer and other conditions. A recently published article compares five different portable external infusion pumps designed for use with insulin.[75] Recent publications on totally implantable infusion pumps discuss their use with the antiarrhythmic drugs bretylium and procainamide[76] and with chemotherapeutic agents in the treatment of cancer.[77,78]

Other controlled release systems of interest include: a "hydrodynamically balanced system" for diazepam which is designed to float on the contents of the stomach while releasing drug for up to 10 hours;[79] a polymeric film (ethylene-vinyl-N,N-diethylglycinate random copolymer) in which the rate of diffusion of non-ionic species varies with the pH of the gastrointestinal environment;[80] the Pennkinetic™ system which utilizes polymer coated drug/ion-exchange resin complexes to provide sustained oral drug delivery for up to 12 hours;[81] and finally, an oral preparation which utilizes an acrylic-based coating to deliver drugs such as prednisolone and 5-aminosalicylic acid to the colon.[82]

III. Routes of Administration

Drugs which are inactivated or poorly absorbed by the gastrointestinal tract and drugs which are subject to extensive first pass metabolism following oral administration are usually administered parenterally. Attempts to overcome these problems have employed various body orifices and organs as alternative routes of administration for certain drugs. For a comprehensive discussion of drug delivery systems with respect to various routes of administration, readers are referred to the recent book, Novel Drug Delivery Systems by Chien.[6]

Recent developments relating to factors affecting drug delivery by these alternate routes will be presented according to the route employed.

Nasal - The administration of progesterone[83] and propranolol[84] by the nasal route was studied by Hussain and co-workers. They found that the nasal route avoided first pass metabolism effects and provided blood levels equal to intravenous administration and higher than obtained with oral administration. Prolonged propranolol blood levels were also achieved through the use of different salts of propranolol. Buserelin acetate, a synthetic nonapeptide with a molecular weight of about 1300, has been employed successfully as a contraceptive when administered by the nasal route.[85]

Navel - The navel appears to be a unique site for the application of drugs to avoid first pass metabolism effects. A recent report indicated that a bandage-type delivery device containing testosterone provided plasma drug levels for up to five weeks. Absorption of drug from the device was more rapid and more extensive when applied to the navel area than when applied to the forearm skin.[86]

Ocular - The Ocusert® Delivery System has been on the market in the United States for several years.[2] Following its introduction, several alternative systems for pilocarpine have been explored. These include the use of pseudo-latex drops which provides 24 hour activity,[87] a fibrin film system which provides 8 hour activity,[88] and a hydrophilic thermoplastic matrix.[89] Formation factors affecting pilocarpine activity include the evaluation of poloxamer gels[90] to promote longer octic contact time and the use of an ionophore, lasalocid, to enhance drug transfer through the ocular membranes.[91]

Readers with a specific interest in ocular delivery systems are referred to the book, Ophthalmic Drug Delivery Systems, by Robinson for detailed discussions.[92]

Oral Mucosal - Tablets containing nitroglycerin in an inert polymer vehicle have been marketed recently. When placed in the buccal pouch the tablets adhere to the oral mucosa and provide activity for up to 6 hours. This system, which can be utilized for other drugs, is described in a recent patent.[93] An oral mucosal dosage form of insulin has been reported to provide a limited degree of oral insulin absorption.[94]

Transdermal - Transdermal absorption was the subject of a recent international symposium. The proceedings of the symposium provide a review of the current state of the art.[95] The three transdermal nitroglycerin devices recently approved for marketing in the United States have been reviewed with respect to differences in their design.[96] Transdermal devices for clonidine[97] and nicardipine[98] have been reported to be under development. The use of adjuvants and modified vehicles to potentiate the transdermal absorption of clindamycin[99] and indomethacin[100] have been reported.

Rectal - The rectal, oral and intra-arterial administration of propranolol were compared by deBoer and co-workers.[101] They found that the rectal route permits drugs to enter the general circulation without first passing through the liver. Higuchi and co-workers studied the use of non-surfactant adjuvants to promote the rectal absorption of theophylline and lidocaine[102] as well as insulin and heparin.[103] Murakami and co-workers studied the use of enamine adjuvants to promote the rectal absorption of β-lactam antibiotics.[104]

Uterine/Vaginal - Intrauterine and intravaginal devices have been in use since the early 1970's to deliver contraceptive steroids to the target organ. The book by Chien contains individual chapters on intrauterine devices, intravaginal devices and devices for esterus control in animals.[6] A recent report by Morimoto and co-workers on the vaginal absorption of insulin from polyacrylic acid gels indicates that this route also has potential for the systemic administration of drugs.[105]

Conclusion - Drug delivery system technology has made remarkable progress in the past few years. Rate controlled drug delivery systems, designed on the basis of the pharmacokinetic and pharmacodynamic properties of the drug, are now a reality.[106] New Drug Applications for advanced delivery systems for several old drugs have been approved recently. The acceptance of these new systems by the medical profession and patients has stimulated interest in the application of this new technology to other drugs. During the next decade we can look forward to the development of new therapeutic systems for many drugs.

As we enter this new era of therapeutics it should be realized that drug delivery system technology will, of necessity, find application in the search for new drugs. A recent publication by Goldman suggests that the principles of rate controlled drug delivery should also be applied to the preclinical evaluation of the safety and efficacy of potential new drugs in animals.[7] In that way promising new drugs, which have poor therapeutic ratios when evaluated by non-rate controlled methods, could find possible acceptance and utility.

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Chapter 29. Enzyme Immunoassay

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Introduction - Enzyme immunoassay (EIA) is a technique closely related in concept to the technique of radioimmunoassay (RIA) developed by Yalow & Berson.[1] It was first described by van Weemen and Schuurs[2] and by Engvall and Perlmann.[3] Like RIA, it relies on the use of substances which bind the analyte of interest with high affinity and specificity. Enzyme immunoassay is one of a group of alternatives to RIA dubbed "non-isotopic" techniques and variations of the basic principle have been described by numerous acronyms: ELISA, EMIT, SLFIA, CELIA, ELA,TELISA, ELIA, IEIA, etc. Several excellent reviews of EIA and its variants are available.[4-10]

EIA was developed to avoid the problems of radioactive waste disposal and limited shelf life of radio-labeled reagents. Radioactive labels are useful because they have an inherently favorable signal-to-noise ratio. Enzymes are attractive alternatives to radioactive tracers because each enzyme molecule generates many molecules of product when exposed to substrate, thereby amplifying the signal and producing a favorable signal-to-noise ratio. Sensitivity in a properly constructed EIA can be comparable to an RIA.

This review will compare enzyme immunoassay to radioimmunoassay which was discussed previously in this series.[11] It will provide an overview of the variations on the technique along with their applicability and limitations. Recently developed alternative non-isotopic immunoassay techniques will be noted.

Basic Principles of Ligand Assay - The methods of RIA or EIA rely upon the binding of an analyte to specific binding substance, usually an antibody. Many RIA reviews are available and the concepts are often applicable to EIA.[11-16] Both assays can be carried out in competitive and non-competitive modes.

In a competitive assay, the analyte (antigen) from the sample competes with a known quantity of labeled analyte (tracer) for a limited number of antibody binding sites. After the competition has been allowed to approach equilibrium, the antibody is removed from the solution along with whatever has bound to it. The level of tracer bound to the antibody is inversely related to the amount of unlabeled analyte in the sample. Results from unknown specimens are compared with a standard curve constructed with known dilutions of unlabeled analyte. Mathematical methods have been developed which linearize the standard curve and allow easy calculation of unknown analyte levels.[15,17]

Engvall and Perlmann[3] and van Weemen and Schuurs[2] first used an enzyme molecule covalently linked to analyte as the tracer in competitive assays for IgG and HCG. Figure 1a describes how this principle is used. Ab is antibody, Ag is the analyte antigen complementary to the antibody and Ag-E is the enzyme labelled tracer. For simplicity, one ligand is shown immobilized. Enzyme label (E) converts substrate (S) to detectable product (P). Figure 1b is also a competitive assay in which antigen is immobilized. In this case enzyme-labelled antibody (Ab-E) is the

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tracer. Numerous methods have been used to couple enzymes to proteins [4,18,19] and techniques used to prepare protein conjugates of small molecules before immunization are applicable to the preparation of enzyme-labelled smaller molecules.[20]

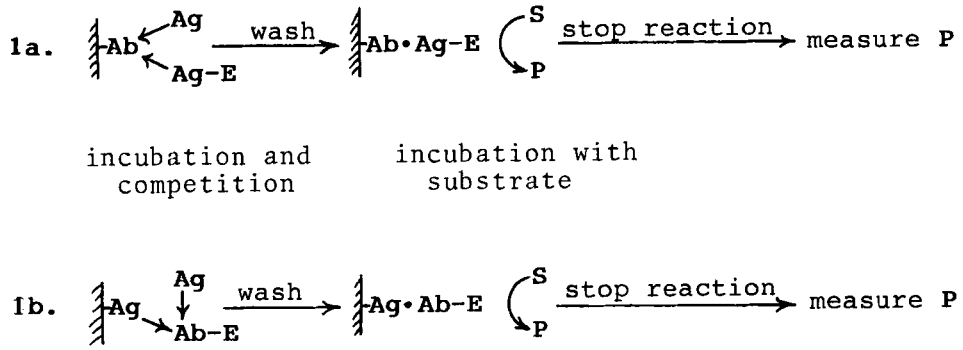

Figure 1: Competitive EIA approaches

The separation of antibody-bound tracer from unbound or free tracer must occur before the tracer concentration is determined. This is done either by immobilizing one of the ligands before the competition so that it can be physically removed afterward, or by precipitation/trapping afterwards.[4] EIA's require one step more than RIA: incubation with substrate. Thus immobilization before the competition is most often used as shown in Figure 1a and 1b.

An alternative to the competitive RIA is the immunoradiometric assay or IRMA technique.[13] In this method, the unknown is allowed to bind to antibody which is present in excess. The antibody-antigen complex is usually separated, and then incubated with a labelled second antibody against the antigen to form an antibody-antigen-labelled antibody "sandwich". In this case the second antibody is the tracer. This assay is frequently termed the "sandwich technique". Figure 2a describes the EIA version of IRMA, which has been applied to many analytes since its first description.[21,22] Version (a) is used to determine antigen such as a protein. Version (b) is used to determine antibody in the sample. Here a second antibody (Ab_g) is raised in goat against human IgG and binds to the analyte, human antibody (Ab_h) against Ag, in the sample.

IRMA or sandwich EIA's also require that the antibody-bound components be separated from the unbound or free components. Most separation methods used for RIA can be used with EIA. If the label is to be determined in the solid phase such as for sandwich methods, the ability to wash the solid phase to remove traces of liquid phase is essential. For such methods it is preferable to attach the first antibody to a solid phase, such as the interior of a plastic tube, the surface of a plastic bead, or to the surface of a readily sedimented particle which can be physically separated from the fluid components. If the label is to be determined in the liquid phase, bound phase must be completely precipitated, leaving an optically clear supernatant.

The selection of the enzyme to be used as the label depends on the sensitivity required for the assay, since the molar absorptivity of the product and the turnover number of enzymes are quite variable. [4,23] Peroxidase has been generally used in spectrophotometric assays, such as hepatitis B surface antigen,

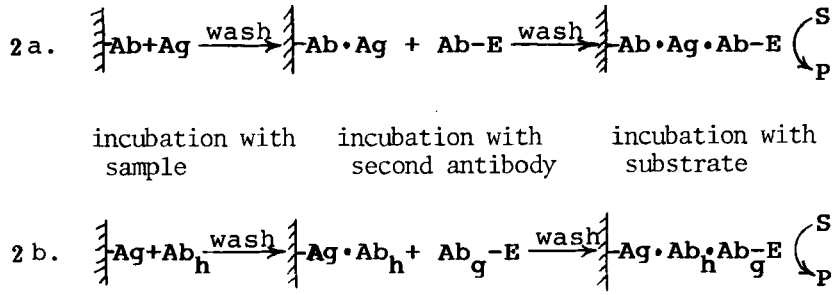

Figure 2: Sandwich EIA.

which require maximum sensitivity.[24,25] The substrate usually used is o-phenylenediamine. For assays requiring less sensitivity alkaline phosphatase/ p-nitrophenyl phosphate is often used. This has the advantage that the substrate is not light sensitive. Glucose oxidase/2,2-azino-di [3-ethylbenzthiazolin sulfonate-6] is reported to be superior to peroxidase/o-phenylenediamine.[26] Substrates which produce fluorescent products [27] have been used with alkaline phosphatase or beta-galactosidase labels.[28]

Determination of Macromolecules - Since high affinity antibodies to macromolecules can usually be easily raised, immunoassays are readily used to determine trace proteins. As the sandwich technique is easier to automate, this has been the method of choice in commercial assays. A good supply of purified antigen is required to synthesize the tracer if a competitive assay is to be used. Linking the analyte with a large enzyme molecule can also significantly alter its immunoreactivity. Moreover, sandwich assays are performed with the antibody concentration in excess. The procedures used to adsorb antibody to the solid phase do not easily produce reproducible antibody concentrations on tubes, beads or other easily washed carriers, [29,30] so it is much more difficult to produce a competitive assay having good precision.

Competitive assays also have thermodynamic limitations when some solid phase-supported antibody is used. Antibody is used in limiting quantities so the amount of tracer bound can be very small when the concentration of analyte in the sample is low. To increase the signal one must increase antibody concentration. The amount of antibody which can be bound to a plastic bead or tube is limited by the surface area of the solid support. Moreover, large solution volume to solid surface ratios result in tests with long incubation times because the slower diffusion rate of proteins leads to a slow approach to equilibrium. Competitive assays are often done with finely divided solid support to increase the surface area available and minimize the diffusion distance. Competitive assays with bound antigen and free antibody have been described for the determination of unbound antigen. The antibody can be labeled as the tracer in this case. [22,31,32]

Homogeneous EIA for macromolecules where no separation is required has been demonstrated using the EMIT technique described below for smaller molecules.[51]

Sandwich assays for macromolecules with separable antigenically distinct subunits have also been described using an antibody against one subunit on the solid

phase, and antibody specific for the other subunit as the tracer.[33] Monoclonal antibodies have also been used in the same way.[34] A variation on this approach for single-subunit proteins has been the use of two monoclonal antibodies directed against different regions of the same molecule.[35] In both cases, the tracer will not interfere with the binding of analyte to the solid phase so one incubation and one washing step can be eliminated.

EIA's have become well accepted tools in microbiology. Microbial antigens have been determined by using sandwich techniques for many medically significant virus infections.[24,26,30,36] Monoclonal antibody can be used to enhance the specificity of the test, [23] since the preparation of pure microbial antigen for immunization is often difficult.[37]

In most viral infections, viral antigen is complexed with excess circulating antibody and infection or immunity is diagnosed by detecting circulating specific antibody. EIA methods have proved useful for detection of antibodies, [26] especially to distinguished IgM antibody produced in response to a recent infection from IgG antibody present from a previous infection or immunization.[38] This is illustrated in Figure 2b. Viral antigen on the solid phase complexes with circulating antibody. A second labeled antibody specific to either IgG or IgM allows the class of the antibody to be determined. These assays are usually semiquantitative, but are a significant improvement over hemagglutination or complement fixation assays. Monoclonal antibodies allow immunization with impure antigen and selection from the resulting array of specific antibodies produced.[35,39]

Immobilized antigen EIA methods have been used to screen clones of hybridoma for antibody production. [40] EIA has also been used to detect carcinogen binding to DNA. Antisera raised to benzo[a]pyrene covalently linked to DNA were used in a non-competitive EIA.[78]

When the analyte is an enzyme, it can be used as its own tracer. The enzyme creatine kinase is composed of two subunits either "M" or "B". It is usually found as either MM or MB. Antibodies have been raised which selectively inhibit the M subunit. [41] The MB activity is estimated as twice the residual activity after inhibition of M activity in a homogeneous system. The MB isoenzyme has also been determined by precipitating it with immobilized non-inhibiting anti-B and then determining the enzyme activity in the precipitated fraction.[42]

Precipitating antibodies to the enzyme lactate dehydrogenase have been used to achieve a similar goal. The enzyme is composed of four subunits of the types M and H. The enzyme is found in the forms MMMM, MMMH, MMHH, MHHH and HHHH. Precipitation of four of the five isoenzyme forms can be achieved by adding anti-M, and the supernatant activity is only that of the HHHH isoenzyme.[43]

Determination of Small Molecules - The determination of small molecules by EIA can only be done by competitive binding assays, since these analytes generally lack the two distinct sites to which antibody can bind without interference. Homogeneous assay systems not requiring separation of bound and free ligands have been developed.

Conventional heterogeneous competitive EIA's have more steps and require more time to perform than RIA's, since at least two incubations are required, one with antibody and a second with substrate. Nevertheless, the applicability of heterogeneous EIA to hormones, drugs and small peptides has been well documented. In principle, EIA is applicable to the determination of any small molecule analyte which can be labeled with an enzyme, so that the tracer and native analyte will compete for the antibody binding site. The length of the bridging group in the tracer, the nature of the carrier molecule used to elicit

antibody production and the position on the analyte where the bridging linkage is attached will affect the binding constants with the antibody.[19,44,45]

As drugs in physiological fluids are frequently determined in clinical laboratories, EIA's have been developed to meet this need.[46] Assays for thyroid hormones have received much attention because of the large volume of testing and the reasonably high concentrations in serum. [47,48,49] An assay to simultaneously determine T-3 and T-4 using two separate enzyme/substrate systems has also been demonstrated.[50] Heterogenous systems for aminoglycoside antibiotics,[28] digoxin,[51] methotrexate,[53] steroids, [53-56] and other hormones [57,44] have been reported. Monoclonal antibodies have been used to reduce cross-reactivity problems.[58]

The ultimate goal of EIA development has been to eliminate the need to separate the tracer which is bound to antibody from the unbound tracer. This would make EIA more convenient than RIA which always requires a separation step. Several such "homogeneous" alternatives have been developed into successful commercial products in part because of their applicability to the determination of circulating therapeutic drug levels. Most therapeutic drugs are found in the microgram per milliliter range in serum; this is well above the level of most hormones, but within the detection range for several homogeneous assay systems.

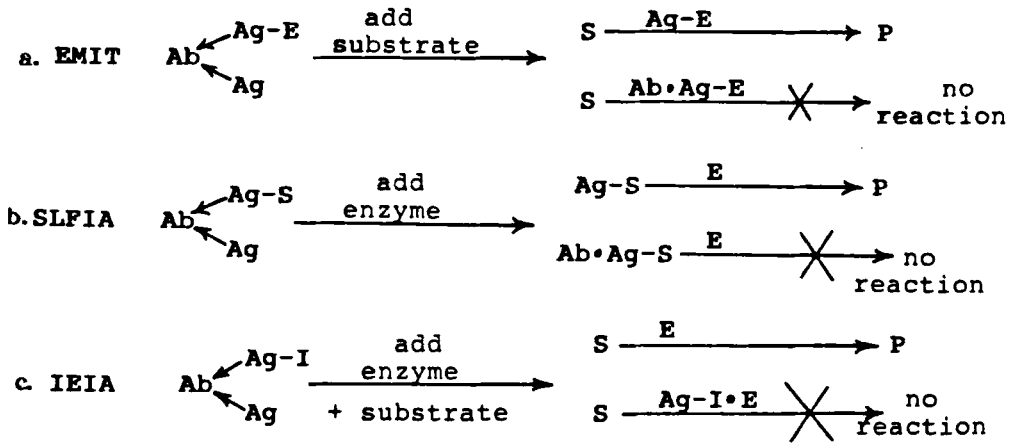

Figure 3: Homogeneous EIA methods.

EMIT (enzyme multiplied immunoassay technique, Figure 3a) is the prototype homogeneous EIA system.[59,60] Analyte molecules are covalently linked to enzyme, such that one or more are near the enzyme active site. In one version, anti-analyte, when mixed with this enzyme, binds to the analyte thus sterically hindering access of substrate to the active site.[61] Free analyte from sample also occupies the antibody binding site, so the amount of active enzyme is proportional to the free analyte in the sample. No separation of bound from free phases is required. The effect can be enhanced by using macromolecular substrates [62] and has even been applied to macromolecule determination. [63] The most extensive application has been to determine circulating or urine levels of therapeutic or abused drugs such as tobramycin, [64] valproic acid, [65] phencyclidine, [66] and cannabinoids, [67] among many others.[60]

Several homogeneous alternatives have been demonstrated. SLFIA (substrate-linked fluorescent immunoassay, Figure 3b) uses drug covalently linked to a substrate. An enzyme is capable of reacting with the drug-substrate to form a

fluorescent product. The enzyme will not react with drug-substrate bound to antibody.[68]

Systems have also been described which rely on an enzyme inhibitor or modulator coupled to the analyte. These systems have been dubbed IEIA (inhibition enzyme immunoassay, Figure 3c) or EMIA (enzyme modulated immunoassay). In these techniques, the complex Ab·Ag-I does not affect the enzyme activity, presumably due to steric hindrance. Unbound Ag-I irreversibly inhibits the enzyme. Its residual activity is inversely proportional to the concentration of free Ag from the sample.[69,70,79]

Analogous assay methods in which coenzyme is coupled to the analyte have been described.[71] In these systems the antibody-bound modulator-analyte is incapable of affecting the activity of an enzyme in the solution, so the enzymatic activity observed is related to the amount of free analyte in competition for the antibody binding site.

Alternatives to EIA - Enzyme immunoassay is a clearly useful technique. It uses readily available equipment, and the amplification properties of the enzyme label give it excellent sensitivity. An enzyme label has the disadvantage that one must incubate it with substrate to obtain a signal, and many alternatives to enzymes as labels have been described including electron spin labels, chemiluminescent and bioluminescent compounds and live virus particles.[5,72,73]

For routine testing in clinical situations, fluorescent labels have the advantage of no radioactivity, extensive shelf life and no need for incubation with substrate. Homogeneous techniques, such as fluorescence polarization for small molecules[74,75] and fluorescence quenching[76,77] for both large and small molecules, appear to be well suited for routine use provided adequate instrumentation is available.

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Chapter 30. Progress in the Development of Radioimaging Agents

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Introduction - The history of the development of radioimaging agents has been reviewed,[1,2,3] and several authors have summarized the mechanisms which operate to explain the in vivo partitioning of radiopharmaceuticals.[4-10] One recent classification system views radioimaging agents as either substrate non-specific (the compound does not participate in a specific chemical reaction) or substrate specific (the substrate must participate in a definite chemical reaction or take part in a specific ligand-substrate interaction).[8] Radioactive gases for lung ventilation, labeled cells as blood pool or spleen imaging agents, tagged particles for delineating capillary beds and radiocolloids for uptake in the reticuloendothelial system are examples of substrate non-specific radiodiagnostics. Enzyme substrates or inhibitors, receptor binding ligands, metabolically trapped biomolecules and their models, and antibodies to site-associated antigens are prime examples of substrate specific radiopharmaceuticals. Although research still continues in the development of new substrate non-specific agents, the attention of synthetic medicinal chemists has been largely directed to the substrate specific class.[9]

Overview of Commercial Radiopharmaceuticals - Since the first NDA (I-131 NaI) was approved in 1951, more than 50 medically diagnostic radiotracers have achieved that status. Today 48 are available. Technetium labeled (Tc-99m) ligands, colloids and particles predominate on the NDA list, but many of these substances would be classified as substrate non-specific agents. A few organic radiopharmaceuticals, both substrate non-specific and substrate specific, have achieved commercialization; these include radioiodinated rose bengal, fibrinogen, oleic acid, human serum albumin, hippuric acid, as well as radiomercury chlormerodrin and Se-75 selenomethionine.

Commercially available items, however, by no means represent the limit of clinically employed radiopharmaceuticals. Many new agents are employed diagnostically in major medical centers, often synthesized and used on-site under the broad investigational prerogatives available to clinical researchers. The prospects for manufacturing and shipment by pharmaceutical companies of agents containing positron emitters (e.g. C-11, $t_{1/2}$ = 20 min; N-13, $t_{1/2}$ = 10 min; F-18, $t_{1/2}$ = 110 min) may be slim indeed, but the growing access to medical cyclotrons and the significant diagnostic value of such agents mean that their clinical use is not only large but expanding.

Contemporary research concerns of radiopharmaceutical chemists are the development of convenient and rapid labeling methods for introducing radiohalogens into biologically important carrier molecules, preparation and evaluation of radiohalogenated imaging agents, synthesis and structural studies on metal ion radionuclide-containing pharmaceuticals, rapid incorporation of positron-emitting isotopes into imaging agents, antibody transport of imaging nuclides to target tissues, and quantitative

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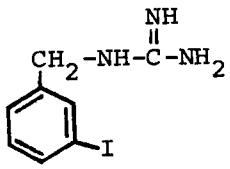
structure-distribution studies of radioactive diagnostics.

Radiohalogenation Methods - In radiopharmaceuticals which localize by receptor-substrate interaction, the specific activity of the administered agent must be as high as possible.[11] Much early work in radiopharmaceutical synthesis was done with radioiodide diluted with sufficient I-127 iodide so that final products could be isolated and purified.[12a] Because image quality is diminished by receptor-competing non-radioactive species, radiopharmaceutical syntheses are now performed with no carrier added. Radioiodination techniques have been reviewed.[12b]

Classical thermal or photolytic halogen exchange methods which yield a product inseparable from unreacted starting material are now largely avoided if an alternative labeling method exists.[12-14] However,

$$Ar\text{-}X + {}^*X^- \longrightarrow Ar\text{-}{}^*X + X^-$$

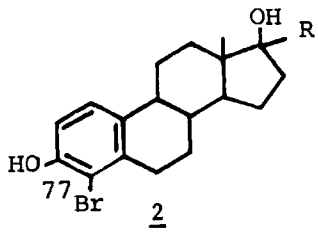
the recent reports that a solid-phase thermally induced exchange between radioiodide and aryl halides can be done with high radiochemical yields by adding ammonium sulfate to the medium renews interest in this simple labeling method.[15] Thermal halide exchange labeling is suitable for non-activated aryl rings and directs the incoming nuclide to a position which might not be accessible to direct electrophilic substitution. Ammonium sulfate catalysis of such exchanges succeeds under sufficiently mild conditions that thermally labile functions in the molecule survive. Specific activities up to 100 Ci/mmol are obtained in the labeling of m-iodobenzylguanidine (1) with I-125 iodide.[15]



1

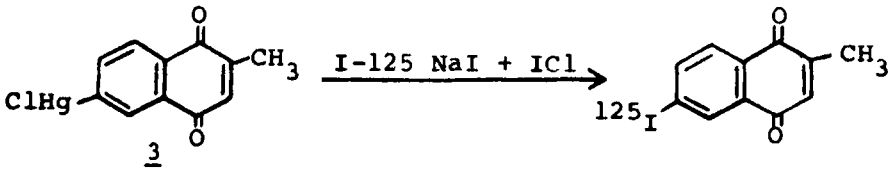
Long-chain, ω-radioiodinated fatty acids have been prepared by a similar exchange from ω-bromo fatty acids. Conditions studied included refluxing in acetone or toluene, melt, or phase-transfer catalysis. Melt condensation gave high specific activity with radiochemical yields of about 80% in five minutes of contact, and appeared the simplest of all methods evaluated.[16]

Reactions in which the labeled products are of different mass and/or polarity than the precursors lend themselves to the preparation of high specific activity product because the "hot" compound can be chromatographically separated. In a study on the radioiodination and radiobromination of anisole catalyzed by several N-chloroamides and imides, chlorination could be suppressed by working in anhydrous solvents.[17] Specific activities of 600-1200 Ci/mmol could be obtained in the synthesis of Br-77 4-bromoestrogens (2) by direct bromination of the steroid with Br-77 NaBr and N-chlorosuccinimide.[18]



2

Electrophilic ipso substitution can direct a radiohalide to a deactivated position, but usually requires the presence of a competing halogen source in the oxidant that can lower the specific activity of the product. Substitution of radioiodine on a chloromercurated quinone (3), and of radiobromine or radioiodine on m-trimethylsilyl toluene (4) gave products which could not be obtained by direct electrophilic substitution.[19,20] Br-82 6-bromocholesterol could not be obtained by Br-for-Br exchange with the non-radioactive precursor in numerous



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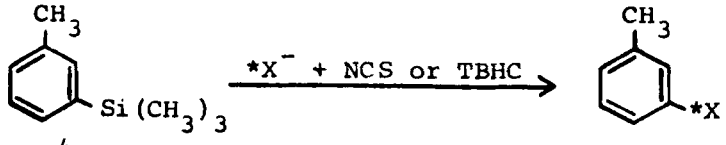
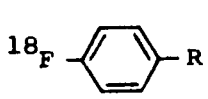


4

NCS = N-chlorosuccinimide
TBHC = t-butylhypochlorite

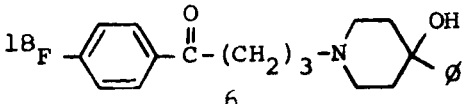
solvents or in a melt, but treatment of 6-chloromercurycholesterol with Br-82 Br_2 resulted in a 46% radiochemical yield and 95% radiochemical purity.[21]

Another route for radiohalogenation at unactivated carbons, which does not require the presence of a non-radioactive halide source, is the Wallach modification of the Sandmeyer reaction.[22-26] Tewson and Welch have shown that simple aryl fluorides (5), haloperidol (6) and spiroperidol (7) can be prepared from the triazenes (8) trapped from the diazonium ion precursors.[22-24] Radiochemical yields were low and variable (0.1 to 50%) with choice of aryl amine, solvent, and acid, but specific activities were high ($\sim 10^5$ Ci/mmol).[22]



R = H, CH_3, CN, $CO(CH_2)_3Cl$

5



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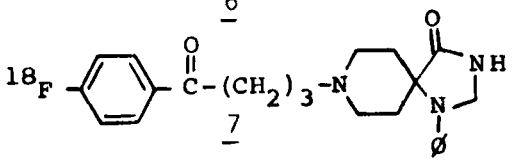

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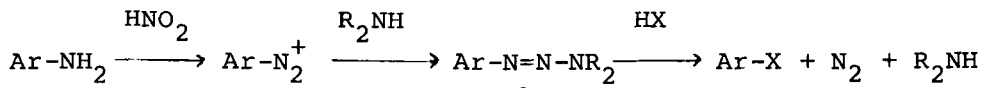
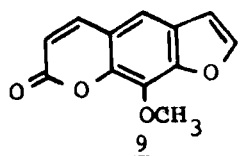


8

Radiofluorination methods[24] and methods of producing F-18 have been reviewed.[25] A radiofluorination exchange on aryl fluorides and a displacement of chloride or trimethylammonium groups from purines by F-18 fluoride have shown utility as labeling methods.[26,27]

The Wallach reaction has been applied to radioiodination of simple aromatics and the pharmaceuticals methoxsalen (9) and 1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (10).[28,29a] Radioiodinated products were obtained at no-carrier added levels which could not be obtained in the parallel Sandmeyer reaction.[29b]



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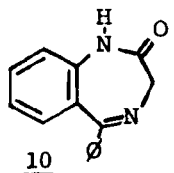
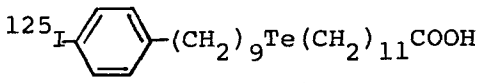


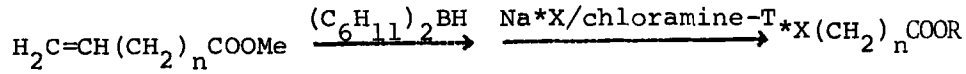
10

I-125-15-(p-iodophenyl)-6-tellurapentadecanoic acid (11), a compound which displays the biodistribution of a typical fatty acid, was prepared by the Wallach technique.[30] 1-Aryl-3,3-diethyltriazenes were radioiodinated and radiobrominated by decomposition of the triazenes with trimethylsilyl halides.[31]



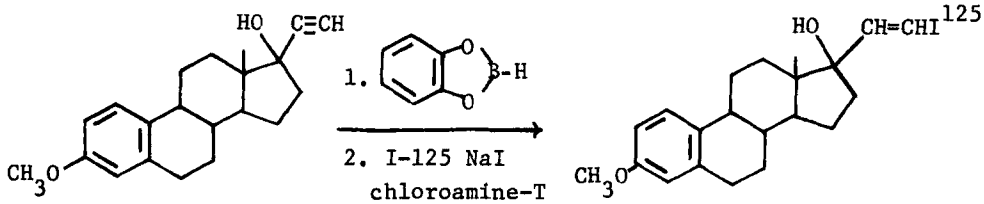
11

For substitution at an aliphatic carbon, a non-carrier-added radiobromination of primary and secondary alcohols was reported with Br-77 and trimethylsilyl chloride and used to prepare Br-77 12-bromododecanoic acid.[32] A versatile synthesis of radioiodinated or radiobrominated alkyl, vinyl, or aryl compounds involves the hydroboration of an olefin[33] and the subsequent treatment with radioiodide or bromide in the presence of chloramine-T.[34,35] Fatty acids (13) and esters (12) were isolated in 46-79% radiochemical yields on the no-carrier-added scale. The precursor vinyl and aryl radiotracers were prepared from vinylboronic acids or



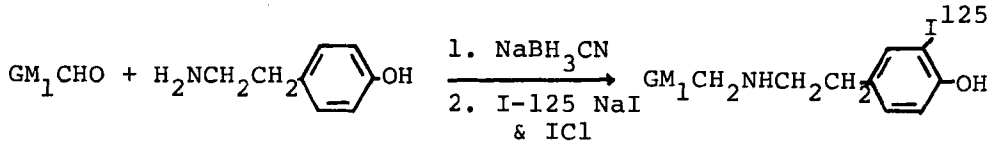
12, n=10, R=Me 13, n=18, R=H

arylboronic acids previously synthesized by transmetallations or catecholborations.[36] A radioiodinated derivative (14) of mestranol was also obtained. Although carrier halide was present in the labeling of these boronic acids, one preliminary experiment at no-carrier-added level was successful.[35]



14

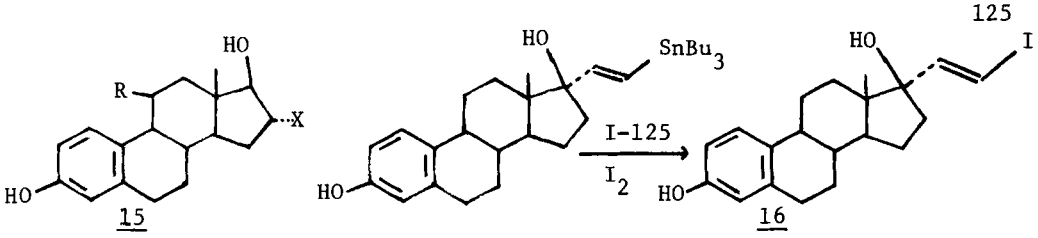
Several indirect radioiodination methods have been developed in which a pendant functionality carries the label. Ganglioside GM_1 was oxidized with galactose oxidase to introduce an aldehyde, condensed with tyramine, reduced, and radioiodinated. The method may have general utility for carbonyl containing biomolecules.[37] Similarly, radioiodinated 2,4,5-triiodobenzoate, phenylalkanoates and iopanoate esters of cholesterol, pregnenolone, and dehydroepiandrosterone were prepared.[38-40] While these compounds did undergo *in vivo* hydrolysis of the label, their biodistribution indicated promise as site-directed imaging agents for ovaries and



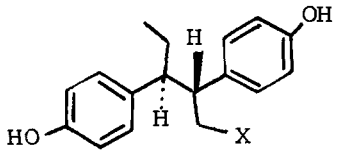
adrenal cortex. Exposure of tyrosine, chloroquine, and bovine serum albumin to Kr-76 and Kr-77 resulted in low efficiency (<1%) excitation labeling with Br-76 and Br-77 respectively.[41]

Radiopharmaceuticals for Imaging the Estrogen Receptor - Several recent reviews have appeared on synthesis and evaluation of estrogenic radioligands.[42-51] The objective of these studies is a receptor specific radiodiagnostic for tumors arising in tissues bearing estrogen receptors. Since the estrogen receptor in vivo is characterized by a high-affinity binding constant ($K_a \sim 10^9$) and a low receptor density (0.1 to 10 nM), a very high specific activity agent is required. A review on receptor-binding radiopharmaceuticals discusses the estrogen and other receptor systems.[52] Non-specific binding is a critical factor in radiopharmaceuticals for the estrogen receptor, and recommendations have been made on steroidal modifications which "design away" from these sites.[43]

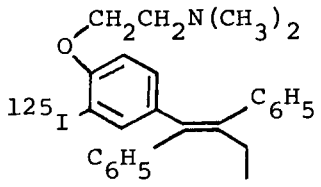
While candidate radiopharmaceuticals have been prepared with radioiodine,[47,48] radiobromine,[45-47] F-18,[53] C-11,[49] radioselenium [54] and other nuclides, the most promising agents to date are 16α-[Br-77]bromoestradiol-17β (15, R=H; X=Br) and 16α-[Br-77]bromo-11β-methoxyestradiol-17β (15, R=OMe; X=Br).[45,46] Earlier studies with labeled estrogens and antiestrogens have been summarized.[44] Preliminary clinical studies with (15, R=H; X=Br) have been encouraging,[45] but the potential advantage of using I-123-labeled agents (shorter $t_{1/2}$, better gamma energy) prompted the synthesis of radioiodinated 16α-iodoestradiol-17β(15, R=H; X=I).[47] Although the biodistribution was favorable, the 24-hour synthesis time precluded the use of an I-123 label ($t_{1/2}$=13 hr). HPLC isolation was required to prepare the analogs of (15). An alternative compound, 17α[I-125]iodovinylestradiol (16) could be prepared in minutes; biodistribution in immature female rats showed a uterine uptake and a uterus-to-blood ratio comparable to or better than 15 (R=H; X=I).[48]



Non-steroidal estrogens (hexestrols) and antiestrogens (tamoxifens) have been prepared and evaluated for uterine uptake.[50,51,55,56] The hexestrols (17) display receptor-mediated uptake into uterine tissue, but proved too labile toward in vivo dehalogenation to merit further investigation.[55] An I-125 iodotamoxifen (18) gave uterus-to-blood ratios in immature female rats of 23/1 at 6 hours postdosing, but the impressive ratio was more the result of low blood concentration than high target tissue uptake.[56]



17: X=I-125; Br-77



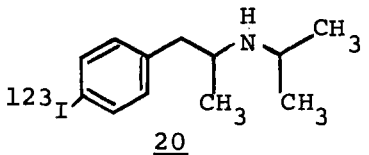
18

Fatty Acid Analogs - From 1975 to the present more than fifty references have appeared to new analogs of fatty acids tagged with radioiodine, bromine, fluorine, selenium, tellurium, technetium and carbon.[57,58] Two recent reviews summarize the pre-1982 studies.[59,60] Two new tellurium-

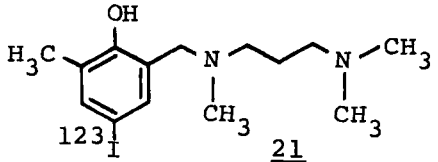
containing acids (11) and (19) appear to be retained in the heart longer than simple ω-iodinated fatty acids.[30,61] A radioiodinated phenylsulfonamide attached to a fatty acid has also been prepared.[60]

$^{131}I-(CH_2)_8Te(CH_2)_7COOH$ (19)

Radiolabeled Aryl-and Aralkylamines for Brain Imaging - Synthesis and evaluation of over 50 aryl-and aralkylamines led to the selection of I-123 N-isopropyliodoamphetamine (20) for clinical trials; these studies indicate that (20) is useful in assessing regional brain perfusion.[63-65] An isotopic alternative, the I-131 counterpart of (20), has been synthesized by a modified Leuckart reaction on p-iodoacetophenone and labeled by exchange with radioiodide.[66] Se-labeled diamines, I-123-labeled N,N,N'-trimethyl-N'-[2-hydroxy-3-methyl-5-iodobenzyl]-1,3-propanediamine (21), and a series of I-125-labeled xylylenediamines have shown marked uptake in rat brains.[67-69]



20



21

Other Enzyme Inhibitors and Substrates - Radiolabeled inhibitors, especially for adrenomedullary and adrenocortical enzymes, remain an active area of investigation.[70-72] Improvements in producing F-18 2-deoxy-2-fluoro-D-glucose have appeared,[73,74] and two new synthetic methods have been advanced.[75,76] This agent is finding clinical utility in detection of tumors, strokes and regions of altered glucose metabolism in man.[77,78] F-18 2-Fluoro-2-deoxy-D-mannose has also been prepared and evaluated as a tumor-seeking radiopharmaceutical.[79]

C-11 Radiopharmaceuticals - The short $t_{\frac{1}{2}}$ and the ability to prepare inherently labeled biological molecules have made C-11 chemistry unique. The early work has been reviewed,[80] and while some improvements are still being made in microscale syntheses,[81] remote control procedures,[82,83] and semiautomated chemical processing,[84] most investigations focus on synthesis of new agents. C-11 Spiroperidol, a dopamine antagonist for brain imaging, has been prepared from C-11 HCN, and C-11 1,3-bis (2-chloroethyl)nitrosourea was obtained from C-11 phosgene.[85,86] Both agents were chromatographically isolated after less than 40 minutes synthesis time. β-Methyl[1-C-11]-heptadecanoic acid was prepared by carbonylation of the requisite Grignard.[87] C-11 S-adenosylmethionine was prepared enzymatically from C-11 methionine and then incubated with methyltransferases to yield C-11-methoxytyramine and [C-11-methyl]adrenaline.[88] Other C-11 biomolecules for which syntheses were reported are [N-7-methyl-C-11]caffeine and [1-C-11]pyruvic acid.[89,90] A racemic C-11 valine, developed as a pancreatic imaging agent, has been resolved on a reverse phase HPLC column using a chiral mobile phase.[91] The method appears adaptable to other C-11 amino acid racemates.

Technetium-99m Radiopharmaceuticals - Technetium-99m is the preferred nuclide of nuclear medicine research due to its short $t_{\frac{1}{2}}$ (6 hr), favorable emission (140 KeV), and simplicity of chelative attachment to ligands. Virtually all of the Tc-99m complexes prepared to date distribute by substrate non-specific processes depending mainly on lipophilicity/hydrophobicity. Those cleared through the kidney have proven useful in evaluating renal disease, while those excreted by a

hepatobiliary pathway are useful in assessing liver function.[92,93] Burns noted that relative liver uptake of a series of Tc-99m iminodiacetate complexes correlated with a modified partition function,[94] and others have reviewed similar correlations which relate charge characteristics to hepatic uptake.[10,95,96]

Technetium-99m chelates of N-pyridoxyl-5-methyltryptophan,[95] of 2-[(9-thioethyl)imino]diacetic acid,[96] and of napthalene-like iminodicarboxylates all displayed biliary excretion patterns.[97] A highly soluble Tc-99m dextran complex has been prepared and proposed as a blood pool imaging agent.[98] Structural studies have been reported on several Tc-99 complexes containing sulfur and nitrogen ligands.[99-101]

Other Metal Ions in Radiopharmaceuticals - Lipophilic complexes of indium have been used to label platelets, leucocytes and liposomes for diagnostic and therapeutic purposes. Acetylacetone,[102] tropolone,[103] and 8-hydroxyquinolone[104] have all been studied as labeling reagents for indium. Tropolone has also been used as a lipid solubilizing agent for Ga-67 and Fe-59.[105] A new efficient method for labeling biologically important proteins with Ga-67 uses deferoxamine as a bifunctional chelating agent. The human serum albumin complex thereby labeled shows less deteriorating effects of conjugation and labeling than does the conventionally labeled radioiodinated agent.[106]

Osmium metallocenes have been prepared by thermal exchange between Os-191 $OsCl_4$ and ferrocene derivatives,[107] and a preparation of Re-186 perrhenate as a candidate imaging agent has also appeared.[108] Metalloporphyrins bearing radioindium and radiogallium have shown promise as tumor and lymph node imaging agents.[109] Syntheses and striking node incorporations with In-111 labeled tetra-(N,N,N-trimethylanilinium) porphyrin and tetra-(N-methyl-4-pyridyl)porphyrin in rats and rabbits have been reported.[110-111]

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Chapter 31. Mathematical Models for Toxicity Evaluation

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Impetus for research on alternate methods of toxicity evaluation arises from two sources--first, from groups desirous of reducing the use of animals in research and testing, and second, because animal testing is costly, the substitution of models for animal testing promises significant economic advantages. Both of these reasons have come to the fore in recent years, and the Division of Research Resources of the National Institutes of Health (NIH) has begun to monitor progress in the general area known as "alternatives to animal research". This has followed a conference sponsored by the National Toxicology Program and NIH[1] held on that subject in 1981.

This review will cover those mathematical models which are under study with the goal of producing results comparable to those obtained by testing in animals. Bacterial cell culture studies, such as the Ames test or the many in vitro cell transformation studies, will not be included. Since several recent reviews in this series have dealt with QSAR and Drug Design, this field will not be covered.[2,3,4]

Methodology - Various statistical methodologies have been applied to the modeling/classification problem encountered when one has a number of objects, which are described by a number of descriptors, to classify the objects in terms of the descriptors. These methods include 2-group multiple regression analysis, principal components, discriminant analysis, and the linear learning machine. Despite individual preferences shown by workers for one or another of these methods, they are all related, have common bases in statistics, and can all be expected to provide similar, although not identical, classifications and to provide similar predictability.[5,6,7] The technological advances which have permitted real progress in these areas are the continuing simultaneous increase in capabilities and reduction in costs of computers.

Toxicity Models: LD_{50} - The first biological test to come under scrutiny, primarily from animal care advocates but also from leaders in toxicology research, was the LD_{50} test. The history of this test, including its development, methodology, limitations, and conflicting legal requirements from country to country, have been thoroughly reviewed.[8,9] A strong case has been made for its replacement by an approximate LD_{50} value, which requires many fewer animals for its determination. The importance of obtaining an LD_{50} value for any compound for which the value is in excess of 1 or 2 g/kg has also been seriously questioned.

Verification of the unreliable nature of experimental LD_{50} values was recently made in a study performed by the Commission of the European Communities, in which rat oral LD_{50} values for the same five test compounds were obtained experimentally by 65 testing laboratories. The results (expressed as the ratio of the maximal/minimal values obtained for each compound) varied from 3.66 to 11.89.[10] More rigorous control of the testing conditions using the same five compounds and 80 laboratories, reduced the ratios from 2.44 to 8.38. Such results bring into serious question the value of requiring oral rat LD_{50} values to be determined for regulatory purposes, by showing the improbability of obtaining or recognizing an "exact" LD_{50}.

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Independent of these developments, beginning in 1976 the first statistical model for estimation of oral rat LD_{50} values by regression analysis, was developed by Craig and Enslein,[11] using LD_{50} values recorded in the 1974 Toxic Substances List[12] for 425 diverse compounds, substructural features obtained from the U.S. Army CIDS system,[13] the molecular weights, and the n-octanol/water partition coefficients. Utility of the prototype model was limited and has been challenged,[14] but it demonstrated the feasibility of this approach. Refinements have resulted in a model based on 1,851 compounds for which LD_{50} values were reported in the computer version of the Registry of Toxic Effects of Chemical Substances (RTECS). This model employed substructural features which were assigned by a modification of the CROSSBOW system.[15] The model contained 83 substructural keys and the molecular weights; the partition coefficient was not used in its development. Results were reported from a study which used this expanded model to estimate oral rat LD_{50} values for 908 compounds, none of which were used to develop the model.[16] Cumulative distribution of residuals is shown in Fig. 1. As can be seen, 90% of the predicted values are within 1 log unit of the observed.

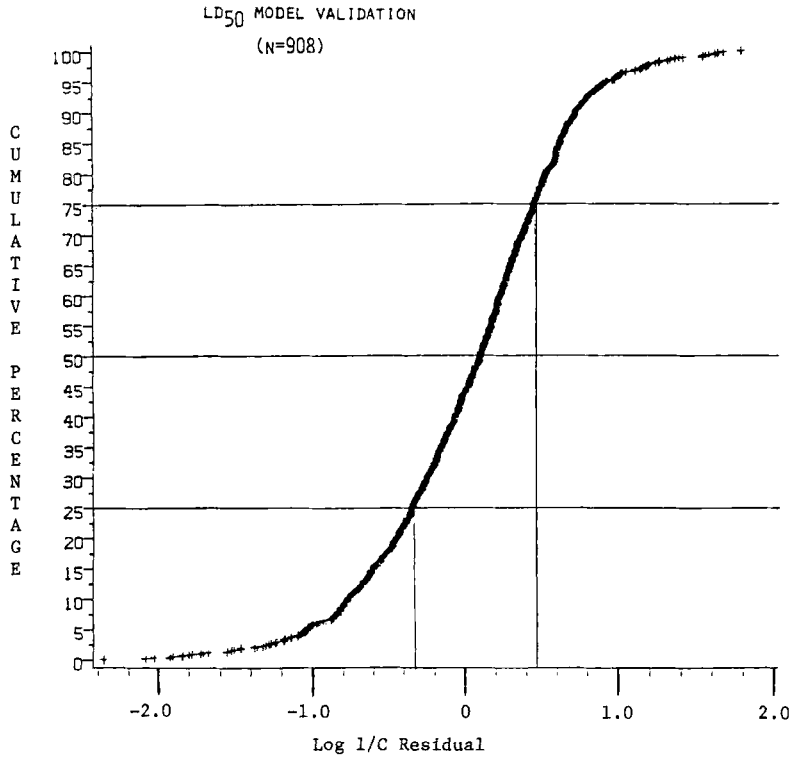

Figure 1.

Keeping in mind the established indefinite nature of experimental LD_{50} values when obtained in different laboratories,[10] the general correspondence between the estimates and experimental values is acceptable. Examination of the original literature for 18 of most poorly predicted values resulted in the identification of six major errors (of from 8- to 1000-fold) in the values reported in the computer version of RTECS (6/18 = 33% error). As a control, the original literature was checked for LD_{50} values for 20 of the 908 compounds for which the estimates were in excellent agreement with the RTECS values; no significant errors were found.

The actual uses to which the LD_{50} values are put should be reviewed in light of these findings. For many situations it may suffice to use the less expensive estimates provided by such a model in lieu of performing the animal tests.

A promising sideline development has been reported, using the same methodology which resulted in the LD_{50} model. With 160 test compounds, a model was obtained which estimates the rat oral LD_{50}, based on oral mouse LD_{50} values, eight substructural features and the partition coefficient.[36] This prototype model resulted in a slight, but statistically significant improvement over the use of the mouse data alone to estimate the rat LD_{50} value. Further refinements promise to assist the difficult problem of species-to-species translation of data.

Carcinogenicity - Many attempts to correlate carcinogenicity of polycyclic aromatic hydrocarbons by electronic indices, calculated by quantum chemical methods, are summarized by Neely[17] and Loew, et al.[18] Yuan and Jurs have reported the development of a useful linear discriminant based on pattern recognition methodologies, which relates the carcinogenicity of some 200 polycyclic aromatic hydrocarbons and heterocyclic analogs to 28 various structural, geometric and calcuated physical properties.[20] Norden, et al., have used the SIMCA (Simple Modeling of Chemical Analogy) principal components approach to relate carcinogenicity of 32 polycyclic aromatic hydrocarbons to 15 calculated quantum chemical indices and eight experimentally measured variables.[21]

Wishnok and Archer reported a simple correlation between the carcinogenicity of 47 nitrosamines and the number of carbon atoms in each molecule.[19] Nitrosamines as carcinogens have been studied by Dunn and Wold,[22] and by Rose and Jurs,[23] who present a useful linear discriminant analysis model based on physical and substructure attributes. Jurs and colleagues have also used the same approach to model carinogenic effects of aromatic amines[24] and diverse compounds.[25] Dunn, et al., have reported the application of the SIMCA method to a series of 4-nitroquinoline-1-oxides.[26] The carcinogenicity of a diverse set of 343 compounds evaluated by the International Agency for Research on Cancer was modeled by Enslein and Craig, using a discriminant analysis approach.[27] All the carcinogenesis models reported appear to correctly estimate from 75 to 90% of the chemicals used in their derivation.

Mutagenesis - Hansch and co-workers have reported the development of a QSAR equation which satisfactorily related the "Ames test" mutagenicity of a series of 15 organoplatinum analogs to an electron withdrawal parameter.[28] Hopfinger and Potenzone obtained a QSAR equation for eighteen 1-phenyl-3,3-dialkyltriazenes, which related well to an equation they developed for the antitumor potency of 24 analogs in the same series. Their analysis indicates the importance of the 3-dimensional shape of these compounds as a useful QSAR parameter.[29] Parodi, et al., have reported a statistical study of 21 compounds for carcinogenicity and mutagenic potency in the "Ames test".[30]

Kier, et al., described a good correlation between mutagenesis and molecular connectivity indices for a series of 15 nitrosamines.[31] Craig and Enslein reported a discriminant analysis model which relates "Ames test" mutagenicity of 416 diverse compounds to 48 substructural features.[32]

Tinker has developed a model for relating "Ames test" results of more than 100 diverse compounds to substructural features.[33] Based on the method of Hodes,[34] the model estimated the mutagenicity of 88% of 34 unknown compounds to within one level of activity out of a range of eight levels.

Teratogenesis - A teratogenesis discriminant analysis model was developed by Enslein and colleagues using substructural features from 426 compounds which were

evaluated by a panel of teratologists from 670 compounds reported as tested for teratogenesis in the literature.[35] The model correctly classified 78% of the compounds used in its development and incorrectly classified 13%.

Conclusion - Statistical estimates have not yet achieved general acceptance in the field of toxicology, but their increasing availability has compelled scientists to recognize that experimental toxicity data are also not exact end points; they are subject to considerable variation due to the many factors enumerated by Zbinden,[8] and are in themselves estimates. It may well be accepted soon that an estimate of an LD_{50}, which is based on a model built from a large database, may be as or more accurate than an experimental test run on only three or four animals at three or four doses.

The major role for these models at present is to aid in the setting of priorities to reduce the thousands of compounds as yet untested to a small manageable set of candidates for animal testing.

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Chapter 32. Applications of Recombinant DNA Technology of Interest to Medicinal Chemists

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Introduction: One of the most exciting scientific achievements of the last decade is the development of recombinant DNA technology. The techniques involved in cloning both eukaryotic and prokaryotic genes were reviewed in Volume 17 of *Annual Reports in Medicinal Chemistry*.[1] Physiologically important proteins, such as insulin, growth hormone and interferon,[2] have been cloned and produced on a large scale. In fact, human insulin obtained by recombinant DNA techniques has been recently introduced for clinical use. However, the most significant impact of recombinant technology on therapy is likely to come from a novel source: an improved understanding of medicinally relevant processes to aid chemists in the design of new types of medicinal agents. We will emphasize in this review the regulatory aspects of gene transcription, RNA processing, and protein post-translational processing, since these processes may offer opportunities for therapeutic intervention at regulatory sites in medicinally relevant systems. We will outline the use of recombinant technology to supplement classical techniques for study of the mechanisms of action of expressed proteins. Finally, we will discuss how recombinant DNA methodologies can improve our understanding of the etiology of, and suggest possible therapy for, inherited metabolic diseases.

Gene Transcription: Eukaryotic gene expression involves a sequential cascade of events beginning with the transcription of DNA into RNA in the nucleus, processing of the RNA into biologically active mRNA, transport of mRNA to the cytoplasm, translation of the mRNA at the ribosome into protein, and processing and/or assembly of the final polypeptide into a biologically active protein. Each step of this generalized cascade represents a point of regulatory control which may afford an opportunity for therapeutic intervention by new medicinal agents. Recombinant DNA technology will help to elucidate structural features of regulatory processes which may prove important to medicinal chemists for the design of such new agents. Aspects of regulation at the level of eukaryotic gene transcription have been recently reviewed.[3] As opposed to prokaryotes, direct regulation of mRNA transcription by protein interactions with specific DNA sites has been established in only a few eukaryotic systems. These include viral SV 40 early gene repression by T antigen protein[4] and puff induction with resultant transcriptional activation in the polytene chromosomes of *Drosophila melanogaster* (fruit fly) by the ecdysterone-receptor complex.[5] In most eukaryotic systems, however, it has been difficult to distinguish such transcriptional activation from the contributions of post-transcriptional RNA processing, mRNA stability, and enhanced translation to the observed increase in protein synthesis. Recombinant DNA technology has been a valuable tool in sorting out these factors, because it enables complete isolation of a gene of interest and measurement of levels of expression in normal and artificial cell systems. Gene fusion experiments have been especially important in this regard. Briefly, thse studies involve excision of the putative regulatory sequences of a cloned gene, their fusion to the structural part of a different cloned gene coding for a selectable marker, and assay of the predicted regulatory behavior of the resultant fusion gene in a transformed host cell. Mapping techniques can then be used in an attempt to delineate the DNA sequence in the regulatory region responsible for the observed behavior.

Some of the most interesting work which tests the role of eukaryotic DNA-protein interactions in regulating gene expression comes from studies of hormone-inducible cell systems. One example is the regulation of the metallothionein gene. Metallothionein is a mammalian protein involved in heavy metal detoxification and homeostasis. Its synthesis in the liver can be induced to very high levels by metal ions and/or glucocorticoids. The gene for mouse metallothionein-1 (MT-1) has been cloned and characterized.[6] The cloned sequence was initially used to show that levels of MT-1

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mRNA increased during induction by either cadmium[7] or the steroid hormone dexamethasone.[8] However, it has also been shown that MT-1 mRNA stability is affected by these agents in the normal cell.[7] Consequently, to completely isolate the components of the hormone/heavy metal induction, the gene and its regulatory regions were transferred to an artificial cell system.[9] It was found that MT-1 synthesis in the resulting cells was regulated by metals but not by glucocorticoids. The same result was obtained using a similar vector to transform mouse L cells, known to contain the appropriate glucocorticoid receptor. In addition, this study constructed a fusion gene using only the promoter region of the MT-1 gene with the Herpes simplex virus thymidine kinase (HSV TK) structural gene. TK production in mouse TK^- L cells transfected with a plasmid carrying the fusion gene was induced by cadmium, but not glucocorticoids, showing that MT-1 mRNA specific RNA processing or stability does not contribute to induction by cadmium. These studies suggest that glucocorticoid regulation of metallothionein is mediated by a trans-acting element, which is not transferred along with the cloned MT-1 gene. Further studies have involved microinjection of the MT-1/HSV TK fusion plasmid into mouse egg cells, with the result that the transformed cells' HSV TK was regulated again only by cadmium.[10] Gene mapping techniques were then used to establish that cadmium regulation depends on a region 90 bases upstream from the MT-1 transcription start site. The egg cells were then used to impregnate female mice. Ten percent of the resulting offspring produced HSV TK in their livers which was regulated by cadmium but not by glucocorticoids, illustrating the independence of the cadmium-regulatory sequence from environmental variables.[11]

A second system which has been studied to elucidate possible DNA sequences mediating steroid receptor binding involves cultured cells that have integrated sequences of the mouse mammary tumor virus (MMTV).[12] It has been shown that transcription of MMTV mRNA from the integrated genome is induced by dexamethasone in cells expressing the dexamethasone receptor.[13] The results of several studies suggest that, unlike the metallothionein gene, the MMTV genome (containing only 3200 bases) carries the steroid receptor binding sequence.[14] One likely candidate is a sequence at each end of the MMTV genome, termed the Long Terminal Repeat (LTR), which is not transcribed, but appears to be necessary for integration. To test its possible involvement in regulation, the MMTV-LTR was fused to the structural gene for mouse dihydrofolate reductase (DHFR).[15] When the resultant plasmid was used to transfect Chinese hamster ovary cells, DHFR production could be increased ten-fold by exposing the cells to dexamethasone. Further studies have measured specific binding of purified glucocorticoid/receptor complex to subfragments of MMTV DNA containing the LTR, in one case mapping the binding site far downstream of the 5' end of MMTV mRNA,[16] in another to within 100 to 150 bases of the transcription start site,[17] and in a third to a fragment covering positions -400 to -50 of the LTR.[18]Work is underway to delineate the exact DNA sequences involved in steroid/receptor binding, and to isolate other factors, such as chromatin, which may also modulate induction.

Similar techniques have been applied to the study of growth hormone gene expression. The rat and human growth hormone genes have been cloned and sequenced along with large stretches of contiguous genomic DNA. Both the rat and human genes are known to be regulated at the transcriptional level by dexamethasone and triiodothyronine.[19] One study has shown that human growth hormone synthesis in mouse fibroblasts transformed with the human growth hormone gene could be induced three to five-fold by steroids.[20] Consequently, a fusion gene was constructed by connecting a 500 base pair segment contiguous to the 5' end of the growth hormone structural gene with the marker HSV TK structural gene. Expression of the resultant TK gene was regulated by steroids, supporting the hypothesis that steroid/receptor binding normally mediates growth hormone induction. These studies do not rule out possible mRNA stabilization effects, however. Another hormone-induced system studied by similar techniques is $\alpha_{2\mu}$-globulin, a protein whose production in the liver is regulated by steroids.[21] Mouse TK^- L cells were cotransformed with cloned rat $\alpha_{2\mu}$-globulin DNA and the HSV TK gene.[22] Both $\alpha_{2\mu}$-globulin mRNA and protein were induced by dexamethasone in the resulting TK+ cells.

Recombinant DNA technology has enabled considerable progress on the elucidation of steroid/receptor DNA binding sequences in the chicken oviduct protein system. Each of the four major steroid-induced proteins, ovalbumin, conalbumin, ovomucoid, and lysozyme, is regulated differentially by the various classes of steroids, each class acting via its own receptor.[23] In the case of lysozyme, a gene fusion experiment similar to those described above indicates that the steroid

receptor binding site resides upstream from the 5' end of the gene.[24] Two groups have been involved in elucidating the sequence(s) required for steroid receptor recognition in these genes. One group isolated a series of restriction fragments from cloned genes for the various oviduct proteins and measured the ability of these fragments to specifically displace calf thymus DNA-cellulose from the progesterone receptor.[25] The smallest fragment shown to be active, 48 base pairs long, was analyzed via computer for sequence homology with the whole genes in an effort to prove its specificity for binding the steroid hormone receptor. One consensus sequence, ATC AT-T TCTG TTGTA, showed homology to a variety of presumptive regulatory sites of these genes, and may thus represent a common binding site for the steroid receptor. The second group measured the binding of oviduct gene fragments to highly purified steroid receptor subunit A, narrowing binding to a region 135 to 247 bases upstream from the start of transcription of the ovalbumin gene.[26] These authors proposed a different, A + T rich, sequence as the steroid receptor binding site. The synergistic combination of the A and B subunits (the latter does not bind DNA with high affinity but rather to a protein component in chromatin) may act to expose the ovalbumin gene to enhanced transcription.

Induction of interferon (IFN) by viruses and viral RNA is well known and recombinant DNA studies have suggested that this induction depends on sequences in the IFN genes. To prove this sequence dependence, a vector containing the human α_1-interferon (IFN-α_1) gene and the HSV TK gene was used to transform TK$^-$ mouse L cells.[27] The resulting TK+ cells produced incorrectly initiated IFN-α_1 mRNA. However, when these cells were induced with Newcastle disease virus (NDV), their IFN-α_1 mRNA was correctly initiated and polyadenylated. Similar studies with human IFN-β have shown that NDV and the duplex poly riboinosine-ribocytidine (rI.rC) induce increased amounts of IFN-β mRNA in mouse cells transformed with cloned IFN-β DNA.[28] In addition, mouse L TK$^-$ cells transformed with a cloned TK gene and a fusion plasmid constructed from the TK promoter and human IFN-β structural gene resulted in TK+ cells showing no constitutive expression of human IFN-β. IFN-β protein could be induced by poly (rI.rC), although its expression could not be subsequently shut off.[29] This result suggests that the IFN-β coding sequence alone directs the inducible synthesis of IFN-β, but not the mechanism that switches it off.

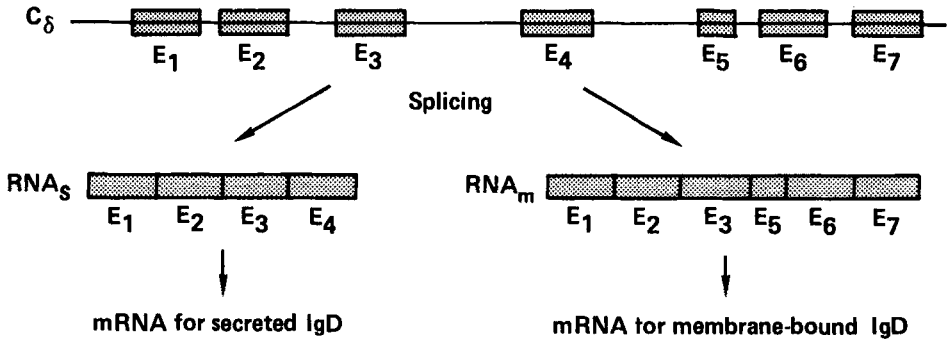
The structural aspects of the chromosome which any DNA binding protein must recognize are greatly influenced by two factors, in addition to the primary DNA sequence: chromatin structure and DNA methylation. Recent reviews have addressed the role of both factors in the regulation of gene expression.[30,31] It should be appreciated that eukaryotic DNA, as it exists in the cell nucleus, is packaged with protein in a highly complex structure, termed chromatin. The protein-DNA complex, comprising both histone and non-histone proteins, is almost completely refractory to the transcriptional machinery. Consequently, the specificity by which regions of DNA, in individual cell types, are decondensed and thereby made available for transcription is largely unknown. Two changes of chromatin structure have been shown to result from such an activation event: increased DNAse sensitivity and acetylation of histones associated with the activated region. Though many examples of both these changes have been documented, such studies have not provided evidence of how the transition is signalled. It may be that recognition of specific regions of chromatin is achieved via non-histone protein molecules which initially interact with incoming induction molecules, as mentioned above in the case of ovalbumin gene activation, by the progesterone receptor protein.

The degree of DNA methylation has long been implicated as a regulatory element in gene expression. The 5 position of approximately 5% of all cytosine residues in DNA is methylated, generally in the sequence CpG. One of the most definitive studies to date linking methylation to transcriptional deactivation involves the use of the hamster adenine phosphoribosyl transferase (aprt) gene cloned into pBR 322 with the HSV TK gene to cotransform mouse L TK$^-$ aprt$^-$ cells.[32] Cells transformed with unmethylated aprt DNA expressed the protein, while those transformed with a methylated aprt gene did not. The connection between methylation and gene expression is not always so clear, however, as is illustrated by studies in the metallothionein system discussed above. Earlier work showed that MT-1 DNA was methylated in non-expressing cells, but unmethylated in those expressing the protein.[33] But in studies discussed above, when the MT-1 genes of mice, whose mothers had been impregnated with egg cells carrying the fusion gene between the MT-1 promoter and the HSV TK structural gene were examined, there was no direct correlation between degree of DNA methylation and TK expression.[10] A recent study of expression of

the albumin gene in rat hepatoma cells found that undermethylation of the 5' end of the gene was necessary but not sufficient for expression.[34] The authors suggest that transcription may hinder methylation of a gene, so that the resulting undermethylation is an effect and not a cause of gene expression. DNAase sensitivity and acetylation of histones also seem to be necessary but not sufficient requirements for induction of transcription, indicating that they are the secondary results of other mechanisms which activate quiescent genes.

RNA Processing: Eukaryotic RNA must be processed before it can leave the nucleus and be translated into protein. It has been known for some time that this processing involves addition of a cap structure to the 5' end and polyadenylation of the 3' end. However, more recent evidence indicates that this processing is much more complex in that most genes require removal of one or more intervening non-coding sequences (introns) and splicing together of the coding regions (exons). RNA sequence information (largely from cDNA cloning) has indicated several common sequences involved in signaling processing events. A polyA tail is added to an RNA 15 to 20 bases downstream of the recognition sequence, AAUAAA. The exon splicing machinery recognizes consensus sequences of AG/GURAG and Y-YYY-CAG/ at the 5' and 3' boundaries between exon and intron, respectively (where / represents the boundary, R is A or G, and Y is C or U). The 5' exon's AG is then joined to the RNA following the Y-YYY-CAG sequence with excision of the intervening intron RNA.[35] A primary transcript may contain many alternate poly A and splice recognition sites, but, at present, the mechanism determining which ones are selected is unknown. It is this possibility of alternate splicing options, the use of 'exon-shuffling',[36] that has been shown to be one mechanism for generating a great diversity of polypeptide products. Control of splicing also affords the possibility of tissue-specific generation of a protein and regulation of its level of expression.[37] The small DNA tumor viruses, where introns were first discovered, are a good example of the use of differential RNA splicing to maximize usage of a limited genome.[38] A case which better illustrates the importance of RNA processing in medicinal systems is found in the expression of immunoglobulins.

The mechanisms which provide the tremendous diversity of antibody expression have been extensively reviewed.[39] The most relevant work in the present context has delineated the construction of the $C\mu$,[40] $C\delta$,[41] $C\gamma 2a$,[42] and $C\epsilon$[43] genes. The figure below illustrates this process in the case of $C\delta$. These heavy chain constant region genes are made up of several exons coding for structural regions (E_1 to E_3), and a C-terminal segment, which determines whether the antibody will be secreted (E_4) or membrane-bound (E_5 to E_7). RNA processing then not only joins the structural regions, but also makes the choice between secreted and membrane forms of the antibody. The process regulating this choice of alternate exon splices is unknown, although one recent study suggested that thymidine-rich regions of the genes may be involved.[44]



Though research in the area is still at an early stage, it is already clear that hormone and enzyme diversity can be generated by differential RNA processing. Recent studies of the processing of the calcitonin gene stem from the observation that mouse medullary thyroid carcinoma continues to make large amounts of calcitonin-reactive nuclear RNA even after it has switched to producing very low levels of the hormone.[45] The normal splicing protocol for this RNA in the thyroid uses the exon coding for calcitonin, which is subsequently cleaved post-translationally from its larger

protein precursor.[46] An alternate splicing event occurs in the hypothalamus, or in thyroid carcinoma tissue, to include a different exon coding for an unrelated polypeptide which is similarly excised from its larger precursor. Although the mRNA for this new polypeptide can be detected in the hypothalamus, the protein's function is as yet unknown. This example illustrates the tissue specific expression of different proteins from a single gene.

RNA processing may also be instrumental in controlling the level of a polypeptide in different tissues, as has been found for amylase in mouse liver and salivary gland.[47] The two amylase mRNAs isolated from these tissues, though transcribed from a common gene and coding for the same enzyme, contain different lengths of RNA at their 5' ends, resulting from the use of alternate exons during RNA processing.[48] This non-translated RNA is postulated to influence processing efficiency and/or RNA stability so as to account for the different levels of enzyme in each tissue. In addition, the mRNAs in each tissue consist of major and minor forms resulting from the use of alternate polyadenylation sites.[49] The fact that the ratio of major to minor mRNA is the same in both tissues suggests the possibility that the RNA sequences determine the frequency at which each polyadenylation site is used.

Variant forms of a known hormone are another possible result of alternate RNA processing events. The cloning of genomic human growth hormone DNA revealed the source of a related, though somewhat smaller, polypeptide found in the pituitary.[50] Splicing of the second exon of the human growth hormone gene to an alternate splice site, 45 nucleotides 3' to the normal end of the second intron, produces an mRNA coding for a polypeptide 15 amino acids shorter than growth hormone, consistent with the observed size of the related protein. The physiological function of this product remains unknown. Two forms of rat prolactin mRNA have been shown to result from splicing to different sites separated by three nucleotides, resulting in proteins differing by one amino acid residue.[51]

Post-translational Processing: As the initial product of transcription requires processing, so the initially translated protein is often proteolytically modified to produce the final polypeptide product(s). Proteins destined for secretion are synthesized with a hydrophobic signal or leader peptide at their amino terminus which guides the nascent polypeptide into and through the endoplasmic reticular membrane as a first step toward storage and ultimate secretion.[52] Once this function is accomplished, the signal sequence is proteolytically removed by signal peptidase. Many proteins are then glycosylated and/or proteolytically modified further before attaining biological activity. By determining the primary structure of protein's mRNA through cDNA cloning and sequence analysis, it is possible to predict the complete structure of precursor peptides and thereby unravel the details of processing events. The elucidation of these steps can also help establish whether they contribute to the tissue-specific expression of the proteins under study.

The precursor molecules for many of the small peptide hormones appear to undergo extensive post-translational processing steps. Since the processing pathway of mammalian insulin precursors had been worked out by classical techniques,[53] recombinant technology only confirmed these findings and established the signal sequence of the preprohormone.[54] However, the cloned gene has proven useful in studying cellular components necessary for the correct expression of the insulin hormone. An SV40 vector which contained a genomic DNA fragment comprising the rat I insulin gene was used to transfect monkey kidney cells.[55] The resultant lytically infected cells expressed the rat proinsulin peptide, indicating that they possessed the enzymes necessary to correctly splice the RNA's one intron, and remove the signal portion of the initial product of translation. However, it is clear that these cells do not possess the proteolytic activity necessary to remove the C-peptide. This post-translational processing activity could account, in part, for the tissue-specific expression of mature insulin in pancreatic B-cells. A similar system described recently, with general applicability to the study of gene expression in mammalian cells in culture, confirmed that processing of cloned rat genomic insulin DNA proceeded only as far as cleavage of the signal sequence.[56]

The cloning of cDNA from mRNA sequences expressed by the fish endocrine pancreas has proven to be a rich source of other pancreatic hormones.[57] The cloned cDNAs have been used to predict the precursor peptide sequences for both the preproglucagon[58] and preprosomatostatin[59] hormones. The somatostatin precursor peptide is 121 amino acids in length, the C-terminal 14

amino acids of which comprise the biologically active hormone. The fish preproglucagon peptide is of similar size (125 amino acids) with the 29 amino acid hormone centrally located within the precursor. Pairs of basic amino acid residues separate the hormone from the rest of the precursor peptide sequence, suggesting that, like insulin, the precursor molecule is processed post-translationally by a trypsin-like protease activity. Of further interest is the disclosure that a heretofore unknown glucagon-like peptide sequence is synthesized as a part of the glucagon precursor, and could also be cleaved from the precursor peptide at dibasic residues.[58] The function and/or biological activity of this peptide is presently unclear, but it could represent the fish counterpart of one of several glucagon-related peptides such as secretin, gastric inhibitory peptide, or peptide PHI. The important fact established by this primary sequence data is that the two hormones, in fish, are coordinately expressed. The combined results from several groups have also shown that, among other cloned sequences from fish endocrine mRNA, are cDNAs which encode other naturally occurring somatostatin analogues [59-61] and a pancreatic peptide-like protein which is closely analogous to peptide PYY. [62] The latter peptide has a glycine preceding its C-terminal dibasic residue cleavage site, an arrangement found in several other biologically active peptides such as progastrin[63] and procalcitonin,[64] which require an amidated carboxy terminal residue for activity. Subsequent cloning of the rat and human preprosomatostatin cDNA sequences has indicated that the large precursor peptide first seen in the fish is probably common to all vertebrates.[65,66] In addition, a larger somatostatin peptide (SS-28) which has been isolated from both porcine gut[67] and hypothalamic tissue[68] comprises the last 28 amino acids of both the human and rat precursors. The fact that both peptides are present in the same tissue suggests the possibility that the precursor molecule is differentially processed. The presence of this 28 amino acid peptide is significant, since it shows greater potency in inhibiting both pancreatic[69] and pituitary[70] endocrine function, possibly through a greater affinity for the somatostatin receptor.[71]

Perhaps the most elaborate post-translational processing of precursor molecules has been demonstrated for the opioid peptides expressed in both the central and peripheral nervous systems. Prior to molecular cloning, the best evidence for a multifactor opioid precursor was the pro-opiomelanocortin (POMC) peptide synthesized in the mammalian adrenal medulla and shown to contain Met-enkephalin, β-endorphin, β-melanocyte stimulating hormone (β-MSH), α-MSH, and β-lipotropin (β-LPH), in addition to adrenocorticotropin (ACTH).[72,73] Sequence analysis of the bovine POMC cDNA confirmed the presence of the above peptides within the single secreted precursor and, in addition, revealed the sequence of the "cryptic" amino terminal region shown to contain a third (γ) MSH.[74] Subsequent sequence analysis of both rat and human genomic fragments indicates the POMC precursor peptide is highly conserved, as is the general structure (the number and location of introns) of the gene.[75,76] However, there is good evidence that the Met-enkephalin sequence found in the POMC peptide represents only a small portion, if any, of biologically active Met-enkephalin expressed in mammals. A search for another Met-enkephalin precursor led to the sequencing of both the bovine [77, 78] and human[79] preproenkephalin cDNAs synthesized from mRNAs of the adrenal medulla. The preproenkephalin peptides contain four copies of Met-enkephalin, a single copy of Leu-enkephalin, and one copy each of two related but previously unknown peptides. A cloned cDNA sequence derived from hypothalamus mRNA has resolved the sequence of a single precursor for the porcine β-neoendorphin and dynorphin opioid peptides.[80] A cloned cDNA for bovine preproArg-vasopressin has revealed that the Arg-vasopressin sequence is located next to a sequence coding for its carrier protein neurophysin II.[81]

The structures of these opioid and pancreatic peptide precursors suggest some general facets of small peptide synthesis in mammals. First, they are almost all cleaved from their respective precursors by a trypsin-like proteolytic enzyme which recognizes dibasic residues. Second, their occurrence on a common precursor provides for the possibility of a coordinated response in effecting a known biological activity (e.g., modulation of pain by preproenkephalin[78]). A common precursor can also be processed in different tissues to afford various biological activities, as in the differential processing of the POMC precursor by the two lobes of the pituitary.[72] Finally, resolution of the primary sequence for several of these precursors has revealed other, often related, peptide sequences whose structure suggests a potential biological activity. The prediction that these previously unknown peptides are coordinately synthesized with known biologically active molecules provides indirect evidence that they may be involved in a particular physiological response. Although the mechanisms regulating post-translational processing are largely unknown,

a recent study using cloned MMTV gene probes has taken the first steps toward delineating the role played by glucocorticoids in this area.[82]

Expressed Proteins: While the classical techniques will continue to provide the basis for study of expressed proteins, recombinant DNA technology can make a substantial contribution in this field as well. An important technique in this regard is *in vitro* mutagenesis, various versions of which have been recently reviewed (see Chapter 26).[83] Briefly, this technology allows the production of new proteins by engineering specific changes in their cloned genes. For example, a single base change resulting in a gene coding for a new protein with a single amino acid substitution can be engineered by hybridizing a synthetic oligonucleotide carrying the new base sequence to single strands of the wild type gene. The remainder of the wild type gene is duplicated and ligated to the synthetic fragment under stringent conditions.[84] Expression of the mutant gene allows study of the effect of a single amino acid change on the activity of the parent protein. A recent example of an application of the technique comes from work on bacterial tyrosyl tRNA synthetase, where a serine residue was substituted for cysteine.[85] The resulting decrease in ATP binding affinity was consistent with the x-ray crystallographic data for the interaction of the cysteine SH group with the bound ATP.

Recombinant DNA technology has proven useful in studying certain proteins normally available only in minute quantities from natural sources. A dramatic illustration of this potential is the wealth of new information gleaned about the human interferons. Extensive reviews of the field have appeared recently.[86,87] Of special interest to this discussion is the total synthesis of the human α_1 interferon gene[88] and its expression in *E. Coli* and *M. methylotrophus*.[89] The gene was synthesized so that it carries restriction enzyme sites which enable maximal flexibility for engineering variant or hybrid structures.[90] One example of such a study involved constructing a variant human β_1 interferon with tyrosine substituted for a single cysteine residue by splicing together two interferon gene fragments at a common restriction enzyme site.[91] The fact that no interferon activity was measured in cells carrying the new gene suggests that the cysteine in the native protein may form a disulfide bond crucial for the active configuration of the molecule. Results from another study, using similarly constructed hybrids which could be highly purified, suggest the presence of at least two types of interferon receptors.[92] Recombinant technology has also aided in obtaining crystals of α-interferon, which may enable x-ray characterization of the protein.[93] Other valuable proteins being studied by recombinant methods include human insulin,[94] human growth hormone,[95] human urokinase,[96] tissue plasminogen activator,[97] and α_1-antitrypsin.[98]

A related application of recombinant DNA technology is the production of vaccines by synthesizing small peptides mimicing the antigenic regions of a foreign protein, as deduced by recombinant methods. For example, while cloning of the genes for antigens of the foot and mouth disease virus (FMDV)[99] has had variable success in providing proteins which could serve as vaccines, smaller polypeptides were synthesized which possessed sufficient antigenicity to be effective in immunizing animals against the virus.[100] Expression of a cloned gene for human influenza virus haemagglutinin has been studied in *E. coli* for possible use as a vaccine.[101] Another approach, similar to the FMDV precedent, involves synthesizing peptide fragments based on the deduced antigenic regions of haemagglutinin.[102] Efforts to produce vaccines for rabies,[103] malaria,[104] cholera,[105] herpes simplex virus[106] and hepatitis B virus[107] by recombinant DNA methods have also been reported.

Hereditary Disease: Recombinant DNA technology has enabled considerable progress in the understanding and diagnosis of certain human hereditary diseases and may suggest possible therapy in some cases. The foremost example is study of the thalassemias. Briefly, the normal hemoglobins (Hb) are composed of two pairs of protein subunits: Hb A, $\alpha_2\beta_2$; Hb A2, $\alpha_2\delta_2$; and Hb F (fetal), $\alpha_2\gamma_2$. Thalassemias result when α or β chain synthesis is defective, resulting in accumulation of single globin subunits and damage to red blood cells.[108] Mutations in the genes coding for the α, β, and δ chains that make up adult hemoglobin are of two basic types: extensive deletions, which remove some or all of a globin chain coding sequence, and small deletions or base substitutions, which result in incorrect RNA splicing or generation of a premature termination codon. The relevance of an understanding of the genetic basis of thalassemia to therapy was recently demonstrated by the successful treatment of a β-thalassemic patient with 5-azacytidine.[109] This compound induces undermethylation of DNA, a process which has been observed to stimulate expression of repressed genes in culture. It was postulated that 5-azacytidine would derepress the

fetal γ-globin genes, allowing γ-globin chain production and pairing with the normal α-globin chains to restore red blood cell function. Indeed, the γ-globin gene was undermethylated and levels of γ-globin peptide increased, leading to clinical improvement of the patient. An alternate approach employs *in vitro* mutagenesis techniques to produce an altered human $tRNA^{lys}$ that could be used to suppress a nonsense mutation (premature termination codon) found in some forms of β-thalassemia, and thus allow expression of normal β-globin chains.[110] The implications of these studies for the therapy of genetic diseases have been discussed.[111]

It is conceivable that diagnosis of more prevalent diseases such as diabetes may be approached with such techniques. Thus the 5 noncoding region of the human insulin gene has been analyzed in several studies, some of which found a correlation between DNA polymorphisms and type II diabetes.[112,113] Other hereditary diseases which may lend themselves to diagnosis by recombinant techniques include sickle cell anemia,[114] Christmas disease,[115] Huntington's disease,[116] and Duchenne muscular dystrophy.[117] The potential and problems of gene therapy have been recently reviewed.[118]

Conclusion: Despite remarkable progress in the last decade, recombinant DNA technology is still an embryonic field. Nevertheless, it has enabled some very far-reaching questions to be asked which will have a significant impact on future drug development. The molecular mechanisms which regulate the expression of genes at the le of transcription and translation are being unravelled, so that the possibility of therapeutic in...vention in these areas can be evaluated. Experiments designed to provide an expression system for genes in mammalian cells, as exemplified in the study of insulin gene expression, are particularly important illustrations of the potential offered. The opportunity to study proteins of interest previously available only in trace amounts and an understanding of the molecular basis for hereditary disease also afford the promise of new therapeutic strategies. Hopefully, this review will stimulate medicinal chemists to consider advances in recombinant DNA technology in the design and development of new therapeutic agents.

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