PROGRESS IN MEDICINAL CHEMISTRY 23

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Preface

We have pleasure in presenting six reviews in this volume, all of which cover important advances in the chemistry and biology of medicinal products.

In Chapter 1, structure-activity relationships within a series of α-adrenoceptor antagonists are discussed, particularly as regards their use in the fields of depression, diabetes and hypertension.

The latest research into cell surface receptors after introducing radioligand binding is covered in Chapter 2 and a similar theme is continued in Chapter 3 where common structural features of drugs, transmitters and peptides in the central nervous system are evaluated.

Chapter 4 covers the biological and chemical aspects of recently-introduced antidepressant drugs. The discovery of acyclovir as a potent and selective anti-herpes agent is described in Chapter 5.

Finally in Chapter 6, the antibiotic sparsomycin is reviewed for the first time in detail. It appears to be an anti-tumour agent of high promise, being an inhibitor of the peptide bond formation step in protein synthesis.

Again, we thank our authors for their efforts, the owners of copyright for permission to reproduce material, and the staff of our publishers for their constant help and encouragement. We also wish to thank Mrs. A.E. Bisgood (Department of Computing, UWIST) for assistance in producing the index to this volume.

G.P. Ellis

December 1985 G.B. West

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Progress in Medicinal Chemistry – Vol. 23, edited by G.P. Ellis and G.B. West

1 Pharmacology and Structure-Activity Relationships of α_2-Adrenoceptor Antagonists

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INTRODUCTION

Since the initial proposal by Ahlquist in 1948 that adrenoceptors (adrenergic receptors) be classified into α- and β-adrenoceptor subtypes [1], a great deal of work has been carried out which has resulted in a further subclassification of these adrenoceptors. Thus, the β-adrenoceptor has been subclassified into β_1- and β_2-adrenoceptors and the α-adrenoceptor has been subclassified into α_1- and α_2-adrenoceptors. Development of selective agonists and antagonists for these adrenoceptor subtypes has led to a number of important clinical agents. For example, the clinical efficacy of β_1-adrenoceptor antagonists in the treatment of cardiovascular disorders and of β_2-adrenoceptor agonists for bronchial asthma is well established. Agents which act predominantly at α-adrenoceptors include α_1-agonists as decongestants, α_1-adrenoceptor antagonists, such as prazosin, for hypertension, and α_2-adrenoceptor agonists such as clonidine, also for the treatment of hypertension. More recently, a substantial amount of effort has been devoted to the discovery of selective α_2-adrenoceptor antagonists in the hope of obtaining similarly useful clinical entities. Several recent reviews cover the classification, pharmacology, and possible clinical utilities of α_2-adrenoceptor antagonists [2–6] and therefore these areas will be only briefly mentioned here. This review will instead concentrate on the α-adrenoceptor antagonist compounds which have been reported and the structure-activity relationships (SAR) which are available on the various series of antagonists. We have not attempted to cover the chemical synthesis of these agents, and for this information the reader is referred to the original publications.

CLASSIFICATION OF α_2-ADRENOCEPTORS

The α-adrenoceptor was originally subdivided on an anatomical basis into pre- and postsynaptic subtypes. It has subsequently been pointed out that the terms pre- and postjunctional are more encompassing [7] and these will be used in

this review. This original classification followed the demonstration of prejunctional α-adrenoceptors that inhibited neurotransmitter release in a wide range of tissues from several species [8–11]. The terms α_1- and α_2-adrenoceptors [12] were subsequently adopted to describe post- and prejunctional adrenoceptors, respectively [13]. However, the demonstration that α_2-adrenoceptors occur postjunctionally in human platelets [14], human adipocytes [15] and vascular smooth muscle [16] has led to the formulation of alternative methods of subclassification. An early proposal for a functional classification system which designated α_1- and α_2-adrenoceptors as excitatory and inhibitory, respectively [17], has also required modification since inhibitory prejunctional α_1-adrenoceptors [18–20] and excitatory and inhibitory postjunctional α_2-adrenoceptors [7, 16] have been described.

More recently, a subclassification of α-adrenoceptors based on the biochemical responses initiated following receptor stimulation has been proposed [21]. According to this method of subclassification, those α-adrenoceptors that stimulate the turnover of phosphatidylinositol are termed α_1-adrenoceptors, while those which inhibit adenylate cyclase are termed α_2-adrenoceptors. However, the finding that the α_1-adrenoceptor of the rat liver can stimulate production of cyclic AMP [22] would be difficult to reconcile within this method of subclassification.

In view of the recent development of a wide range of specific and selective agonists and antagonists for the α_1- and α_2-adrenoceptor [5], and also based on its consistency with other methods of receptor classification, an alternative pharmacological subclassification would appear to be preferable [23, 24]. This system is based on the order of activities and affinities of agonists and antagonists for adrenoceptors, irrespective of the location or function of the adrenoceptor. The subclassification of α-adrenoceptors has been the subject of numerous reviews (Ref. 2 and references cited theirein).

PHARMACOLOGICAL EVALUATION OF α_2-ADRENOCEPTOR ANTAGONISTS

The α_2-adrenoceptor has been examined in a wide range of experimental systems using a diversity of methods. Direct-binding studies in membrane preparations have enabled an accurate assessment of the affinity of both agonists and antagonists for the α_2-adrenoceptor [24], while the ability of α_2-adrenoceptor agonists to attenuate the activity of adenylate cyclase [25] has provided a more functional and biochemical means of examining these receptors. The demonstration of a proaggregatory response to α_2-adrenoceptor

agonists in human platelets [14] makes this preparation a useful intact cell system in which to examine α_2-adrenoceptors using a functional approach. Isolated tissue preparations have been widely used to investigate the influence of α_2-adrenoceptors on the nerve stimulation-evoked release of neurotransmitter either directly, by measuring the overflow of neurotransmitter [26, 27], or indirectly, by measuring contractile [28, 29] or electrical [30] end-organ responses.

In vivo, the pithed rat has proved a useful preparation to examine both pre- and postjunctional α_2-adrenoceptors in the cardiovascular system [16, 31, 32]. The ability of compounds to attenuate the sedation [33], inhibition of locomotion [34] or mydriasis [35] induced by α_2-adrenoceptor agonists in conscious animals has also been used to assess α_2-adrenoceptor antagonist activity.

Despite the diversity of methods available for examining the α_2-adrenoceptor, this review will concentrate on those methods that provide the comparative and quantitative data required by a pharmacological classification of α_2-adrenoceptors and will therefore be restricted to a consideration of those functional studies in isolated tissue preparations and direct-binding studies on membrane preparations and intact cells that provide such quantitative data.

The most widely used isolated tissue preparations for functionally evaluating the potency of α_2-adrenoceptor antagonists are the transmurally stimulated rat vas deferens [28, 36] and the transmurally stimulated guinea-pig ileum [29, 37]. The rat vas deferens and guinea-pig ileum have proved particularly suitable for characterizing prejunctional α_2-adrenoceptors for two reasons. Firstly, in both of these preparations, activation of prejunctional α_2-adrenoceptors has been shown to decrease, in a concentration-dependent manner, both the release of neurotransmitter and the associated contractile response of the tissue following nerve stimulation [38]. This has enabled the end-organ response of the tissue to be utilized as an indirect index of neurotransmitter release.

The second, and perhaps major, reason for utilizing these preparations has been the predominantly 'non-adrenergic' nature of neurotransmission in the vas deferens [39, 40] and the cholinergic nature of neurotransmission in the guinea-pig ileum. Thus, in contrast to the wide range of vascular preparations used for examining prejunctional α-adrenoceptors [26, 27], in the rat vas deferens and guinea-pig ileum the motor response of the tissue is not mediated through postjunctional α-adrenoceptors. This enables the prejunctional α_2-adrenoceptor antagonist actions of both selective and non-selective α-adrenoceptor antagonists to be examined in these preparations.

More recently, the prostatic portion of the transversely bisected, rat vas deferens preparation [40–42] has been used in preference to intact vasa

deferentia, since the influence of the 'adrenergic' component of neurotransmission in this portion of the tissue does not contribute significantly to the measured end-organ response [43].

The most commonly used method for quantifying antagonist potency in functional studies on the α_2-adrenoceptor involves determining the potency of an α_2-adrenoceptor agonist in inhibiting responses of the vas deferens or guinea-pig ileum to transmural nerve stimulation both in the presence and absence of a range of concentrations of the putative α-adrenoceptor antagonist [44]. In this manner, the ability of a compound to antagonize the inhibitory effects of the α_2-adrenoceptor agonist can be expressed in terms of a 'dose-ratio' (the ratio of the agonist potency in the presence and absence of the antagonist). These 'dose-ratios' are subsequently utilized in the Arunlakshana and Schild analysis from which an estimate of the potency of the antagonist in terms of a pA_2 value can be obtained [44]. In addition to providing an estimate of antagonist potency, this analysis can also provide information as to the competitive, or otherwise, nature of the interaction between the agonist and antagonist [45].

The use of cardiac tissue should provide an alternative preparation for the study of α_2-adrenoceptor antagonists, since both the basal chronotropic and inotropic end-organ responses in this tissue are predominantly mediated through postjunctional β-adrenoceptors. Despite the demonstration of prejunctional α-adrenoceptors in guinea-pig atria [46], cardiac tissue has, however, not been extensively used for evaluating prejunctional α-adrenoceptor antagonist potencies.

Ligand-binding studies provide an alternative, more direct, means of determining the affinity of a compound for a neurotransmitter receptor. These methods have become firmly established in the study of α-adrenoceptor subtypes and a wide range of radiolabelled agonists and antagonists have been used to label α_2-adrenoceptors directly [24]. In these studies, antagonist affinities are determined from their ability to compete with a radiolabelled ligand in binding to the α_2-adrenoceptor. Selectivity in the binding of the radioligand to the α_2-adrenoceptor is achieved either by utilizing a tissue source in which the α-adrenoceptor population is predominantly of the α_2-adrenoceptor subtype (human platelet) or, more commonly, by utilizing subtype-selective radioligands in a concentration range at which they label only the α_2-adrenoceptor.

The α_2-adrenoceptor subtype-selective agonist ligands [^{3}H]clonidine [47], *p*-[^{3}H]aminoclonidine [48], [^{3}H]guanfacine [49], and [^{3}H]UK 14304 [50] have been shown in direct-binding studies to identify sites that display the pharmacological profile of the α_2-adrenoceptor. It has been shown, however,

that agonist ligands preferentially label an affinity state of the α_2-adrenoceptor that displays high affinity for agonists [51]. Since antagonists possess low affinity for this state of the receptor [24], the use of agonist radioligands may not be appropriate for determining the affinity of antagonist compounds.

In this respect, the α_2-adrenoceptor subtype-selective antagonist ligands [^{3}H]yohimbine [52, 53], [^{3}H]rauwolscine [54] and [^{3}H]RX 781094 [55, 56] have been shown in direct-binding studies to label sites that display the pharmacological profile of the α_2-adrenoceptor. In particular, the affinity estimates for the antagonists determined in binding studies with these radioligands have been in close agreement with those determined in functional studies [57], thereby indicating the suitability of assessing antagonist affinities using ligand-binding techniques.

The problem of discriminating between agonist and antagonist ligands has been raised as a possible drawback with respect to interpreting the results from direct-binding studies on several neurotransmitter receptors [58]. In the case of studies using [^{3}H]yohimbine [52] and [^{3}H]rauwolscine [54], the demonstration that agonist and antagonist ligands produce displacement isotherms with Hill coefficients less than unity and close to unity, respectively, may provide a crude test for differentiating agonist from antagonist properties in ligand-binding studies on the α_2-adrenoceptor.

In the tables refering to ligand-binding studies, the data are presented in terms of $-\log IC_{50}$ or K_i values. The $-\log IC_{50}$ is the antilog of the concentration of competing drug displacing specific radioligand binding by 50%. The K_i is the apparent dissociation constant determined from the IC_{50} by the method of Cheng and Prusoff [59].

POTENTIAL CLINICAL USES OF α_2-ADRENOCEPTOR ANTAGONISTS

While there is clearly a therapeutic rôle for agonists of α_2-adrenoceptors, such as in the treatment of hypertension with centrally active agents such as clonidine, guanabenz and guanfacine, clinical applications of α_2-adrenoceptor antagonists have not yet been identified. This is probably due to the relatively recent advent of specific and selective α_2-adrenoceptor antagonists and the fact that results of clinical trials on these agents have not yet been forthcoming.

Several physiological rôles for prejunctional α_2-adrenoceptors may be postulated [2, 60]. They may serve as targets for circulating catecholamines or for transmitters secreted from neighbouring axon terminals. Prejunctional α_2-adrenoceptors may also serve an autoinhibitory rôle in preventing further

release (negative feedback) [27] of endogenous noradrenaline (NA, norepinephrine). At present, functional rôles for these prejunctional α_2-adrenoceptors, or the physiological consequences of their antagonism have not yet been firmly established in man. Although there is substantial evidence for the existence of postjunctional α_2-adrenoceptors (reviews: Refs. 7, 61), a physiological rôle for these adrenoceptors in man has not been clearly identified, either. Thus, the hypothetical nature of the potential clinical uses for α_2-adrenoceptor antagonists outlined in the following sections must be emphasized.

DEPRESSION

According to the catecholamine hypothesis of affective disorders, depression in associated with decreased noradrenergic transmission in the central nervous system [62]. Since prejunctional α_2-adrenoceptors mediate NA levels through the negative feedback mechanism, antagonism of these adrenoceptors should lead to increased levels of NA and an antidepressant effect. There is now a growing body of experimental data to support this hypothesis. It is well established that chronic administration of antidepressants such as the tricyclics, which inhibit amine-reuptake, results in a desensitivity of prejunctional α_2-adrenoceptors [63–69]. For example, depressed patients who underwent long-term treatment with desipramine showed decreased sensitivity to the effects of clonidine on blood pressure and NA levels [70]. It is felt that the reason such antidepressants require several weeks to show clinical efficacy may be due to the slow development of this α_2-adrenoceptor subsensitivity. Development of this subsensitivity has the net effect of attenuating the negative feedback response and hence increases NA levels. The hypothesis that supersensitive α_2-adrenoceptors may play an important rôle in depression has also been proposed [71].

Another manifestation of chronic therapy, with not only the tricyclics, but also with monoamine oxidase inhibitors, atypical antidepressants [72], and electroconvulsive therapy (ECT), is a decrease in the sensitivity of cortical β-adrenoceptors [73]. This is presumably also due to increased levels of NA, which result from the gradual reduction of the effectiveness of the α_2-adrenoceptor negative feedback mechanism. It has been shown in the rat that combined treatment with either tricyclics or monoamine oxidase inhibitors and the irreversible α_2-adrenoceptor antagonist phenoxybenzamine accelerates and intensifies the desensitization of β-adrenoceptors [74, 75]. These results would imply that α_2-adrenoceptor antagonists, either on their own or in combination with classical antidepressants, should produce a more rapid onset of β-adrenoceptor densensitization and hence a more rapid onset of antidepressant activity than is available with current therapeutic agents.

DIABETES

The stimulation of α-adrenoceptors inhibits the release of insulin from pancreatic islet cells [76–78]. These adrenoceptors were identified as being postjunctional α_2-adrenoceptors, since yohimbine, but not prazosin, increased plasma insulin and inhibited adrenaline (epinephrine)-induced hyperglycaemia in mice [79, 80]. Studies have also indicated that islet-activating protein prevents adrenaline-induced hyperglycaemia in rats by increasing insulin release. This action has been associated with antagonism of α_2-adrenoceptor-mediated inhibition of adenylate cyclase activity [81]. Thus, there may be a rôle for α_2-adrenoceptor antagonists in the treatment of diabetes, since this mechanism is different from that of the more classical sulphonylureas and biguanides.

OBESITY

Based on the effects of a number of agonists and antagonists on theophylline-stimulated lipolysis in human fat cells, it was concluded that postjunctional α_2-adrenoceptors inhibit lipolysis in these cells [82]. Adrenaline was identified as the major agonist for these α_2-adrenoceptors [83]. Since human gluteal fat cells tend to have a preponderance of α_2-adrenoceptors – as opposed to β-adrenoceptors, as found in other types of fat cells – α_2-adrenoceptor antagonists may be useful in increasing the metabolism of these fat cells in obese individuals [84].

HYPERTENSION

Postjunctional α_2-adrenoceptors have been identified in the smooth muscle of several species [7, 61]. Stimulation of these adrenoceptors leads to vasoconstriction. However, the physiological rôle of these postjunctional adrenoceptors is not known and consequently their selective blockade would be expected to lead to an antihypertensive effect only if they make a significant contribution to peripheral resistance.

Another possible application of α_2-adrenoceptor antagonists to the treatment of hypertension derives from the observation that there is an increased number of renal α_2-adrenoceptors in Dahl sodium-sensitive rats as compared with resistant rats [85]. It has been hypothesized that, since these α_2-adrenoceptors are located in proximal tubules where NA induces sodium reabsorption, their increased number leads to enhanced sodium retention and possibly high blood pressure [85]. Were this hypothesis to be correct, there

would be a rôle for antagonism of these α_2-adrenoceptors in the treatment of this form of hypertension.

PLATELET DYSFUNCTIONS

The demonstration that α-adrenoceptor agonists stimulate aggregation of human platelets [86] and that these receptors are of the α_2-adrenoceptor subtype [14] may indicate the therapeutic usefulness of α_2-adrenoceptor antagonists in preventing aggregation in disease states characterized by excessive levels of circulating adrenaline. While in preliminary studies [87] yohimbine did not inhibit disseminated intravascular coagulation induced by thrombin in rats, the effects of more selective α_2-adrenoceptor antagonists have not been examined.

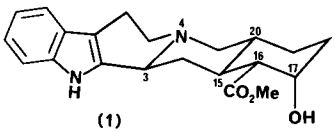
IMPOTENCE

A study in which patients with organic impotence were treated with yohimbine indicated that the drug may have had beneficial results in a number of cases [88]. It was suggested that this may be due to α_2-adrenoceptor antagonism, although there is no clear evidence to date to prove this assertion.

α-ADRENOCEPTOR ANTAGONISTS

YOHIMBINE AND ITS STEREOISOMERS AND ANALOGUES

The finding in 1975 that the indole alkaloid yohimbine (1) was more potent in antagonizing the prejunctional than the postjunctional α-adrenoceptor of the



(1)

rabbit pulmonary artery [89] provided the impetus for much of the work which followed in the next decade, both on the α_2-adrenoceptor antagonist properties of yohimbine and its isomers and on the synthesis and evaluation of newer α_2-adrenoceptor antagonists. Since this initial discovery, yohimbine has been extensively utilized as a pharmacological tool in the investigation and identification of α_2-adrenoceptors using both ligand-binding techniques and studies of

their functional rôles in a wide variety of tissues. A comprehensive review [90] has described both the ligand-binding and functional assay work, as well as the large amount of human and animal pharmacological data that have been published on yohimbine and several stereoisomers. The pharmacological and biochemical properties of the isomeric yohimbine alkaloids have also been reviewed [91]. This review will concentrate on the structure-activity relationships (SAR) of yohimbine and its stereoisomers and on the implication of those relationships toward structural requirements for α_2-adrenoceptor antagonism within this series.

After the initial discovery of the α_2-adrenoceptor selectivity of yohimbine [89], a number of investigations into the α-adrenoceptor antagonist properties of naturally occurring and semi-synthetic yohimbine isomers (and analogue) were forthcoming [53, 92–98]. The structures of these compounds have been represented in two dimensions to present a clearer picture of the stereochemical relationships involved.

The results of ligand-binding studies on a number of yohimbine isomers and analogues from two references are presented in *Table 1.1.* In the first study [92], [^{3}H]yohimbine was used to label α_2-adrenoceptors in human platelets and

Table 1.1. α-ADRENOCEPTOR BINDING FOR YOHIMBINE AND RELATED COMPOUNDS

	Binding K_i(nM) [92][a,b]			*Binding K_i(nM) [93]*[c]		
Compound	α_1	α_2	α_2/α_1	α_1	α_2	α_2/α_1
(1) Yohimbine	127	2	63.5	216	49	4.4
(2) Rauwolscine	336	3	112	800	19	42
(3) Corynanthine	20	557	0.036	19	810	0.023
(4) Alloyohimbine	280	6	46.6			
(5) Pseudoyohimbine	820	928	0.88			
(6) Epi-3-α-yohimbine	1600	1450	1.1			
(7) 11-Methoxyrauwolscine	5290	160	33			
(8) Apoyohimbine				20	14	1.4
(9)				230	122	1.9
(10)				170	149	1.1
(11)				54	42	1.3

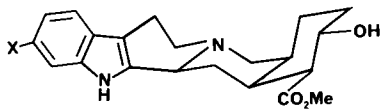
[a] Determined in rat liver membranes with [^{3}H]prazosin.

[b] Determined in human platelet membranes with [^{3}H]yohimbine.

[c] Determined in calf cerebral cortex with [^{3}H]prazosin (α_1) and [^{3}H]clonidine (α_2).

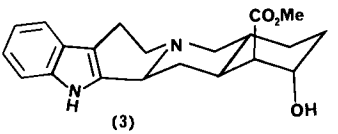
[^{3}H]prazosin was used to label α_1-adrenoceptors in rat liver membranes. In the second [93], [^{3}H]clonidine and [^{3}H]prazosin were used as labels for α_2- and α_1-adrenoceptors, respectively, in calf cerebral cortex membranes. The results of the latter report are in good agreement with other studies in rat cerebral cortical membranes in which [^{3}H]clonidine and [^{3}H]WB-4101 [94] and [^{3}H]clonidine and [^{3}H]prazosin [95] were used as ligands.

The binding data in *Table 1.1* indicate that rauwolscine (α-yohimbine) (2) is considerably more selective than yohimbine (1) for the α_2-adrenoceptor. On the other hand, corynanthine (3), the C-16 epimer of yohimbine, exhibits weak



(2)X=H

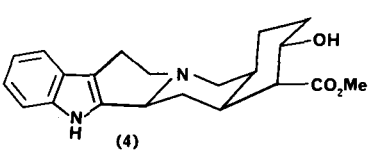
(7)X=OMe



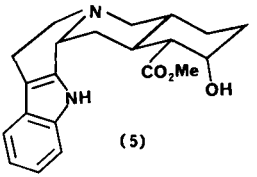
(3)

affinity for the α_2-adrenoceptor and strong affinity for the α_1-adrenoceptor and hence is a selective α_1-adrenoceptor antagonist. The relative selectivities of these three isomers established by these binding studies has been confirmed in a number of in vitro and in vivo functional assays. Both yohimbine and rauwolscine (1–10 μg/kg) dose-dependently antagonized the central hypotensive response to the α_2-adrenoceptor agonist clonidine in anaesthetized cats [95]. Considerably higher doses (30–100 μg/kg) of corynanthine were required to elicit the same response. Yohimbine and rauwolscine (0.01–1 mM) were more effective than corynanthine (10 μM) in increasing stimulation-evoked overflow of tritium from rat occipital cortical slices [96]. Similar results were obtained in a study of the effects of these three compounds on noradrenergic transmission in the pulmonary artery of the rabbit [97]. In the anaesthetized dog, yohimbine and rauwolscine were approximately 30-times more potent at α_2-adrenoceptor than α_1-adrenoceptor antagonists, while corynanthine was found to be 10-times more potent at α_1-adrenoceptors than at α_2-adrenoceptors [98]. Additional studies which establish the α_2-adrenoceptor selectivity of rauwolscine and yohimbine and the α_1-adrenoceptor selectivity of corynanthine have been summarized [90].

On the basis of the data in *Table 1.1* a number of structure-activity (or structure-affinity) relationships in the yohimbine series have been drawn [92, 93]. It has been suggested for yohimbine that the indole nucleus, nitrogen atom (N-4), and the methoxycarbonyl group at C-16 are important for α-adrenoceptor binding [7, 99]. In the α_2-adrenoceptor-selective compounds yohimbine (1), rauwolscine (2), and alloyohimbine (4), these three binding sites



(4)



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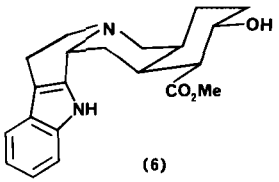

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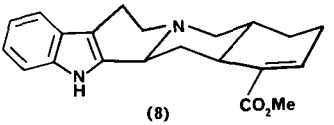
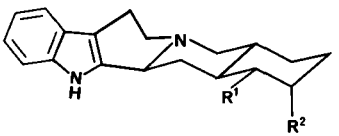


(8)

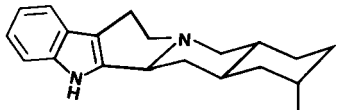
are located approximately in the same plane. In corynanthine (3), the methoxycarbonyl group is oriented out of the plane of the other two sites, and therefore it is the position of this C-16 methoxycarbonyl group which appears to be the determining factor in the specificity of these four isomers [92]. Apoyohimbine (8), in which the methoxycarbonyl group is in a position intermediate between yohimbine and rauwolscine, binds tightly to both adrenoceptors. Pseudoyohimbine (5) and epi-3-α-yohimbine (6), which deviate from an overall planar structure, have very low binding affinities at either adrenoceptor. A methoxy group in the A-ring of rauwolscine gives (7) with reduced affinity for both adrenoceptors, presumably due either to electronic effects which lead to a decreased binding capability of the indole nucleus or to steric factors.

The results on the semi-synthetic analogue (9–11) further point out the importance of the C-16 methoxycarbonyl group, since reduction of this group



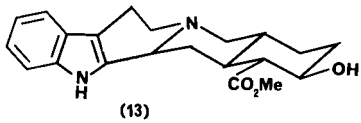
(9)R^1= CH_2OH R^2= OH
(10)R^1= Me R^2= OH
(11)R^1= CO_2Me R^2= H

leads to decreased α_2-adrenoceptor affinity. It is suggested that this is due to removal of a favourable dipole-dipole interaction between the C-16 methoxycarbonyl group and the α_2-adrenoceptor, rather than removal of hydrogen-binding capability, since the hydroxymethyl group of (9) should be expected to hydrogen-bond more favourably than the ester [93]. It should also be pointed out that removal of the methoxycarbonyl group of yohimbine to give yohimbol



(12)

(12) results in more of a decrease in affinity for the α_2-adrenoceptor than for the α_1-adrenoceptor [100]. The C-17 hydroxy group also appears to play an important rôle in determining selectivity for α_1- and α_2-adrenoceptors [93]. Compound (11), in which the C-17 hydroxy group has been removed from yohimbine, displays an enhanced affinity for the α_1-adrenoceptor while the α_2-adrenoceptor affinity is not affected. Apoyohimbine (8), which also lacks this hydroxy group, also binds strongly to both adrenoceptors. These results suggest that the C-17 hydroxyl may cause a severe repulsive interaction at the α_1-adrenoceptor, leading to decreased affinity, while no such interaction is



(13)

present at the α_2-adrenoceptor [93]. Results with β-yohimbine (13), in which epimerization of the C-17 hydroxy group has relatively minor effects on α_1- or α_2-adrenoceptor affinity, would tend to support this conclusion [100].

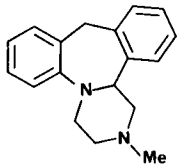
Thus, taking into account these SAR, it is apparent that what would appear to be rather subtle changes in the yohimbine molecule lead to pronounced effects on selectivity for α_1- and α_2-adrenoceptors.

A great deal of data on the *in vivo* activity of yohimbine, as well as of rauwolscine and corynanthine, has been reported [90]. However, attribution of these various activities to α_2- (or α_1)-adrenoceptor antagonist effects must be done with caution (or not at all), since these compounds are also known to possess both serotonergic and dopamine antagonist activity [90]. Due to these effects on other receptors, yohimbine and its stereoisomers will probably remain as useful tools for characterizing and studying α_2-adrenoceptors rather than as useful α_2-antagonist therapeutic agents.

MIANSERIN, CGS-7525A AND RELATED COMPOUNDS

The tetracyclic compound mianserin (14) is a clinically effective antidepressant [101–103] which appears to differ in mechanism of action from the more classical tricyclic antidepressants. In addition to its monoamine neuronal

uptake inhibiting properties, mianserin possesses activity as an α_2-adrenoceptor antagonist [104–107]. This finding was initially based on the demonstration that mianserin increased [^{3}H]NA release from field-stimulated rat cortical slices [104] and increased the electrically evoked overflow and contractile



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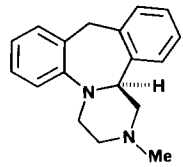


(15)

response in mouse vas deferens [105]. However, mianserin has been shown to be rather non-selective in antagonizing α_2-adrenoceptors as opposed to α_1-adrenoceptors [108–111].

The antidepressant activity of mianserin appears to be stereospecific for the (*S*)-(+)-enantiomer (15) based on the results obtained in a number of animal models [112–113]. Evaluation of the displacement of [^{3}H]prazosin and [^{3}H]clonidine from binding sites in rat isolated brain membranes by racemic,

Table 1.2. α-ADRENOCEPTOR BINDING OF MIANSERIN AND ITS ENANTIOMERS[a] [107]

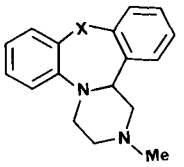
Compound	*– log IC_{50} α_1 (M)*	*– log IC_{50} α_2 (M)*
(14) (+)-Mianserin	6.42	6.49
(15) (+)-(*S*)-Mianserin	6.58	6.42
(–)-(*R*)-Mianserin	5.65	6.03

[a] Determined in rat isolated brain membranes with [^{3}H]clonidine (α_2) and [^{3}H]prazosin (α_1).

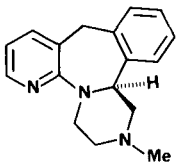
(*S*)-(+)- and (*R*)-(–)-mianserin revealed that the (*S*)-(+)-enantiomer was more potent in displacing both ligands, but that the difference between the two enantiomers at the α_2-adrenoceptor-binding site was not pronounced (*Table 1.2*) [107, 114]. (*S*)-(+)-Mianserin was also found to be considerably more potent than its (*R*)-(–)-antipode in causing K^+-induced [^{3}H]NA release from rat cerebral cortex slices [115] and in inhibiting neuronal NA reuptake [113]. Based on these data as a whole, it is difficult to assess the contribution which α_2-adrenoceptor antagonism may make to the antidepressant activity of mianserin.

A structure-activity relationship study of a number of mianserin analogues

was performed in which α_2-adrenoceptor antagonism was determined by measuring the potentiation of depolarization-induced NA release from rat cerebral cortex slices [113]. The most active compounds in this assay were those in which the overall bent, tricyclic structure of mianserin was retained (e.g., 16–21). The activity for 6-azamianserin was stereospecific for the (+)-enantiomer (19), as had been found for minaserin itself.



(16) X = O
(17) X = S
(18) X = NMe



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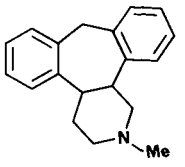

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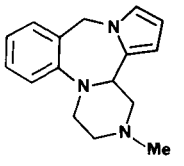


(21)

The D-pyrrolidino analogue (21), designated CGS-7525A, has been the subject of further evaluation and has been compared with mianserin as a potential antidepressant clinical candidate [116–117]. In binding studies (*Table 1.3*), CGS-7525A was slightly more potent than mianserin in displacing [^{3}H]clonidine from the low-affinity site of calf brain membranes and signifi-

Table 1.3. EFFECTS OF CGS-7525A AND MIANSERIN ON [^{3}H]PRAZOSIN AND [^{3}H]CLONIDINE BINDING TO BRAIN MEMBRANES [116]

		[^{3}H]Clonidine[b]	
Compound	*[^{3}H]Prazosin*[a] *– log IC_{50} (M)*	*Low affinity – log IC_{50} (M)*	*High affinity – log IC_{50} (M)*
(21) CGS-7525A	5.9	8.3	8.4
(14) Mianserin	6.1	8.1	7.9*
(1) Yohimbine	5.5	7.3*	7.2*

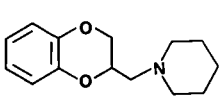
[a] Rat brain.
[b] Calf brain.
* Significantly different from CGS-7525A.

cantly more potent in displacing [^{3}H]clonidine from the high-affinity site. Both CGS-7525A and mianserin were considerably less potent in displacing [^{3}H]prazosin from rat brain membranes, although the selectivity implied by these data is not in accord with previous studies for both mianserin (*Table 1.2*) and yohimbine (*Table 1.1*).

CGS-7525A prevented clonidine-induced antiwrithing activity in the mouse and antagonized the ability of clonidine to suppress locus coeruleus neuronal firing rate [116]. In both of these *in vivo* assays, CGS-7525A was approximately 10-times more potent than mianserin. Thus, it was concluded that this compound may represent a useful tool for investigating brain α_2-adrenoceptors and the involvement of those receptors in antidepressant therapy [116].

BENZODIOXANS – IDAZOXAN, IMILOXAN AND RELATED COMPOUNDS

The benzodioxans are a class of α-adrenergic antagonists which have been known for over 50 years. Piperoxan (22), for example, is a high-affinity α_2-adrenoceptor antagonist which has approximately a 10-fold selectivity for α_2-adrenoceptors relative to α_1-adrenoceptors. As part of a programme directed towards the preparation of α_2-adrenoceptor antagonists as potential antidepressants, structural features of piperoxan were combined with those of



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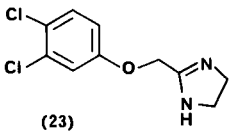

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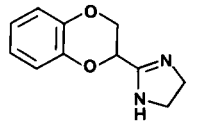

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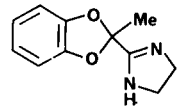


(25)

the weak α_2-antagonist, femmetazole (23), to produce (24), (RX 781094) [118, 119]. Compound (24), now known as idazoxan, proved to be a highly potent and selective α_2-adrenoceptor antagonist, with an α_2/α_1 selectivity ratio of 288 in the initial evaluation [119]. Independent reports from two other groups on the activity of this compound appeared during the same time period [120–122]. Some interesting chemistry was uncovered during the preparation of (24). A patent describing the synthesis of (24) had been issued a number of years earlier [123], but attempts to reproduce this synthesis led to the benzodioxole (25) instead [124]. A different route gave the correct benzodioxan structure (24) [119, 122, 124].

Systematic modification of the idazoxan structure (24) has revealed interesting structure-activity relationships as well as producing several analogue with increased α_2-adrenoceptor antagonist potency and selectivity

[125–127]. Substitution in the aromatic ring, at C-3 of the benzodioxan, or in the imidazoline ring of (24) led to compounds with greatly reduced α_2-adrenoceptor antagonism [119]. Replacement of the imidazoline with various other heterocycles also gave substantially less active compounds. The C-2 position of (24) proved to be the only position in which potency and selectivity was not reduced upon substitution. Thus, substitution with lower

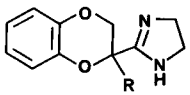
Table 1.4. α-ADRENOCEPTOR ANTAGONISM OF IDAZOXAN ANALOGUES IN ISOLATED TISSUE EXPERIMENTS

Compound	pA_2 (α_2)[a]	pA_2 (α_1)[b]	*Ratio* α_2/α_1	*Ref.*
(24) Idazoxan	8.50	6.32	151	126
(26) (2-methyl)	7.96	5.30	457	126
(27) (2-ethyl)	8.69	6.40	195	126
(28) (2-propyl)	8.92	6.56	229	126
(29) (2-isopropenyl)	8.21	5.42	617	126
(30) (2-methoxy)	9.41	6.91	316	127
(31) (2-ethoxy)	8.92	6.74	151	127
(32) (2-benzyloxy)	8.68	6.39	195	127

[a] Determined in rat vas deferens against UK-14,304-induced inhibition of electrically evoked contraction.

[b] Determined in rat anococcygeus against norepinephrine-induced contraction.

alkyl groups as in (26–28) led to increased potency and selectivity, while an isopropenyl group (29) led to increased selectivity both *in vitro* (*Table 1.4*) and *in vivo* [125, 126]. Substitution with alkoxy groups also led to compounds (30–32) with increased selectivity and potency [127]. Even a group as large as



(26) R= Me

(27) R= Et

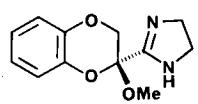
(28) R= *n*-Pr

(29) R=

(30) R= OMe

(31) R= OEt

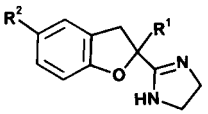
(32) R= OCH_2Ph



(33)

benzyloxy is tolerated in position 2 (compound 32). Preparation of the enantiomers of methoxy compound (30) and evaluation in a number of test systems (binding and *in vivo*) showed that the (*S*)-enantiomer (33) was much more potent as an α_2-antagonist [128]. For example, in displacement of [^{3}H]idazoxan from rat cerebral cortex membranes, enantiomer (33) was 640-times more potent than the (*R*)-enantiomer. It is interesting to note that only a 3-fold separation in the activities of the enantiomers of idazoxan was reported [129].

Further SAR work on the idazoxan series in which the benzodioxan ring was modified led to dihydrobenzofurans (34–36) with α_2-adrenoceptor antagonist potencies and selectivities comparable with those of idazoxan [130]. The 2-ethyl and 2-propyl compounds (34, 35) possessed greater potency and selectivity *in vitro*, while the 5-chloro derivative (36) was twice as potent as idazoxan when administered orally as an antagonist of guanoxabenz-induced mydriasis in the anaesthetized rat [130]. An independent report on the α_2-adrenoceptor selective antagonist activity of the parent compound (37), as well as the related benzocyclobutane (38), has been published [131].

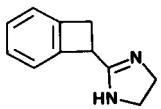


(34) R^1 = Et R^2 = H

(35) R^1 = *n*-Pr R^2 = H

(36) R^1 = H R^2 = Cl

(37) R^1, R^2 = H



(38)

Because of its high potency and selectivity as an antagonist of α_2-adrenoceptors, idazoxan has been the subject of many studies both to assess the potential of the drug as a clinical entity and to explore the effects of α_2-adrenoceptor antagonism in pharmacological models. Since the results of most of these studies have already been reviewed [132, 133], they will not be dealt with here. Several studies comparing idazoxan with other α_2-adrenoceptor antagonists have also been reported [134, 135]. These studies have confirmed the potency and selectivity of idazoxan as an α_2-adrenoceptor antagonist and have demonstrated that idazoxan has a high degree of specificity for the α_2-adrenoceptor [133]. Tritiated idazoxan has been introduced as a specific α_2-adrenoceptor antagonist radioligand [55, 56].

There have been reports that in certain models, idazoxan demonstrated partial agonist activity at α_1-adrenoceptors [136, 137] and at α_2-adrenoceptors

[138, 139]. In one study, it was noted that the weak α_1-adrenoceptor agonism of idazoxan was eliminated in the 2-substituted analogues (26, 27 and 29) [126]. In a number of other models, however, no α_1- or α_2-adrenoceptor agonist effects were noted [135].

Idazoxan has been advanced to human clinical trials as an antidepressant, and the results of those trials, and possibly of trials in other disease states, will help to establish both the clinical potential of this drug and of α_2-adrenoceptor antagonists as therapeutic agents in general.

In a synthetic programme similar to that which produced idazoxan, a series of 2-substituted benzodioxanyl imidazolines and imidazoles were prepared and evaluated for α_2-adrenoceptor antagonism [122]. Similar results were obtained in the imidazoline series where (24) (idazoxan) was identified as the most potent α_2-adrenoceptor antagonist (*Table 1.5*). Among the benzodioxanyl imidazoles, the *N*-ethylimidazole (39) (RS-21361, imiloxan) was identified as

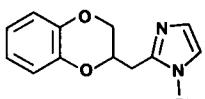
Table 1.5 α-ADRENOCEPTOR ANTAGONISM OF IMILOXAN [122]

Compound	pA_2 (α_2)[a]	pA_2 (α_1)[b]	*Ratio* α_2/α_1
(39) Imiloxan	6.71	<4	>513
(24) Idazoxan	7.80	5.80	100
(2) Rauwolscine	7.92	6.27	45
(1) Yohimbine	7.71	6.21	32

[a] Determined using isolated rat vas deferens against xylazine-induced inhibition of electrically evoked contraction.

[b] Determined using isolated rat vas deferens against amidephrine-induced contraction.

the most selective α_2-adrenoceptor antagonist, although its potency was considerably less than that of idazoxan. SAR in this series were probed by varying the substitution on the imidazole nitrogen and the chain length between the imidazole and benzodioxan rings. Lower alkyl groups on the imidazole



(39)

nitrogen and either one or two methylene groups between the two rings were found to give maximal potency and selectivity.

In a number of *in vitro* and *in vivo* models, imiloxan has been characterized as a selective, albeit weak, α_2-adrenoceptor antagonist. For example, in the

pithed rat, imiloxan antagonized the inhibitory effect of clonidine on the tachycardia produced by cardiac sympathetic nerve stimulation [140]. After acute and chronic dosing, imiloxan also reversed the effect of clonidine on locomotor activity [141]. A down-regulation of β-adrenoceptors in rat cerebral cortex was observed after chronic dosing of imiloxan over a 10 day period [142]. And in a comparative study of imiloxan, idazoxan and rauwolscine, imiloxan was again characterized as a weak but selective α_2-adrenoceptor antagonist [134].

2-SULPHONAMIDOBENZOQUINOLIZINES

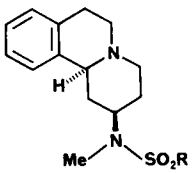
Several 2-sulphonamidobenzoquinolizines (40–42), originally prepared as antihypertensive agents [143], were found to be potent and selective α_2-adrenoceptor antagonists [144, 145]. The data in *Table 1.6* indicate that (40)

Table 1.6 α-ADRENOCEPTOR ANTAGONISM AND AFFINITY OF SUBSTITUTED BENZOQUINOLIZINES

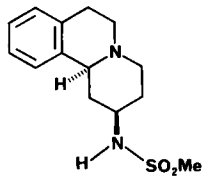
	Isolated Tissue pA_2 *[147]*			*Binding* K_i *(nM) [146]*[c]		
Compound	α_2[a]	α_1[b]	α_2/α_1	α_2	α_1	α_2/α_1
(40) WY 25309	7.81	6.04	59	7.4	3495	474
(41) WY 26392	8.08	6.34	55	5.5	1751	320
(42) WY 26703	8.46	6.49	93	9.2	1951	212
(43) WY 24965	6.27	5.33	9			
(1) Yohimbine	7.58	6.58	10	11.1	1015	92
(24) Idazoxan				9.2	1928	209

[a] Determined in the isolated rat vas deferens against clonidine-induced inhibition of electrically evoked contraction.
[b] Determined in the isolated rat anococcygeus against methoxamine-induced contraction.
[c] Determined in rat cerebral cortical membranes using [^{3}H]rauwolscine (α_2) and [^{3}H]prazosin.

(WY 25309), (41) (WY 26392) and (42) (WY 26703) were more potent and selective *in vitro* than yohimbine as α_2-adrenoceptor antagonists. In receptor-



(40) R = Me
(41) R = *n*-Pr
(42) R = *i*-Bu



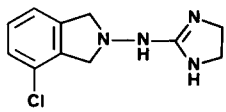
(43)

binding studies (*Table 1.6*), all three of these agents were more potent than yohimbine and at least as potent as idazoxan in displacing [^{3}H]rauwolscine from rat cerebral cortical membranes [146]. Compounds (40) and (41) were more selective than idazoxan for the α_2-adrenoceptor. The only SAR that can be drawn from these four benzoquinolizines is that *N*-methylation as in (43) led to a more potent and selective α_2-adrenoceptor antagonist (40 vs. 43).

A number of *in vitro* and *in vivo* models confirmed the selective α_2-adrenoceptor antagonism of (40–42) [147–150]. All three compounds antagonized clonidine-induced inhibition of electrically evoked contraction of the rat isolated vas deferens [147] and (41) reversed clonidine inhibition of tachycardia evoked by cardiac nerve stimulation in the anaesthetized dog [148]. Compound (41) was also active in increasing sympathetic tone in the anesthetized cat [149]. In the pithed rat, (40), (41) and (42) were active in reversing the B-HT 933 (α_2-adrenoceptor agonist)-induced inhibition of electrically evoked tachycardia and in reversing clonidine-induced hypotension in the rat by both central and peripheral administration [150]. On the basis of the latter experiments, it was concluded that (40), (41) and (42) readily penetrate the blood-brain barrier and antagonize α_2-adrenoceptors in the central nervous system [150].

4-CHLORO-2-(2-IMIDAZOLIN-2-YLAMINO)ISOINDOLINE (BDF-6143)

Originally prepared as a potential 'clonidine-like' antihypertensive agent, the isoindoline (44) (BDF-6143) instead turned out to be a potent α_2-adrenoceptor antagonist [151, 152]. BDF-6143 antagonized the hypotensive effects of



(44)

clonidine in anaesthetized rabbits at a significantly lower concentration (10^{-8} M) than yohimbine (10^{-7} M) [153]. Isolated tissue and ligand-binding experiments (*Table 1.7*) confirmed the high affinity and selectivity of this compound for α_2-adrenoceptors and demonstrated that this compound is more selective for α_2-adrenoceptors than either yohimbine or rauwolscine [153, 154]. However, the results of several studies showed that BDF-6143 is a partial agonist at the α_1-adrenoceptor. In a pithed rat preparation, BDF-6143 failed to antagonize the effect of phenylephrine at doses of up to 10^{-6} M, whereas prazosin was effective at 10^{-8} M [154]. In both the rabbit aorta and pulmonary

Table 1.7 α-ADRENOCEPTOR ANTAGONISM AND AFFINITY OF BDF-6143 [153]

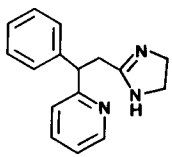
	Isolated Tissue pA_2			*Binding* K_i *(nM)*[c]		
Compound	α_2[a]	α_1[b]	α_2/α_1	α_2	α_1	α_2/α_1
(44) BDF-6143	9.00	7.58	26	1.66	30	18
(2) Rauwolscine	7.93	6.72	16			
(1) Yohimbine	7.58	6.71	7			

[a] Determined in the isolated rat vas deferens against clonidine-induced inhibition of electrically evoked contraction.
[b] Determined in rat anococcygeus against phenylephrine-induced contraction.
[c] Determined in rat cerebral cortex membranes using [^{3}H]rauwolscine (α_2) and [^{125}I]HEAT (α_1).

artery, BDF-6143 was shown to be a partial agonist at the postsynaptic α_1-adrenoceptor and an antagonist at the α_2-adrenoceptor [151]. In addition, an inhibitory effect on the release of NA was also noted which was ascribed to a non-α-adrenoceptor mechanism [151]. BDF-6143 is under development for the treatment of hypertension and chronic heart failure.

2-[2-(4,5-DIHYDRO-1*H*-IMIDAZOL-2-YL)-1-PHENYLETHYL]PYRIDINE (DG-5128)

In a search for new drugs for the treatment of diabetes, compound (45) (DG-5128) [155] was identified as an orally active hypoglycemic agent in animal models [156–158]. The activity of DG-5128 was found to be due to



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stimulation of insulin release from the pancreatic islets [157, 158]. However, the mechanism by which this stimulation of insulin release is achieved was found to be different from that of the sulphonylureas, such as tolbutamide. It is known that α-adrenoceptor agonism suppresses insulin release, whereas β-adrenergic agonism stimulates it. DG-5128 and the non-selective α-adrenoceptor antagonist, phentolamine, effectively blocked the inhibitory effect of adrenaline on glucose-primed insulin release in the rat, while tolbutamide did

not. Evidence that α_2-adrenoceptor antagonism was responsible for the activity of DG-5128 was obtained from the reversal of clonidine-induced suppression of insulin release by both DG-5128 and yohimbine. The α_1-adrenoceptor antagonist prazosin was inactive in this regard. Thus, DG-5128 appears to exert its hypoglycemic effect through stimulation of insulin release following antagonism of the α_2-adrenoceptors on the β cells of the pancreatic islets [158]. Subsequent studies to establish the α_2-antagonist profile of DG-5128 were performed in the dog mesenteric artery and rat vas deferens [159]. In the dog mesenteric artery, DG-5128 augmented the contractile response and [^{3}H]NA release evoked by electrical stimulation of the sympathetic nerve. DG-5128 also suppressed the inhibitory effect of the α_2-adrenoceptor agonist, guanabenz, on this sympathetic contraction and [^{3}H]NA release. In the rat vas deferens, DG-5128 and yohimbine had pA_2 values against clonidine of 6.7 and 7.7, respectively. DG-5128 did not attenuate the contractile response to phenylephrine in the epididymal portion of the vas deferens, indicating a high degree of selectivity for presynaptic α_2-adrenoceptor antagonism [159].

Table 1.8 α-ADRENOCEPTOR AFFINITY OF DG-5128

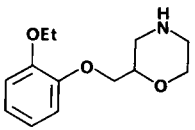
	Binding pK_i [160][a]			*Binding pK_i [161]*[b]		
Compound	α_2	α_1	α_2/α_1	α_2	α_1	α_2/α_1
(45) DG-5128	6.28	5.41	7.4	6.20	<5	>16
(1) Yohimbine	7.45	6.89	3.6			
(39) Imiloxan				7.10	4.50	398

[a] Determined in rat cerebral cortex membranes using [^{3}H]clonidine (α_2) and [^{3}H]prazosin (α_1).
[b] Determined in rat cerebral cortex membranes using [^{3}H]yohimbine (α_1) and [^{3}H]prazosin (α_1).

Data in *Table 1.8* represent results of ligand-binding studies on DG-5128 in comparison with yohimbine [160] and imiloxan (39) [161]. Both studies confirmed that DG-5128 has moderate selectivity for the α_2-adrenoceptor but is of lower affinity than yohimbine or imiloxan. It has also been shown that DG-5128 produces α_2-adrenoceptor antagonism through selective interaction with the low-affinity state of the adrenoceptor [162]. It was suggested that, due to the hydrophilicity of DG-5128, central effects would be minimized and thus DG-5128 would be a useful probe for pharmacological evaluation of peripheral α_2-adrenoceptors *in vivo* [160]. The compound is currently undergoing clinical trials for the treatment of diabetes.

α-ARYLOXYBENZYL MORPHOLINES

As mentioned in the section on potential clinical uses, a molecule which would be capable of antagonizing α_2-adrenoceptors and inhibiting the reuptake of monoamines should show a rapid onset of antidepressant activity in man [75]. From a series of α-aryloxybenzylmorpholines related to the antidepressant, viloxazine (46) [163], compound (47) was identified as an agent which could



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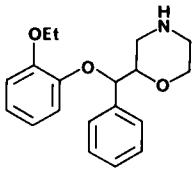

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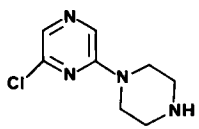
have this kind of pharmacological profile [164]. This compound reversed reserpine-induced blepharospasm and hypothermia in mice at 0.5 mg/kg, p.o. and was more potent than imipramine and viloxazine in inhibiting reuptake of [^{3}H]NA in rat hypothalamic synaptosomes. The evidence for α_2-adrenergic antagonism was based on a test for antagonism of clonidine-induced hypothermia in mice. At 10 mg/kg, p.o., (47) gave an 85% inhibition of the clonidine-induced hypothermia, whereas imipramine and viloxazine were inactive. However, this compound has not yet been evaluated in more classical α_2-adrenoceptor antagonism assays and therefore it is difficult to assess its actual activity in this regard.

PIPERAZINYLIMIDAZO[1,2-*a*]PYRAZINES, PYRIDINYLPIPERAZINES AND PYRIDINYLTETRAHYDROPYRIDINES

The serotonin agonist (48) (MK 0212) was found to possess affinity for α_2-adrenoceptors in binding studies [165]. This prompted the synthesis of a number of substituted piperazines as potential α_2-adrenoceptor antagonists. Among a series of piperazinylimidazo[1,2-*a*]pyrazines, the parent compound (49) was found to possess affinity for the α_2-adrenoceptor equal to that of



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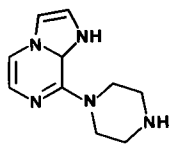

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Table 1.9. α-ADRENOCEPTOR AFFINITY OF PIPERAZINYLIMIDAZO[1,2-*a*]-PYRAZINE (49) [165]

	Binding K_i *(nM)*[a]		
Compound	α_1	α_2	$K_i(\alpha_1)/K_D(\alpha_2)$
(48) MK-212	2400	150	16
(49)	3100	19	160
Mianserin	43	19	2.3

[a] Determined in calf neo-cortical membrane homogenates using [^{3}H]prazosin (α_1) and [^{3}H]clonidine (α_2).

mianserin with approximately 70-times the selectivity of mianserin (*Table 1.9*). Substitution on the imidazo[1,2-*a*]pyrazine led to greatly decreased affinity and selectivity for the α_2-adrenoceptor binding site.

More potent and selective α_2-adrenoceptor antagonists were found among a series of pyridinylpiperazines [166]. *Table 1.10* gives results of evaluation of these compounds by ligand binding and *in vitro* studies in the rat vas deferens [166]. Reversal of clonidine-induced mydriasis was used as an *in vivo* model for the ability of compounds to act as central α_2-andrenoceptor antagonists.

Table 1.10. α-ADRENOCEPTOR ANTAGONISM AND AFFINITY AND RAT MYDRIASIS RESULTS FOR PYRIDINYLPIPERAZINES [166]

			Binding K_i *(nM)*[a]			*Isolated tissue* pA_2[b]			*Mydriasis*[c]
Compound	R^1	R^2	α_2	α_1	α_2/α_1	α_2	α_1	α_2/α_1	AD_{50} *(mg/kg)*
(50)	H	H	37	2400	65	6.4	5.7	5	1.28
(51)	F	H	8.2	2500	305	6.9	5.2	53	0.23
(52)	Cl	H	7.9	1800	228	6.9	5.7	16	0.44
(53)	Br	H	11	1480	135	6.6	5.8	6	0.68
(54)	F	Me	5	490	98	7.4	6.2	16	0.67
(55)	Cl	Me	2.7	215	80	7.4	6.5	8	0.56
(56)	Br	Me	2.9	160	55	7.6	6.5	13	0.74
(1) Yohimbine			49	220	4.5	7.7	6.5	14	1.04
(2) Rauwolscine			18	940	52	7.9	6.0	79	1.10
(24) Idazoxan			1.5	500	333	7.7	6.1	43	0.05

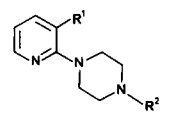
[a] Determined in calf cerebral cortex using [^{3}H]clonidine (α_2) and [^{3}H]prazosin (α_1). The ratio α_2/α_1 is K_i (prazosin)/K_i (clonidine).

[b] Determined in rat vas deferens for antagonism of clonidine (α_2) and methoxamine (α_1).

[c] Reversal of clonidine-induced mydriasis by i.v. administration.

Maximal activity was associated with the 3-halogenated pyridinylpiperazines (51–53). Although not the most potent compound in the ligand-binding and vas deferens assays, the 3-fluoropyridinylpiperazine (51) was, however, the most selective α_2-adrenoceptor antagonist. In reversing clonidine-induced mydriasis, (51) was the most potent compound in the series and was also more potent than yohimbine and rauwolscine. Based on the above data, (51) was chosen for detailed pharmacological studies as an α_2-adrenoceptor antagonist.

In related work, a series of pyridinyltetrahydropyridines was evaluated as potential antidepressants with combined α_2-adrenoceptor antagonist-monoamine neuronal reuptake inhibitor activity [167]. Compound (57) was the most potent and selective compound in the series in both ligand-binding and rat vas



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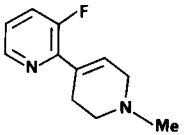

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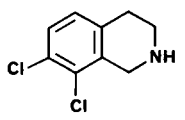
deferens evaluations. In the vas deferens, (57) had a pA_2 value of 6.8 compared with that of (51), which had a pA_2 of 7.4. In antagonizing tetrabenazine-induced ptosis in mice, (57) was 5-times less active than desmethylimipramine, whereas (51) was inactive. The relevance of the latter finding to antidepressant activity remains to be established [167].

1,2,3,4-TETRAHYDROISOQUINOLINES

7,8-Dichloro-1,2,3,4-tetrahydroisoquinoline, (58) (SKF 64139), originally developed as a potent inhibitor of phenylethanolamine-*N*-methyltransferase (PNMT) [168], was subsequently shown to be an α_2-adrenoceptor antagonist [169, 170]. Among a series of PNMT inhibitors tested for inhibition of



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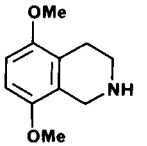

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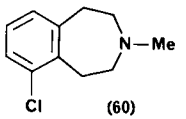
[^{3}H]clonidine binding, SKF 64139 had the lowest IC_{50} (4×10^{-8} M) [171]. The related dimethoxy compound (59) (SKF 72223), which was not a PNMT inhibitor, was also shown to have significant α_2-adrenoceptor affinity [169]. SKF 64139 and SKF 72223 inhibited [^{3}H]clonidine binding to rat cerebral cortex membrane homogenates with K_i values of 89 and 312 nM, respectively

[169]. In a test for antagonism of peripheral α_2-adrenoceptors, SKF 65139 and 72223 competitively antagonized the inhibitory effect of clonidine on the isolated guinea-pig atrium with dissociation constants (K_B) of 380 and 100 nM, respectively. When evaluated in spontaneously hypertensive rats (SHR) and DOCA-salt hypertensive rats [172], both SKF 64139 and SKF 72223 were found to lower blood pressure. SKF 72223 was the more effective hypotensive agent of the two in both models. In DOCA-salt hypertensive rats pretreated with prazosin, treatment with SKF 72223 caused a further decrease in blood pressure. Administration of clonidine slightly decreased the antihypertensive action of SKF 72223. Thus, it was suggested that antagonism of peripheral vascular α_2-adrenoceptors might be in part responsible for the antihypertensive activity of SKF 72223 and SKF 64139, although other possible mechanisms were not excluded [172].

2,3,4,5-TETRAHYDRO-1*H*-3-BENZAZEPINES

The 2,3,4,5-tetrahydro-1*H*-3-benzazepine (60) appears to be a considerably more potent and selective α_2-adrenoceptor antagonist than the related tetrahydroisoquinolines discussed in the previous section [173, 174]. In the isolated guinea pig atrium, (60) competitively antagonized the inhibitory effect of the



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α_2-agonist BHT 920 on neurotransmission [173]. The compound was also effective in increasing the release of [^{3}H]NA from electrically stimulated dog splenic arteries. SKF-86466 was found to be a weak α_1-antagonist based on its ability to antagonize the constrictor response to NA in the isolated rabbit ear artery. Based on these data, and those obtained in the guina-pig atrium, an α_2/α_1 selectivity ratio of 53 was calculated for SKF-86466, compared with ratios of 16 and 165 for phentolamine and yohimbine, respectively, in the same preparation [173]. When SKF-86466 was evaluated using ligand binding, it was found to a remarkably potent and selective α_2-adrenoceptor antagonist (*Table 1.11*) [161]. In fact, the compound had virtually no affinity for the α_1-adrenoceptor of rat cerebral cortical membranes. Additional studies will be needed to resolve the discrepancy between these results and the previously described in vitro results which showed the compound to be considerably less selective.

Table 1.11. α-ADRENOCEPTOR AFFINITY OF SKF 86466 AND SKF 72223 [175]

	Binding pK_i[a] *(nM)*		
Compound	α_1	α_2	α_2/α_1
(59) SKF 72223	5.54	6.80	18
(60) SKF 86466	<4	8.40	>10,000
(24) Idazoxan	6.10	7.90	63
(2) Rauwolscine	6.27	7.92	45

[a] Determined in rat cerebral cortex membranes using [^{3}H]prazosin (α_1) and [^{3}H]yohimbine (α_2).
[b] The α_2/α_1 selectivity ratio is the antilog of the difference between the affinity values (K_i).

SKF-86466 has demonstrated antihypertensive activity in several animal models [175]. The most pronounced antihypertensive effect was seen in anaesthetized DOCA-salt hypertensive rats, a model characterized by high catecholamine levels. In this model, SKF-86466 by i.v. infusion was about 10-times more potent in reducing blood pressure than in anaesthetized normotensive rats [175]. In general haemodynamic evaluation in the anaesthetized dog, SKF-86466 (100 μg/kg per min) decreased mean arterial blood pressure and increased myocardial contractility (dp/dt) [176]. The decrease in mean arterial blood pressure was similar to that observed with prazosin (10 μg/kg per min), while the increase in myocardial contractility was similar to that observed with rauwolscine (10 μg/kg per min). On the basis of these results, it was suggested that SKF-86466 has both inotropic and afterload-reducing properties [176]. The inotropic effect was suggested to be due to release of cardiac NA. However, it is difficult to explain the lack of a significant increase in heart rate if in fact increased levels of cardiac NA were released. In the conscious rabbit, SKF-86466 did not induce significant orthostatic hypotension, unlike prazosin, which demonstrated a considerable orthostatic hypotensive effect [177]. Hence, it was concluded that it may be possible to lower blood pressure through antagonism of α_2-adrenoceptors without the orthostatic liability of α_1-adrenoceptor antagonists.

SKF-86466 has been shown to produce concentration-dependent reductions in intraocular pressure (IOP) when administered topically to the eyes of rabbits [178]. The ocular hypotension did not appear to be secondary to a reduction in systemic blood pressure. Although it was suggested that the decrease in IOP could be due to antagonism of prejunctional α_2-adrenoceptors, the possibility that α_1-adrenoceptor antagonism could be involved was not discounted [178]. The situation regarding this mechanistic question could obviously use some clarification, since α_2-adrenoceptor agonists (such as

B-HT 933 and B-HT 920) have been shown to be very effective in lowering IOP upon topical administration to rabbit eyes, and this decrease in IOP was antagonized by yohimbine [179].

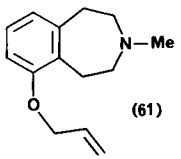
The 2,3,4,5-tetrahydro-1*H*-3-benzazepine structure of SKF-86466 (60) served as a useful scaffold for the preparation of an affinity ligand for the purification of the protein which contains α_2-adrenoceptors from human platelet membranes [180, 181]. The allylated derivatives (61–64) were prepared and their affinities for α_2-adrenoceptors in human platelet membranes and guinea-pig atria were measured [181] (*Table 1.12*). These compounds were then attached via the allyl group to a sulphydryl group on functionalized

Table 1.12. α_2-ADRENOCEPTOR AFFINITY OF 2,3,4,5-TETRAHYDRO-1*H*-3-BENZAZEPINES [100]

Compound	K_i *(nM)*[a]	K_β *(nM)*[b]
(60) SKF 86466	16.7	13
(61)	338	206
(62)	232	–
(63)	6.1	60
(64)	48	150

[a] Determined in human platelet membranes using [^{3}H]yohimbine.
[b] Determined in guinea-pig atria by measuring the inhibition of clonidine-induced depression of neurotransmission.

Sepharose CL-4B by a free-radical reaction. Columns prepared from the resulting modified Sepharose gels were then tested for adsorption of α_2-adrenoceptor-binding activity from solubilized human platelet membrane preparations. Only the column prepared from ligand (64) was found to adsorb protein



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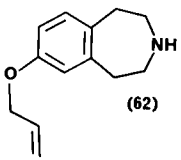

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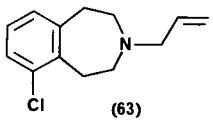

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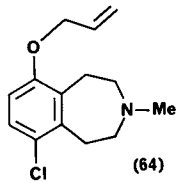

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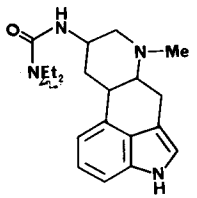
which had [^{3}H]yohimbine-binding activity. This column was able to adsorb 70–80% of the initial [^{3}H]yohimbine binding activity and recovery of the adsorbed material from the column resulted in a 200-fold increase in specific binding activity for [^{3}H]yohimbine. Thus, the Sepharose CL-4B affinity

adsorbant prepared from (64) was proposed to be a useful tool for purification of the protein which contains the α_2-adrenoceptor of human platelets [180].

The results of the binding activity of compounds (60–64) and the affinity adsorbants prepared therefrom furnished useful SAR information [181]. Since removal of the chlorine atom resulted in ligands (61) and (62), which did not adsorb binding activity when coupled to the column, the chlorine apparently provides an important lipophilic interaction with the receptor. The finding that the column prepared from (63) did not adsorb this material implies that the area in proximity to the ring nitrogen cannot tolerate a great deal of steric hindrance.

MISCELLANEOUS COMPOUNDS

A number of miscellaneous compounds have been reported to have α_2-adrenoceptor antagonist activity. Among a number of ergolines which were evaluated for α-adrenoceptor antagonism, the dopamine agonist lisuride (65) was found



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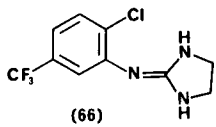
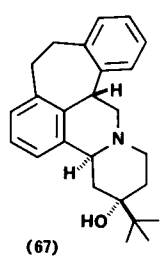


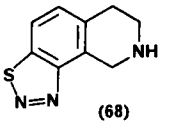
(66)

to be selective for the α_2-adrenoceptor [182]. Lisuride had a pA_2 value of 9.70 in guinea pig ileal (α_2) and 7.47 in guinea pig splenic (α_1) preparations. Rauwolscine had pA_2 values of 8.48 and 7.80, respectively, in the same preparations [182]. The α_1-adrenoceptor agonist (66) has also been reported to possess α_2-adrenoceptor antagonist properties in several models [183]. The dopamine antagonist (+)-butaclamol (67) was approximately equipotent to yohimbine as an α_2-adrenoceptor antagonist as measured by antagonism of the inhibitory effects of clonidine on the contractile response to field stimulation of the guinea pig ileum ((+)-butaclamol), $pA_2 = 8.0$; yohimbine, $pA_2 = 8.1$) [184]. The selectivity of (+)-butaclamol for the α_2-adrenoceptor as opposed to the α_1-adrenoceptor was found to be approximately 10-fold in this study.

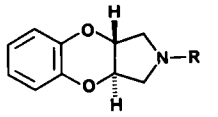
A number of compounds (and related generic structures) have been claimed in the patent literature to be α-adrenoceptor antagonists. These include (68) [185], (69) [186], (70) [187], (71) [188], (72) [189], (73) [190], and (74) [191]. Compound (75) (napamezole) has recently been chosen for clinical



(67)

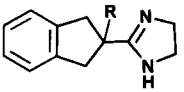


(68)

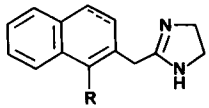


R=H,Me

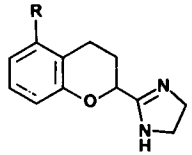
(69)



(70)

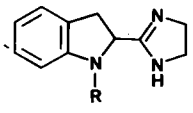


(71)

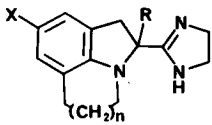


R=H, F

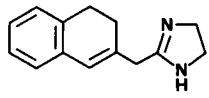
(72)



(73)



(74)



(75)

evaluation as an antidepressant [192]. In the rat vas deferens, (75) had a pA_2 against clonidine of 7.76 (idazoxan, $pA_2 = 8.11$). The selectivity of (75) for the α_2-adrenoceptor was 7.8-fold (idazoxan, 49-fold) on the basis of pA_2 values.

Table 1.13. α_2-ADRENOCEPTOR AFFINITY OF COMPOUNDS (76, 77) [193, 194]

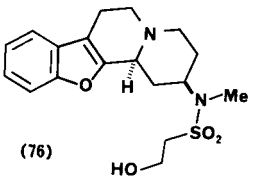
	Binding K_i *(nM)*[a]		
Compound	α_2	α_1	α_2/α_1 *ratio*[b]
(±)-(76)	1.6	210	130
(2*R*,12b*S*)-(76)	1.1	110	100
(±)-(77)	1.3	314	242
(2*R*,12b*S*)-(77)	0.35	115	330
Rauwolscine	25	940	40

[a] Determined using [^{3}H]rauwolscine (α_2) for (76), [^{3}H]clonidine for (77) (α_2) and [^{3}H]prazosin (α_1).

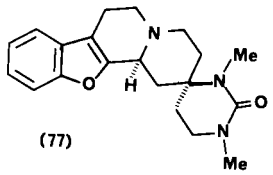
[b] The selectivity ratio α_2/α_1 is $K_i(\alpha_1)/K_i(\alpha_2)$.

Compound (75) was also reported to exhibit selective seratonin reuptake inhibition whereas idazoxan did not exhibit this activity.

Also very recently, several series of *N*-(1,3,4,6,7,12b-hexahydro-2*H*-benzofuro[2,3-*a*]quinazolin-2-yl)sulphonamides, as exemplified by (76) [193] and the spirocyclic congener (77) [194], have been reported to be highly potent and selective α_2-adrenoceptor antagonists (*Table 1.13*). The adrenergic properties of (76) and (77) were found to reside almost completely in the enantiomers with the absolute stereochemistry as drawn. This absolute stereochemistry corresponds to the absolute configuration of yohimbine and rauwolscine.



(76)



(77)

MODELS OF THE α-ADRENOCEPTOR

The first model of the α-adrenoceptor was proposed by Easson and Stedman and is known as the Easson-Stedman Hypothesis (ESH) [99]. Based upon the structure activity studies with a series of phenylethanolamine ligands, a three point attachment model of the adrenoceptor was proposed. The points of attachment are the basic nitrogen, the aromatic group with its *meta*- and *para*-hydroxyl groups, and the alcoholic hydroxyl group of the phenylethanolamine. Variants of this model have been proposed [195, 196] as has the concept of specific ionic interactions between ligands and the α-adrenoceptor [197, 198]. A synthesis of the ESH and the ion-pair model has also been proposed [199].

The development of quantum-mechanical methods for determining the preferred conformation of α-adrenoceptor agonists has been applied in the development of models of the α-adrenoceptor. The preferred conformations of phenylethanolamines have been determined and a model of the α-adrenoceptor has been proposed based on these calculations [200, 201]. This approach has been extended with respect to both phenylethylamine and phenylethanolamine compounds [202]. Using a similar approach, a model of the α-adrenoceptor was proposed based on a conformational analysis of imidazoline ligands [203]. In terms of models specifically designed to describe the interaction of ligands with the α_2-adrenoceptor, the ESH has been shown to be suitable in describing the interactions of phenylethylamines [204] but not imidazolines [205] with the

α_1- and α_2-adrenoceptor. A putative fourth point of attachment in the binding of phenylethylamines to the α_2-adrenoceptor has been proposed to accommodate α-substituted phenylethylamines [204].

A model of the α-adrenoceptor has been proposed based upon structure-activity studies with the yohimbine stereoisomers [7]. In this model, the apoyohimbine structure (*Figure 1.1*) is used as a template for ligands interacting

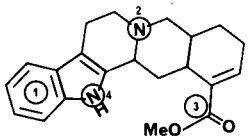


Figure 1.1. Apoyohimbine as a template for the α-adrenoceptor [7]

with α-adrenoceptors. Four important binding sites are identified and it is suggested that interaction with sites 1, 2 and 4 (*Figure 1.1*) may be more important for α_2-adrenoceptor antagonists when compared with α_1-adrenoceptor antagonists [7]. This model, and one based on structure-activity studies with tetramine disulphides [206], represent a significant departure from those mentioned previosuly since they are based on the structure of antagonist rather than agonist ligands. Further refinement of these models of the α-adrenoceptor should be facilitated by taking into account the structures and selectivities of the newer α_2-adrenoceptor antagonists discussed in this chapter.

CONCLUSION

This chapter has summarized the work that has been reported on α_2-adrenoceptor antagonists since the initial report of the α_2-adrenoceptor selectivity of yohimbine [89]. A number of selective and potent agents have been developed with widely varying chemical structures. On the basis of animal models, some of these compounds show promise as potential therapeutic agents for the treatment of depression and diabetes and possibly for obesity, impotence and hypertension. It will be of great interest to watch the progress of those α_2-adrenoceptor antagonists which have been advanced to clinical trails to see if in fact this promise will be realized and result in useful clinical entities.

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2 Radioligand-Receptor Binding in Membrane Receptor Research

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INTRODUCTION

In the last decade, we have witnessed significant advances in our understanding of the biochemical mechanism of drug action and the emergence of the specific sites of drug-action receptors from 'fiction' into 'biochemical reality'. This has been achieved due mainly to two factors: the collaborative efforts of the investigators from the different fields of research (classical and molecular pharmacologists, biochemists, cell and molecular biologists, endocrinologists, geneticists) and the development of the increasingly sensitive and sophisticated analytical procedures now at our disposal which allow a direct approach to drug-receptor interaction studies at the molecular level. This advance has promoted changes in our concept and definition of the receptors.

The observation which in the past led different investigators to the hypothesis that cells possess specific sites (receptors) for drug action was the ability of many cells to respond in a highly selective way to a minute concentration of particular chemical or drug with striking characteristics: the low concentration of drug required to elicit the effect (high potency), the chemical specificity (shown by the marked differences between the potency of drugs or optical isomers), and the biological specificity of the response – specific for the tissue and the receptor activated. Langley, studying the action of nicotine and curare on the voluntary smooth muscle, already in 1905 [1] postulated the existence of specific receptors or 'receptive substance' on the cell, which receive the stimulus from the drug and transmit it to the cell, causing the effect. Ehrlich [2], in his immunological studies on toxin-antitoxin interactions, came to similar conclusions about the existence and rôle of the receptors. Hill [3] formulated the equation describing the formation of the drug-receptor complex in terms of mass-action law, before Michaelis and Menten [4] published their enzyme kinetic theory on the quantitative enzyme-substrate interaction.

The impact of the receptor theory of Ehrlich and Langley on pharmacology was very limited during their lifetimes. The science of pharmacology had not advanced far enough to permit the theory, dealing essentially with molecular mechanisms, to be proven by experimental means. The interaction between the drugs and receptors received quantitative expression some 20 years later in the work of A.J. Clark [5], who showed that the drug has first to bind to the receptor and that the binding is reversible (noncovalent) and highly specific at low ligand concentrations and obeys the law of mass action. But it was not until 1950s and later that the receptor theory became a major area of research interest in pharmacology [6–8]. (For more background of the receptor theory, see also Refs. 9–11.)

The synthesis of structurally related analogues of drugs and endogenous

substances further expanded the field of the pharmacological receptor studies to work on the more detailed structure-function relationship. These studies established the structural requirements for the agonist (receptor stimulatory molecule) and antagonist (receptor inhibitory molecule) and led to distinguishing different classes of antagonists (such as competitive and non-competitive) for many of the receptors. This had an enormous impact on the development of the more pharmaceutical aspects of the research – rational drug design. The discovery of several recent groups of therapeutic drugs is at least partly attributed to this line of research, examples being anti-H_1 and anti-H_2 antihistamines and β-receptor blockers.

The use of different agonists and antagonists acting on different *in vivo* and *in vitro* preparations was also the beginning of the important taxonomic work on receptors which disclosed different classes of receptors, their localization and, for many classes, also the subtypes of the receptors. The earliest was the distinction between the muscarinic and nicotinic cholinergic receptors, followed by others such as β_1- and β_2-adrenergic, pre-postsynaptic receptors.

The classical pharmacological studies, based on the observation of the effect of a particular drug and, in particular, primary neurotransmitter agents acting on different organ models (mostly nerve-muscle preparations) where the receptor was still *in situ* have allowed important pharmacological, biological and therapeutic properties to be evaluated, but these could provide only limited information on the molecular nature of the receptors.

The beginning of the search for the receptor entities themselves was made after 1965 with the first direct and partly successful labelling of the muscarinic cholinergic receptor sites in slices of guinea-pig ileum by [^{3}H]atropine, carried out by Paton and Rang [12]. This was followed by the application of the ligand-binding technique to the opiate receptors [13] and nicotinic cholinergic receptors [14], which attracted enormous scientific interest and promoted similar studies on other receptors.

The initial attempts at the direct labelling of the receptors in tissue homogenates, isolated cells, membranes and other subcellular preparations with the specific markers were severely hampered by the difficulties in preparing suitable ligands which possessed biological activity without a high degree of binding to structures other than the specific receptors, and those of differentiating the receptor binding from that to other acceptor sites. Today, the basic technical difficulties for the biochemical identification and characterization of different membrane and intracellular receptors have been overcome. Well-validated radioligand-binding assays for labelling of almost all known receptors are available and a wide variety of high-affinity, highly specific radioactivity ligands are available for investigating them.

Table 2.1. USES OF RADIOLIGAND-RECEPTOR BINDING

1. BIOCHEMICAL STUDIES OF THE RECEPTOR:
   identification, characterization
   isolation (solubilization, purification, reconstitution)
2. LOCALIZATION, IDENTIFICATION AND CHARACTERIZATION:
   receptor subtypes
   pre- and post-synaptic receptors
   spare receptors
3. STUDIES OF RECEPTOR REGULATION:
   homospecific regulation
   heterospecific regulation
   (desensitization, supersensitivity, hormonal modulation)
4. RECEPTOR DEVELOPMENTAL AND AGEING STUDIES
5. STUDIES OF THE RECEPTOR ALTERATIONS IN DISEASE
6. DRUG SCREENING:
   mechanisms of drug action
   side-effects
   differentiation of agonists from antagonists
7. RADIOASSAY FOR NEUROTRANSMITTERS AND DRUGS

So far, the *in vitro* binding studies have facilitated several aspects of receptor research, as shown in *Table 2.1*, after their existence in broken cell preparations of the target organs had been established.

LIGAND-RECEPTOR INTERACTION

INFORMATION TRANSFER

The cell membrane, as an interphase between the cell and the surrounding environment, plays an important rôle in the transfer of information into the cell. This information transfer is accomplished in many cases through the interaction between the membrane (cell-surface) receptor and the particular chemical or drug acting on the cell. Receptors for many regulatory agents, including peptide and other hormones, neurotransmitters, prostaglandins, antigens, plant lectins and some bacterial toxins, are plasma-membrane components. (A summary of more important groups of membrane receptors is presented in *Table 2.2.*) It should be mentioned here that the cell possesses, in addition to

Table 2.2. MAJOR CLASSES OF MEMBRANE RECEPTORS FOR NEUROTRANSMITTERS, HORMONES OR DRUGS
Multiple receptor subtypes exist for most of the receptors mentioned below.

1. NEUROTRANSMITTER RECEPTORS
   a. opioid receptors
   b. receptors for acetylcholine:
      nicotinic
      muscarinic
   c. receptors for excitatory amino acids:
      L-glutamic acid
      L-aspartic acid
   d. receptors for inhibitory amino acids:
      γ-aminobutyric acid, GABA
      glycine
      β-alanine
   e. substance P receptors
   f. purine receptors
   g. receptors for miscellaneous peptides:
      angiotensin II
      neurotensin
      bombesin
      vasoactive intestinal peptide (VIP)
      thyroliberin
      somatostatin

2. RECEPTORS FOR BIOGENIC AMINES (HORMONES AND/OR NEUROTRANSMITTERS)
   a. catecholamine receptors
      α-adrenergic receptor
      β-adrenergic receptor
      dopamine receptor
   b. histamine receptors
   c. serotonin (5-hydroxytryptamine) receptors

3. HORMONE RECEPTORS
   a. receptors for glycoprotein hormones (glycoprotein of high mol wt. 28,000–43,000)
      gonadotropin receptors (follitropin (FSH); lutropin (LHN))
      thyrotropin (TSH) receptors
      chorionic gonadotropin receptors
   b. insulin and glucagon receptors
   c. receptors for insulin-like hormones growth hormone, prolactin, somatomedins (A, B, C)
   d. corticotropin (ACTH) receptors
   e. melanotropin (MSH) receptors
   f. oxytocin and vasopressin receptors

4. DRUG RECEPTORS
   a. benzodiazepine
   b. antidepressant

the hydrophobic membrane receptor, a soluble cytoplasmic receptor for steroid hormones and a receptor for thyroid hormones confined to the nucleus. The water-soluble intracellular receptors [15] for steroid and thyroid hormones differ from the hydrophobic membrane receptors in many characteristics and are not the subject of this review, but the basic principles of the specific drug binding responsible for the specific effect, which will be discussed here, are valid for both types.

The common property of membrane receptors is that they are macromolecules or macromolecular complexes embedded in the lipid bilayer of the cell membrane, mostly oligomeric proteins or glycoproteins belonging to the class of intrinsic (integral, endomembrane) membrane proteins, which differ in their properties from the loosely membrane-bound, extrinsic (peripheral, exomembrane) proteins located on the surface of the cell membrane (see *Figure 2.1*).

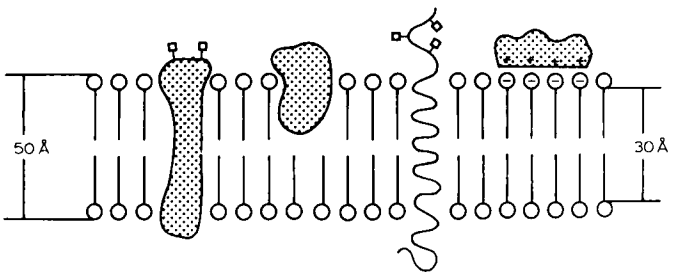

Figure 2.1. A schematic cross-section of a plasma membrane showing the disposition of lipids and membrane proteins. From left to right, examples of: intrinsic globular transmembrane glycoprotein, intrinsic globular protein, fibrous transmembrane glycoprotein and extrinsic membrane protein.

The main property of all the intrinsic types of membrane protein, which comprise 70–80% of all cell membrane protein, is that they are firmly bound to the membrane. Thus, they can not be extracted from the membrane easily and are involved in many regulatory and metabolic functions of the cell such as cell-cell interaction, hormone stimulation, vectorial transport of ions and metabolites, oxidative and photophosphorylation, and lipid biosynthesis. They can be amphipathic, with the hydrophobic ends embedded within the nonpolar interior of the lipid bilayer and the hydrophilic ends protruding from the membrane. The external region of this type of protein is particulary important for their function, since it contains the binding site or recognition site (enzymes, receptors) [16]. Some of the intrinsic proteins are transmembrane, as they intercalate the lipid bilayer. The bilayer-intercalated parts are arranged as one or more helices traversing the membrane and are formed by long stretches of hydrophobic amino acids which are typical only for the membrane proteins and are not found in the water-soluble proteins. Some of the intrinsic proteins are glycoproteins in which the carbohydrate moiety is covalently bound to the protein. So far, no intrinsic membrane protein has been isolated and crystallized in a form available for high-resolution X-ray crystallography. Our present picture of them has also to be based on the inferences drawn from

the structure of water-soluble proteins and from the low-resolution structural data collected from a few purified examples [17].

In order to understand fully the behaviour of the membrane receptors, it is necessary to determine their organization in the membrane, their dynamic arrangement within the membrane, and their physical-chemical properties. The following characteristics of the membrane receptors in the view of the general characteristics of the intrinsic membrane proteins have been demonstrated experimentally:

(1) The protein nature of the membrane receptors has been demonstrated by the use of proteolytic enzymes which have been shown to destroy the membrane receptor function. (2) The intrinsic nature of the membrane receptors has also been demonstrated; their extraction from the membrane requires the use of detergents necessary for all the intrinsic proteins [16]. (3) The presence of the carbohydrate moiety on the extramembrane surface of some of the membrane receptors has been demonstrated by binding with lectins and naturally occurring carbohydrate-binding proteins, such as concanavalin A and wheat-germ agglutinin. Nicotinic cholinergic receptors, insulin and glucagon receptors have been shown to belong to this class of glycoproteins. (4) It was of particular importance, considering the receptor function, to demonstrate that the recognition site of the membrane receptors is exposed on the outer non-cytoplasmic side of the membrane. This was shown for insulin and glucagon receptors in two typical sets of experiments: (a) treatment of intact cells (adypocites) with non-lytic concentrations of trypsin or agarose-immobilized trypsin destroys the response of the cell to insulin and the ability of the cell to bind hormone, and (b) agarose-bound insulin and glucagon, which do not enter the membrane, have been shown to stimulate the cell and provoke the response [18]. (5) The transmembrane topography has been demonstrated for some of the membrane receptors (nicotinic receptors, insulin receptors). For some of them, the structure has been elucidated in detail (for example, the oligomeric structure of nicotinic receptors) [19]. (6) The least-studied part of the membrane receptor is its dependence on the overall membrane environment. It was shown that the acidic phospholipids and the fluidity of the membrane appear to be important for the binding. There is also good evidence now that these receptors are not static, but have a degree of mobility in the membrane, such as the possibility of the free lateral diffusion in the plane of the membrane. This motion of the receptor in the plane of the membrane plays a physiologically important rôle and is involved in the communication among various receptors, and assembly of multimolecular structures [20]. From studies on intrinsic membrane proteins it is also apparent that the membrane receptors possess no unique gross physical or chemical properties that distinguish them from other

membrane proteins of which they constitute only a small fraction (about 1%). However, they differ from them in their remarkable and unique ability to bind specific ligands with high affinity and to translate this binding into the relevant biological response.

According to the present 'consensus statement', membrane receptor mediated information transfer into the cell proceeds in several steps involving membrane and intracellular molecular mechanisms which can be divided into six consecutive stages: (1) recognition; (2) generation of the signal; (3) transmission of the signal to the effector system; (4) decoding of the signal by the effector; (5) response; (6) return to the basal state, involving removal of the signal and attenuation of the response.

Schematic representation of these events is given in *Figure 2.2.*

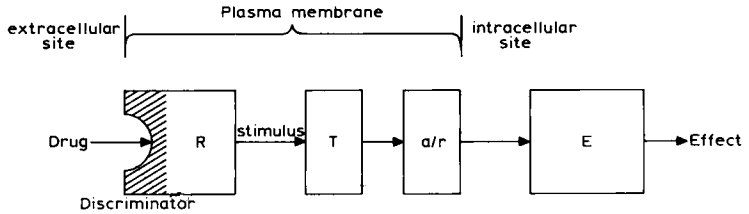

Figure 2.2. Schematic representation of the membrane receptor-mediated signal-effector coupling proceeding by the cascade of processes: R, receptor; T, transmission; a/r, amplification or reduction of the signal, and E, effector system.

Any attempt to elucidate the process of signal-effect coupling will require the study of each step of the information transfer [21].

The first steps in the receptor activation – recognition of the extracellular messenger by the membrane-bound receptor (by definition, the receptor is the component capable of discriminating the information from similar molecules encoding different messages) – are accomplished through highly specific binding of the regulatory ligand (D) to the membrane receptor (R) (by definition, the receptor contains at least one binding site specific for a natural ligand). The ligand-receptor binding is then translated into the generation of the signal, which depends on the ligand and membrane receptor activated. In the case of slow-acting extracellular ligands (such as insulin or epidermal growth factor (EGF), the process of signal generation involves micro-aggregation, redistribution and/or internalization of the hormone-receptor complex. The subsequent events of the signal transmission to the effector system are not clear yet, but they probably involve an intracellular coupling component [21].

In the case of fast-acting extracellular messengers (such as neurotransmitters or catecholamines), more information on signal generation and transmission is available and this proceeds in a cascade of processes. The first step after the formation of the ligand-receptor complex is believed to be a change in conformation of the receptor. The conformation change in the receptor is followed by the specific 'primary response' such as the regulation of the membrane enzyme activity, resulting in the formation of a product called the secondary chemical messenger (to be differentiated from the first or extracellular messenger), which then modulates other cellular systems and is thus responsible for the transmission (T) of the signal via amplification (a) or reduction (r) of the signal and further activation of the intracellular effector system (E) (*Figure 2.2*). The process of activating the effector system through the cascade of the molecular events is entirely contained in the membrane receptor, not in the ligand, and the ligand only acts as a trigger for the receptor-mediated effect. Receptors can interact with one or several effector systems in the cell for which they must also possess the recognition site and, on the contrary, a single effector system may connect with different receptors [22].

THE SECOND-MESSENGER CONCEPT

The definition of the second messenger (the molecule for communication between the membrane receptor-activation and intracellular response) was first proposed by Sutherland in 1956 [23]. It implies that a first messenger (hormone, neurotransmitter or drug) transmits its extracellular message to the cell via another molecule, the 'second messenger', produced in response to the first or extracellular messenger, which is then responsible for a series of specific intracellular events leading to the effect, specific for the hormone and receptor activated. Sutherland discovered the first second messenger, cyclic adenosine 3′,5′-monophosphate (cAMP), by studying the induction produced by hormones (adrenaline and glucagon) of liver glycogenolysis. He observed that, in response to the addition of the hormone to liver homogenates, a product which he called 'heat-stable factor' was formed in the particulate fraction. In the second stage, this factor stimulated the formation of the phosphorylase in the supernatant fraction, in which the hormones themselves were inactive. This effect was more pronounced at lower hormonal concentrations. He also showed that the relative activity of the hormone was similar in the experiments on liver homogenates, liver slices and intact animals. Sutherland was thus the first to show the link between the receptor stimulation by the hormone and activation of the next membrane regulatory mechanism leading to the effect. The 'heat-stable factor' was later characterized as cAMP and the membrane

enzymatic activity responsible for the formation of cAMP was shown to be the membrane enzyme, adenylate cyclase.

The scientific community was reluctant for almost a decade to appreciate the biological importance of this nucleotide. Only in the 1970s did the significance of the second messenger concept become apparent and new development on this field started to increase exponentially. Most of the progress came from the biochemical and genetic studies from different laboratories [24,25]. It was shown that the hormone-dependent adenylate cyclase system is composed of at least five functional units which are involved in bidirectional regulation of adenylate cyclase: the adenylate-cyclase-stimulating receptor (R_s) and the adenylate-cyclase-inhibiting receptor (R_i); two regulatory units of adenylate cyclase the guanine nucleotide- (GTP-) dependent regulatory proteins (N_i and N_s proteins) and the catalytic moiety of the adenylate cyclase (C) responsible for the conversion of ATP into cAMP (*Figure 2.3*).

Information on the receptor-dependent cyclase system is still far from complete, but all five components of the system are extensively studied. It has been shown that a large variety of agonists including small molecules such as L-noradrenaline, histamine, serotonin, small peptides such as vasointestinal peptide (VIP), opiates and large polypeptide hormones such as glucagon, adrenocorticotropic hormone (ACTH), and thyrotropin (TSH) activate the adenylate cyclase through their receptors. Radioligand-binding studies have allowed characterization of the various membrane receptors which are part of the system. They also established that receptors are physically separable from the catalytic unit [26]. The elegant genetic reconstruction work of Ross and Gilman in 1977 [27] led to identification of a previously unidentified part of the receptor adenylate cyclase system: the GTP-dependent regulatory proteins (N_s and N_i) which are essential in the agonist-induced enzyme activation process. Cloning the variants of S 49 lymphoma cell lines, they observed that the CYC^- mutant of S 49 lymphoma cell lacks the whole working β-receptor adenylate cyclase system responsive to hormone, GTP and fluoride ions. It was shown that CYC^- cells are deficient in a component distinct from the β-receptor and catalytic unit. They achieved the restoration of the complete functional β-receptor-adenylate cyclase system by adding detergent extracts of wild-type S 49 lymphoma cells to the CYC^- cells which supplied the missing component, subsequently classified as GTP-binding regulatory N_s protein.

The bidirectional regulation of adenylate cyclase is obtained via two distinct types of receptor: β-adrenergic receptor (β_1, β_2 subtype), dopamine D_1, adenosine A_2, vasopressin V_2, and histamine H_2 stimulate the adenylate cyclase through the N_s protein, whereas an equally large group of receptors

including α_2-adrenergic, adenosine A_1, prostaglandin, somatostatin, opiates, dopamine D_2, vasopressin V_1, etc., receptors mediate their effects through inhibition of adenylate cyclase activity, utilizing the N_i binding protein (*Figure 2.3*). The two coupling components (N_s and N_i) exhibit some

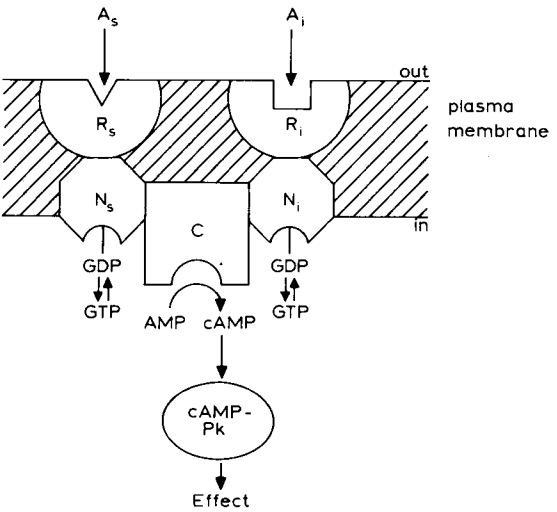

Figure 2.3. Scheme of regulatory pathways in the receptor-adenalyte cyclase system: Five components of the bidirectional regulation of adenylate cyclase. Agonist (A_s) binding to receptor (R_s) stimulates adenylate cyclase (C) through GTP-binding regulatory (N_s) protein. Agonist (A_i) binding to receptor (R_i) inhibits adenylate cyclase through the GTP-binding regulatory (N_i) protein. Conversion of ATP into cAMP by C is thus either enhanced or inhibited. cAMP activates cAMP-dependent protein kinase(cAMP-Pk) leading to the effect characteristic for the cell and receptor activated.

similarities and also important differences in structure and functional properties. N_s and N_i binding proteins are both heterotrimeric in structure (N_s–α_s, $\beta\gamma$; N_i–α_i, $\beta\gamma$ subunits) and have been successfully purified [25]. (The catalytic unit of adenylate cyclase, on the other hand, has not been purified, but it seems likely that its structure is also oligomeric.) Many studies also indicate that N_s and N_i are part of the whole family of N-proteins which exist in the membranes and have multiple rôles in addition to activation of adenylate cyclase (N_i is possibly involved in the receptor-mediated Ca^{2+} mobilization) [28,29]. Both proteins undergo an activation-deactivation cycle. As proposed by Abramson and Molinoff [30], the activation is initiated by the agonist-receptor interaction followed by binding of the receptor-agonist complex to the

N-protein. This interaction induces the conformation changes in the N-protein that allow the exchange of bound GDP with GTP in the presence of Mg^{2+} (the sensitivity of N_i to Mg^{2+} is higher than the sensitivity of N_s) [31]. The GTP activated N-protein can then interact with the catalytic unit of adenylate cyclase to convert ATP into cAMP (see *Figure 2.3*).

In the further process of receptor-effector coupling, cAMP activates membrane and/or intracellular enzyme protein kinase responsible for protein phosphorylation.

It is now well established that two membrane-bound and two soluble cAMP-dependent protein kinases and their isozymes mediate most of the intracellular effects of cAMP on enzyme function, protein synthesis and very likely gene expression in eucaryotic cells. These protein kinases have been designated as 'general', since they can regulate the activities of a large number of different protein substrates [32].

Sutherland and co-workers observed that only a limited number of hormones (catecholamines in various tissues, glucagon in liver, histamine in brain, and ACTH in adrenal cortex and adipose tissue), acting on their receptor were able to stimulate the membrane enzyme adenylate cyclase to produce cAMP, and correctly predicted that, although cAMP was the only second messenger known at that time, it seemed likely that other second messengers would be discovered [23].

Ca^{2+} was proposed next as being a 'second messenger', since a dramatic increase in the intracellular free Ca^{2+} by either mobilization of intracellular Ca^{2+} stores and/or influx from extracellular sites, elicited by diverse extracellular signals including neurotransmitters and hormones, by unknown mechanisms independent of cAMP formation, was observed. Since part of the increase in the free intracellular Ca^{2+} results from the mobilization of Ca^{2+} from the intracellular pools, whereas the receptors are at the cell surface, it was postulated that the receptor occupation must generate a diffusible messenger molecule capable of releasing Ca^{2+} from the internal stores. A significant amount of information accumulated which suggested that the inositol lipid break-down catalyzed by phospolipase(s) C might be involved in the Ca^{2+} signalling [33,34]. The evidence in favour of this idea came very recently, when the membrane lipid whose break-down is controlled by the receptor was identified [35,36]. The proposed lipid is the phosphoinositide-phosphatidylinositol 4,5-biphosphate (PIP_2). It was also shown, that, in contrast to the receptor-adenylate cyclase system, regulation of intracellular Ca^{2+} levels by receptors is not mediated via a united mechanism [37]. The PIP_2 lipid break-down alone, upon the action of phospholipase(s) C, produces two potent cellular signals by yielding two biologically active products: (a) 1,2-

diacylglycerol (1,2-DG) and (b) the recently discovered *myo*-inositol 1,4,5-triphosphate (IP_3). 1,2-Diacylglycerol is responsible further for direct stimulation of protein kinase C, whereas IP_3 activates protein kinase C (or other Ca^{2+}-dependent protein kinase(s)) by mobilizing Ca^{2+} from the intracellular stores (*Figure 2.4*).

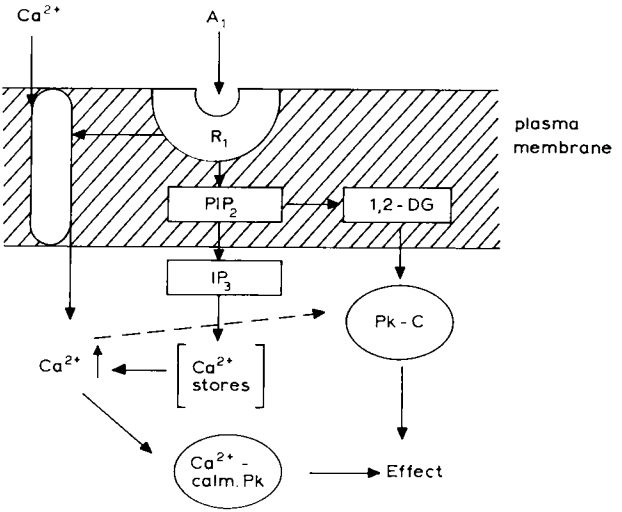

Figure 2.4. Scheme of regulatory pathways in the receptor-Ca^{2+} mobilizing system. Agonist (A_1) binding to receptor (R_1) activates: (a) Ca^{2+} influx, (b) initiates phospholipid breakdown (PIP_2, phosphoinositide-phosphatidylinositol 4,5-biphosphate; IP_3, myo*-inositol 1,4,5-triphosphate; 1,2-DG, 1,2-diacylglycerol), which is followed by the release of Ca^{2+} from the internal Ca^{2+} stores and activation of protein kinases (Pk-C = protein kinase C; Ca^{2+}-calm Pk, = calcium, calmodulin-dependent protein kinase), leading to the effect.*

A number of experiments showed that IP_3 can cause release of Ca^{2+} from a wide spectrum of cell types, which strengthens the view that IP_3 acts as the link between receptor occupation and intracellular Ca^{2+} mobilization. Some scientists even ascribe a 'second messenger' function to this molecule. They believe that the release of IP_3 is a very early response of the cell to various stimuli such as vasopressin V_1, adrenergic α_1 and muscarinic agonists in several tissues [38]. Although details of how IP_3 may function as a second messenger remain unclear, the findings on the involvement of phosphoinositide breakdown represent a recent major breakthrough in our understanding of the coupling of receptor with the Ca^{2+}-dependent response of the cell [38].

In addition to Ca^{2+} mobilization through phospholipid break-down, some hormones, drugs, neurotransmitters, by stimulating the receptors, are able to raise the cytosolic Ca^{2+} independent of phospholipid break-down (see *Figure 2.4*).

In the studies of the rôle of Ca^{2+} in receptor-mediated Ca^{2+} signalling, general agreement about the question of where the receptor-mobilized Ca^{2+} comes from, has not been reached. The studies on hepatocytes as a cellular model have shown that the Ca^{2+} mobilization includes rapid Ca^{2+} release into the cytosol from the cell-associated stores (mitochondria and/or endoplasmic reticulum and plasma membrane) and slower entry of Ca^{2+} from the exterior [36]. Another aspect still under investigation is the intracellular origin of the Ca^{2+} released from the Ca^{2+} stores by IP_3. The results obtained in different cell types strongly indicate that IP_3 releases Ca^{2+} from endoplasmic reticulum but not from mitochondria [39]; but at the moment, neither of the above internal Ca^{2+} stores can be ruled out.

Further transmission of the Ca^{2+} signalling in the cell is mediated, as in the case of the receptor-adenylate cyclase system, by the specific group of protein kinases responsible for the protein phosphorylation, but dependent on Ca^{2+}, not on cAMP. Two Ca^{2+}-dependent protein kinases have been discovered: (a) protein kinase dependent on Ca^{2+} and calmodulin (Ca^{2+}-binding protein); and (b) protein kinase C dependent on Ca^{2+}, phospholipid and 1,2-diacylglycerol. Both types have broad protein-substrate specificity, which suggests that Ca^{2+} activates protein phosphorylation directly [32].

In spite of the experimental difficulties in correlating the ligand-receptor interaction with the Ca^{2+} mobilization, the number of reports on receptors transmitting their information by the above mechanism is constantly increasing: these include α_1-adrenergic, cholinergic-muscarinic, histamine H_1, 5-hydroxytryptamine–5 HT, vasopressin V_1, substance P and angiotensin II receptors in several tissues.

To show the functioning of some of the membrane receptors and the rôle of the 'second messenger' molecule in them, we discussed in detail two examples of the membrane-receptor-mediated information transfer systems, both proceeding by a cascade of events (the receptor-adenylate cyclase and the receptor Ca^{2+}-mobilizing system). As shown, the studies of the molecular mechanisms involved are far more advanced in the receptor-adenylate cyclase system than the others and, from the standpoint of the structural features, we are within sight of the molecular description of five essential components of the system (R_s, R_i, N_s, N_i and C). Rodbell [40] proposed, in addition, a separate putative regulatory unit for cations (mono- and/or divalent) (M unit) as part of the receptor-adenylate cyclase and possibly other receptor-effector

systems. Its rôle in the regulation of signal transduction needs to be further clarified.

Knowledge of the receptor Ca^{2+}-mobilization system is in a progressive state. It appears that this system is more complex, and the relationship of receptor, Ca^{2+} and phospholipid-metabolism is still full of contradictions, partly since no sufficiently good experimental possibility has been found to differentiate the various receptor-mediated extra- and intracellular Ca^{2+}-mobilizing mechanisms and partly since the responses to the receptor activation vary and are highly dependent on the location of the receptor.

Knowledge on other receptor-effector systems is even more scanty, ranging from receptors where knowledge on the molecular structure of the receptors is in advanced state without comparable understanding of the signal-transducing mechanisms, to receptors where both parts are almost unknown. In both instances, the 'second messenger' molecule has not been identified.

An example of the first type of receptor mentioned above is the insulin receptor and some insulin-like receptors. Many of the structural features of the insulin receptor at the molecular level are known (the subunit structure of insulin receptor) [41], but the detailed molecular mechanism by which insulin regulates cellular metabolism and development is not known. Some workers believe that insulin action is mediated by an as yet unknown soluble 'second messenger' molecule (peptide or peptide-like material, material having lipid or carbohydrate components or a family of mediators?) released from the plasma membrane upon exposure to insulin [42], whereas others think that this would be too simple a mechanism to explain various insulin actions in the cell. Houslay and Heyworth [43] recently proposed three distinct but closely-related routes (a multi-pathway mechanism) through which insulin may elicit the effects on target cells after binding to its membrane receptor: (a) endocytosis of the insulin receptor (after insulin binds to the receptor/receptor clusters and internalizes, which transfers the signal into the cell – at this point receptor is either destroyed by lysosomes or recycles to the plasma membrane), which could be responsible for translocation of specific membrane proteins; (b) activation of the specific protein kinase (specific for the insulin receptor, tyrosine) and (c) activation of GTP-regulatory N-binding protein and associated protein kinases, eliciting the alteration in the phosphorylation of membrane and cytosolic proteins. This proposed multi-pathway mechanism offers a better possibility of explaining the various actions of insulin on a wide variety of processes within the cell [44].

With a large variety of neuropeptides, the mechanism of receptor-mediated signal transfer is not known, since the biochemical as well as the pharmacological studies in this area are in an early phase. The radioligand-binding studies

of most neuropeptide-receptors are still restricted by the limited knowledge of peptide pharmacology, whereas studies of the molecular mechanism of action are complicated by the ability of neuropeptides to influence (regulate or modulate) several membrane systems (ionic channels, cyclic nucleotide metabolism, Ca^{2+}-mobilization, etc.).

There appears to be also a third group of receptors, such as the nicotinic-acetylcholine receptor (AChR) and possibly receptors for some amino-acid transmitters (GABA receptor), where the receptor is closely linked to an ionophore and no other component and/or changes have been detected between receptor and ion flux. AChR mediates the transmission of the impulses at certain cholinergic synapses in many parts of the nervous system. This is achieved by the interaction of neuronally released acetylcholine (ACh) with its postsynaptic membrane receptor. AChR, upon agonist binding, activates a gated cation channel and triggers the selective rise in the permeability of the postsynaptic membrane for small ions (primarily Na^+, K^+ ions). Na^+ and K^+ ions move across the membrane, producing a decrease in membrane potential. The resulting local depolarization, when sufficient, gives rise to an action potential and produces a general response in the postsynaptic cell. The cation channel is an integral part of the ACh receptor itself with the gating region on the extracellular site of the channel structure. This information is based on the evidence that binding of ACh to the receptor followed by the conformational change in the receptor opens the cationic channel. It still remains to be clarified how the various parts of the AChR, which is a transmembrane glycoprotein consisting of five protein subunits, contribute to the receptor functional domains (channel structure, gating mechanisms) [19].

GABA is an inhibitory amino-acid transmitter which produces changes in neuronal excitability by the opening of chloride ion channels in the cell membrane. The GABA-receptor appears to be unique among the receptors, since its function can be modulated by another receptor – the benzodiazepine drug receptor. Benzodiazepines (widely prescribed anxiolytic, sedative-hypnotic anticonvulsant and muscle relaxant drugs), by binding to their receptor, have been shown to increase the frequency of chloride-channel opening in response to GABA stimulus (there is no evidence to suggest that benzodiazepines can control gating of the chloride channel directly) [45].

The aim of the above description of some of the molecular mechanisms involved in the receptor-effector coupling is to show the complexity and importance of these studies, but, due to limited space and limited knowledge of some receptor-effector systems, they cannot be discussed in detail for each receptor-effector system separately. It shows also that much greater insight into the structure and function of all components of the receptor-effector system,

not only the ligand-binding domain, will be needed to understand the functioning of the receptors.

QUANTITATIVE ASPECTS OF THE LIGAND-RECEPTOR INTERACTION

The sequence of the first event in receptor activation – binding of the ligand to the receptor macromolecule – is an exact physicochemical interaction, the precise nature of which is not known due to our limited knowledge of receptor structure. We have to rely on the theoretical assumption that the ligand-receptor interaction, similar to enzyme-substrate interactions, is a simple biomolecular process and obeys the mass action law. Under the assumption that a single class of equivalent independent binding sites exists on the receptor and the biological effect directly correlates with the fractional occupancy of the binding sites (the second assumption in some systems may be incorrect due to the presence of spare receptors), the interaction between the ligand and the receptor proceeds according to *Scheme 2.1*:

(a) interaction of an agonist:

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} LR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} LR^* \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} L + R^* \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \underline{R} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} R$$

(b) interaction of an antagonist:

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} LR$$

(c) the affinity or equilibrium constant for the reaction:

$$K_A = \frac{[LR]}{[L]\,[R]} \text{ or } K_D = \frac{1}{K_A}$$

(d) biological effect: the final biological effect (E) is directly related to the strength of the signal, thus to the number of [LR] complexes formed, or, considering equation (c), on K, [L], [R]:

$$E = (f)\,[LR] \text{ or } E = (f)K_A\,[L]\,[R]$$

Scheme 2.1. L, free ligand; R, free receptor; LR, ligand-receptor complex; K_A*, equilibrium association constant;* K_D*, equilibrium dissociation constant;* k_n*, association rate constant;* k_{-n}*, dissociation rate constant; LR*, activated complex; R*, activated receptor;* $\underline{R}$*, receptor in the intermediate step.*

In the simple ligand-receptor interaction, the ligand (L) (drug, neurotransmitter, hormone) reversibly binds to the receptor forming the complex (LR). Agonists, in contrast to the antagonists, are able to initiate the stimulus to the cell by forming an activated complex (LR*). The nature of the activation is complex, as shown in the previous section, and in some cases

involves the conformational change in the receptor followed by coupling to the next membrane component (see *Figures 2.3* and *2.4*) and other mechanisms. When the complex dissociates into L + R, the receptor very likely switches (conformation changes of the receptor macromolecule and uncoupling of the receptor from the other membrane components) from the activated state (R*), first to the intermediate-regenerative state (R), which could explain the loss of receptor responsiveness in the case when agonist is repeatedly applied, and finally to the resting state (R). The ligand (L) is inactivated either by the catabolic or by the uptake mechanisms. The differences in the interactions of agonists, antagonists and partial agonists with their receptor are explained on the basis of the differences in the kinetics of the reaction (equations (a) and (b)). For the agonist, the relationship between the association (k_n) and dissociation rate (k_{-n}) constants in the reaction sequence must be $k_2 \gg k_{-1}$, thus allowing the reaction to proceed (equation (a)); for competitive agonists $k_2 \ll k_{-1}$, thus limiting the reaction to the first step (equation (b)); and for partial agonists $k_2 \approx k_1$ [46].

The affinity of the ligand-receptor interaction is expressed as affinity or equilibrium constant K (K_A or K_D) measured in the equilibrium (equation (c)) or from the kinetic parameters where:

$$K_A = \frac{k_n}{k_{-n}} \quad \text{or} \quad K_D = \frac{k_{-n}}{k_n} .$$

The magnitude and rate of the stimulus into the cell basically depends on three determinants: affinity of the ligand for the receptor (K), concentration of the ligand [L], and concentration of receptor, [R] (equation (d)). Accordingly, all changes in the stimulus are the consequence of the changes in any of the above parameters.

The above main considerations of the quantitative aspects of the ligand-receptor interaction should serve only as an introduction, since the application of the law of mass action to the measurement of the receptor-ligand interaction has been worked out and reviewed extensively [47,48].

RADIOLIGAND-RECEPTOR-BINDING ASSAY

The basic principle of the radioligand-receptor-binding assay is to follow the first step in the receptor activation – binding of the ligand to the receptor in a broken cell preparation – and determine the quantitative parameters (thermodynamic and kinetic) which define the receptor, the ligand and receptor-ligand interaction.

The method of the ligand-binding assay employed to the receptor-related studies does not differ from the techniques used to measure ligand binding in other biochemical systems (radioimmunoassay or other competition binding assays), but could not be successfully applied to the receptor studies for several reasons, typical for all membrane receptors – exceedingly low receptor number in the broken cell preparations compared with other membrane constituents, strict chemical specificity required for the binding of the ligand to the receptor, poor biological activity in addition to low specific radioactivity of the ligands, rapid degradation of both ligand and receptor, and high nonspecific binding of the ligand to structures besides the receptor – which invalidated the suggestion that ligand binding to the target tissue is biologically relevant. The pioneering studies in the 1960s eliminated these problems, so that the method now used allows the precise measurement of ligand-receptor interaction on the molecular level. The problem of defining the requirements for binding to the receptor (for example, distinguishing specific receptor binding from nonspecific binding) was overcome to a great extent by developing the basic criteria to aid the investigators [49]. The criteria and conditions for the receptor identification by radioligand binding are summarized in *Table 2.3.*

DETERMINATION AND EVALUATION OF THE RADIOLIGAND-RECEPTOR BINDING PARAMETERS

Radioligands

In designing a radioligand-receptor binding study, the selection of the radioligand is most important for successful receptor identification. The radiolabelled ligand (tritiated or radioiodinated) chosen for the study should possess high specificity for the receptor, and sufficient radioactivity to detect the expected number of receptor sites. Many commercially available radioligands satisfy these criteria, but more selective and highly radioactive ligands will still be needed. Target cells possess approximately 10^4–10^5 binding sites per cell; in membrane preparations from the receptor-rich tissues, we could expect a receptor binding of 0.2–20 pmol/mg membrane protein and in membrane preparations from other tissues, receptor binding would be less than 0.2 pmol/mg protein. This puts some limitations on the use of tritiated ligand (where the degree of radioactivity varies between 5 and 90 Ci/mmol) in the system where the density of receptor is extremely low or small amount of tissue is available. Iodinated ligands can be prepared with higher radioactivity (2000 Ci/mmol), but the use of iodinated label presents other problems: relatively short half-life (60 days for ^{125}I and 8 days for ^{131}I); storage over a

Table 2.3. SPECIFICITY CRITERIA AND CONDITIONS FOR RECEPTOR IDENTIFICATION BY THE RADIOLIGAND BINDING

1. RADIOLIGAND – RADIOACTIVELY LABELLED RECEPTOR AGONIST OR ANTAGONIST
   Criteria: pharmacologically active
   chemically pure
   specific radioactivity: 5–90 Ci/mmol, preferentially in the range above 20 Ci/mmol for the tritiated products; in the range of 2000 Ci/mmol for iodinated products

2. BINDING – SATURABILITY AND AFFINITY OF THE RECEPTOR-BINDING SITES
   Parameters: number of receptor-binding sites (B_{max})
   equilibrium dissociation constant (K_D) for the receptor-radioligand complex, a measure for the affinity

3. KINETICS OF BINDING REACTION – REVERSIBILITY OF THE RECEPTOR-LIGAND INTERACTION
   Parameters: association rate constant k_1 – determined from the time course of ligand-receptor binding
   dissociation rate constant k_{-1} – determined from dissociation of ligand-receptor complex

   equilibrium dissociation constant $K_D = \frac{k_{-1}}{k_1}$

4. DRUG DISPLACEMENT IN COMPETITION BINDING STUDIES – ability of nonradioactive agonist, antagonist or drug belonging to different chemical and pharmacological classes to compete for the radioligand binding site. To determine affinity, specificity, or stereospecificity of the competitor

   Parameters: $K_i = \frac{IC_{50}}{1 + [L]/K_D}$

   K_i = dissociation constant for the receptor-inhibitor complex, a measure for the affinity
   IC_{50} = concentration of unlabelled ligand (inhibitor) giving half maximum displacement of radioligand
   $[L]$ = concentration of radioligand in the assay
   K_D = dissociation constant for the receptor-radioligand complex

5. CORRELATION BETWEEN DRUG AFFINITY FOR THE RECEPTOR-BINDING SITES WITH PHARMACOLOGICAL POTENCY EXPERIMENTS
   Parameters: K_D and K_i in correlation with K_A and K_B

   K_A = dissociation constant for receptor agonist in pharmacol. experiments
   K_B = dissociation constant for receptor antagonist in pharmacol. experiments

6. SUBCELLULAR DISTRIBUTION OF THE RECEPTOR SITES – identification of receptor in the preparation by the radioligand binding, should correlate with the function of the receptor in the tissue.

7. REGIONAL DISTRIBUTION OF THE RECEPTOR AND TISSUE SPECIFICITY

short period of time even in the cold (2–3 weeks) may sometimes not prevent the degradation of the ligand (iodinated peptides); and the relatively bulky radiolabel in some cases could cause the alteration of biological activity. An important consideration is also the integrity of the radioligand, where ideally 100% of the radioactivity should be present as the active ligand. In selecting the appropriate ligand, it also appears that in many instances the use of labelled antagonists has some advantages over agonists. Most neurotransmitters (unlike the hormones) have low affinity (generally 10–100-fold less than antagonists) for their receptors and, in addition to chemical degradation, bind more extensively to the non-receptor binding sites (such as recognition sites, catabolic enzymes) [50] and are thus, in many systems, less appropriate ligands for binding studies [51].

Determination of specific receptor binding

In a series of binding experiments to determine the total radioligand binding, different concentrations of radioligand are incubated with fixed aliquots of membranes, cells or tissue homogenates for an appropriate time which allows the binding reaction to proceed to equilibrium at constant temperature (approximately 30–40 min at 24° C, 10–20 min at 37° C), After the incubation, the ligand-tissue-complex is rapidly chilled to 4° C and quantitatively separated from the free (unbound) ligand by one of the methods of choice: centrifugation or vacuum filtration through cellulose or glass-fibre filters. The pellets are quickly rinsed with cold buffer and bound radioactivity in the particular material is measured after solubilization (^{14}C, ^{3}H) or directly (^{125}I). In the ideal situation, the method should be rapid and gentle, so that the equilibrium conditions are preserved throughout the assay. Binding studies performed at the desired physiological temperature 37° C, due to potent proteinase activity present in intact cells as well as in membrane preparations, may lead to the degradation of the receptor-protein and thus to underestimation of both ligand-binding capacity and ligand affinity. In many instances, this can be avoided by performing the assays at 24° C, which still permits sufficiently rapid equilibration of the system in addition to minimizing the degradation of the receptor. The same applies also for the storage of the membranes, where even at 0° C some proteolytic activity is observed and the storage at lower temperature in some cases destroys the receptor.

Once the total radio-ligand tissue binding is demonstrated, the interaction must be further characterized in order to establish which part of the binding truly reflects the specific ligand-receptor interaction. For this purpose, in the parallel experiments, nonspecific nonreceptor binding is determined: different

concentrations of radioligand are incubated with the membranes under the same conditions as in the determination of the total binding, but in the presence of high concentrations of non-radioactive ligand which binds to the same receptor. Bound radioactivity is determined as above.

Determination of nonspecific binding is based on the assumption that the competing non-labelled ligand, present in high concentration in the assay, will displace the radioactive ligand, present in low concentration in the assay, from essentially all high-affinity receptor sites and saturate the receptor (displaceable binding) but will not interfere appreciably with the binding of radioligand to low-affinity nonsaturable nonspecific sites (nondisplaceable binding). Concerning the concentration of a displacing drug, the lowest concentration consistent with displacement of at least 90% of specific binding should be used. Calculation of the correct molar excess of the displacing drug over the concentration of radioligand is based on the ratio of the K_D of receptor-ligand and receptor-competitor complex. In the case where both ligands have equivalent affinities, 10–100-fold excess is sufficient to displace 90 or 99%, respectively, of the ligand from the high-affinity sites. Huge excesses of unlabelled ligand, however, could result in additional displacement of nonspecific sites.

The specific receptor binding is calculated for each radioligand concentration used as the difference between the total and nonspecific binding. Determination of specific binding according to the above principle (displaceable binding = specific binding, and nondisplaceable binding = nonspecific binding), which is at present the only approach to differentiate receptor from nonreceptor binding in the radioligand-binding assay, has led to mistakes and contradictions in several receptor systems. Two major problems have become apparent: (a) Nonspecific binding of the ligand does not necessarily represent only low-affinity binding, but can also exhibit high affinity for the ligand. It was shown that [^{3}H]spiperone binds with high affinity not only to the dopaminergic and serotoninergic receptors, but also to the nonspecific spirodecanone sites [52]. Several groups observed also high-affinity displaceable radioligand binding to tubings, filters and glassware [53]. (b) Specific high-affinity displaceable binding could represent either the receptor site or recognition site for compounds having a similar chemical structure or having a chemical moiety identical to the radioligand. High-affinity displaceable binding of [^{3}H]cimetidine, [^{3}H]ranitidine, [^{3}H]histamine and [^{3}H]imipramine was shown to be not to the receptor sites, but to the recognition site. [^{3}H]Cimetidine, instead of binding to histamine H_2-receptors, was shown to bind to imidazole recognition sites, whereas [^{3}H]ranitidine binding was to furan recognition sites [48,52].

The best way to prevent these mistakes is to determine specific binding by

using a displacer which possesses the same receptor profile and/or pharmacological properties as the radioligand, but belongs to a different chemical class of drugs. It is generally agreed that this should be adopted as a strict and most important rule in setting up the binding assay [54].

In the ideal binding assay, the nonspecific binding should be low in comparison with the specific binding. However, this is difficult to achieve and it depends greatly on tissue source and number of receptor sites per unit of tissue. Subcellular fractionation in comparison with tissue homogenate cells normally allows further reduction in nonspecific binding.

Once the specific binding is demonstrated, the binding must further satisfy the criteria summarized in *Table 2.3.*

Saturability of binding, high affinity of binding (K_D), number of receptor sites (B_{max})

The specific receptor binding in contrast to nonspecific binding of the radioligand should be saturable, indicating a finite number of receptor-binding sites. Under equilibrium conditions, specific binding should saturate with increasing concentrations of radioligand in the incubation medium (see saturation isotherm of [^{3}H]mepyramine binding data on *Figure 2.6*).

To analyse the ligand-binding data (determination of the maximal number of receptor-binding sites (B_{max}) and equilibrium dissociation constant for the receptor-ligand complex (K_D)), the method of Scatchard [55] is usually

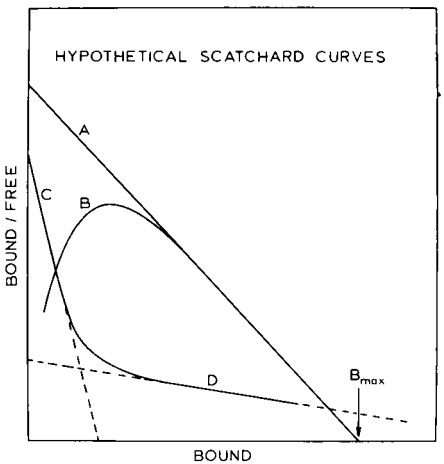

Figure 2.5. Hypothetical Scatchard curves determining the different receptor-ligand interactions: (A) ligand interaction with homogeneous sites; (B) ligand interaction with homogeneous sites, showing positive cooperativity; (C + D) ligand interaction with heterogeneous sites.

employed. The binding data from the saturation experiments are plotted according to the method of Scatchard, where the bound/free ([RL]/[L]) ligand ratio is plotted against bound ligands [L]. The receptor concentration equals B_{max} when the bound/free ratio approaches zero and the slope of the line equals $-K_D^{-1}$ (*Figure 2.5*).

The linear transformation of the binding data, using the Scatchard analysis, indicates that the radioligand binds to a single class of homogeneous binding sites, all having equal affinity for the ligand (*Figure 2.5* line A), whereas a nonlinear plot, after it is resolved into the linear components, is an indication of heterogeneous sites with different affinities for the ligand and different number of binding sites (*Figure 2.5* curves C,D). The mixed system could be interpreted as: (a) two affinity states (high and low) of the same receptor; (b) two different receptors; (c) two different receptor subtypes; (d) negative co-operativity at the homogeneous receptor sites. Curve B on *Figure 2.5* represents positive co-operativity of the homogeneous sites [55a].

The resolution of the mixed binding systems is usually a difficult task for the investigator, and the decision as to which of above situations applies for the system under investigation should be carefully made by use of multiple radioligands (ligands with high selectivity, capable of discerning among different binding sites) and multiple nonlabelled competing ligands. In the opioid receptor, by analysing the mixed system, only the radioligands of at least 100-times higher affinity for the preferred site in comparison with the next preferred site were able to differentiate between the two opioid receptor subtypes (introducing curvature in the Scatchard plot), whereas differences in the affinity of the radioligand up to 10-fold showed an inaccurate picture of the binding to the homogeneous sites (linear Scatchard plot) [56].

When using the method of Scatchard to avoid false conclusions and methodological artifacts, the bound and free ligand must be accurately determined at equilibrium. The nonlinearity in the Scatchard plot interpreted as multiple state, multiple site and/or co-operativity of the receptor-binding sites, shown in *Figure 2.5*, can simply result in methodological mistakes, such as nonequilibrium conditions in the assay, presence of endogeneous ligand, inaccurate estimation of 'free' or 'bound' ligand in the system with high nonspecific binding (errors in either of them are magnified, since ratios of these parameters are used in Scatchard plots), ligand-ligand and receptor-receptor interactions, and degradation of the ligand (for critical review see Ref. 57).

Other methods often used to analyse the saturation binding data are: (a) the double-reciprocal plot, where reciprocal of bound (B^{-1}) (y axis) is plotted against free ligand (F^{-1}) (x axis). The intercept on the y axis gives the reciprocal of the number of receptor sites ($1/n$), whereas the intercept on the

x axis represents the reciprocal of the dissociation constant ($-1/K_D$); (b) the application of the Hill equation (Hill plot), where the ratio of $\log_{10} B/B_{max} - B$ is plotted against $\log_{10}$ free ligand (B = bound ligand at different ligand concentration in the incubation media, B_{max} = maximum number of binding sites determined by the method of Scatchard). The slope of the line gives the Hill coefficient (n_H). A Hill coefficient close to the value of 1 is an indication of the radioligand interaction with the homogeneous binding sites whereas $n_H < 1$ or > 1 is an indication of the heterogeneous interaction or cooperativity of the binding sites. The Hill binding constant, K_D, is calculated as the abscissa value when $B/B_{max} - B = 0$ and is the analogue of the K_D determined by the method of Scatchard [58].

Reversibility of binding

The kinetics of association and dissociation of the radioligand with and from the receptor should be followed in the binding assay, when using reversible ligand. In two sets of experiments, the time-course of the interaction of different concentrations of ligand with the receptor binding site and dissociation of the radioligand from the receptor must be followed. The association rate constant, k_1, and dissociation rate constant, k_{-1}, are calculated or obtained graphically. The ratio of k_{-1}/k_1 gives the equilibrium dissociation constant, K_D, which should agree with the K_D determined by the equilibrium method of Scatchard or by other methods (such as the double-reciprocal plot or the Hill plot).

Drug displacement and determination of the affinity constant (K_i) for competitor

Generally, a receptor site is expected to display a selectivity for a given class of ligands. This specificity of binding enables the initiation of the response specific for the given receptor. In the binding assay, the unlabelled agonists and antagonists of the given receptor should thus effectively compete with the radioligand for the receptor binding sites, whereas agents with no pharmacological activity for the receptor should not compete for the receptor site. In a series of binding experiments, membranes are incubated with a fixed concentration of radioligand in the presence of different concentrations of competitor. A competition curve is constructed for each competitor: % bound radioligand, $\%B$ (y axis) is plotted against the molar concentration of competitor (100% binding represents the radioligand binding in the absence of competitor). The IC_{50} value is determined from the curve and the affinity or dissociation constant (K_i) of each competitor for the receptor according to the equation of Cheng and Prusoff [59] on *Table 2.3*. The equation to determine K_i values (*Table 2.3*) is

valid only when the total number of receptor sites (R_t) in the assay is significantly lower than K_D ($R_t < 0.1\ K_D$). The affinity constants for a series of competitors give then the rank order of specificity and/or stereospecificity for the receptor. In the investigations of receptors on the molecular level, using the radioligand-binding technique, this is the most important and decisive criterion for defining the binding sites as the receptor site (see *Figure 2.7*). The resulting data from the displacement experiments may also be analysed by Hill plot or subjected to the linearization (Hofstee plot) to determine the nature of the competitor-receptor interaction [58,60].

Correlation of binding (K_D, K_i) and pharmacological parameters

The binding site can be definitely equated with the receptor site only when the binding sites can be shown to be associated with a pharmacological or physiological event. When identifying the receptor by the radio-ligand binding technique, it is not possible to follow directly the final effect in the cell elicited by the ligand-receptor interaction. The binding parameters of the radioligand and its competitors should thus be compared with their potency *in vivo* and *in vitro* obtained in the various pharmacological procedures. (1) The rank order of potency of a series of agonists and antagonists to inhibit radioligand receptor binding should be comparable with their potency to elicit or antagonize the biological response in the intact tissue. (2) The K_i for the receptor-inhibitor complex obtained from the binding studies should correlate with drug potency from the pharmacological studies [61]. (3) Compounds, inactive on the receptor *in situ*, should not inhibit radioligand binding. (4) The binding must correlate with some readily measurable biochemical activity elicited or inhibited by the ligand-receptor interaction. If the second messenger of the receptor is known, the binding of a drug to the receptor and its effect on second messenger production should be studied simultaneously and affinities compared. (Good correlation exists between the binding affinities of a series of adrenergic drugs for the β-adrenergic-receptor and their activation of adenylate cyclase.) (5) When quantitative correlation between binding and pharmacological parameters is made, it is preferred that pharmacological data and binding data be obtained on the same system under identical conditions (this criterion could be satisfied to a certain extent for periphery; in the CNS, however, the quantitative correlation is difficult to achieve).

Although it is desirable to compare the affinity constants of the radioligand (K_D) or competing drug (K_i) determined in the radioligand-binding assay with the affinity constants, dissociation constants (K_A for agonists or K_B for antagonists) or the relative efficacies obtained in the pharmacological

procedures (*Table 2.3*), in most structure-activity relationship studies on isolated tissues these values (for most drugs) are not available and one is forced to use the less meaningful ED_{50} values (the concentration of drug eliciting half-maximal response) and intrinsic activity (ratio of maximal response of drug relative to that of a standard reference agonist), which is a measure for efficacy. The ED_{50} value, depending on the receptor reserve, which may be large in some tissues, is usually a poor estimate of the true dissociation constant, and a compound with low efficacy may show the same intrinsic activity as a full agonist in the system with high receptor reserve [61,62].

The tissue localization and distribution of the receptor binding sites

The receptor binding sites may be expected to be found in target organs where the ligand has a biological rôle.

In general, when all the previously mentioned criteria are satisfied, it is likely that the binding of the radioligand to the subcellular fraction of a target organ represents the true receptor binding.

RADIOLIGAND BINDING IN THE BIOCHEMICAL IDENTIFICATION AND CHARACTERIZATION OF THE MEMBRANE RECEPTORS

The biochemical studies of membrane receptors are oriented towards the final goals: the isolation of the receptor macromolecule, which then can be studied in detail; and reconstitution of the receptor back into the working system in the membrane where it could again show its basic functional properties – the signal initiating and transducing functions.

The molecular characterization of the various membrane receptor systems was initiated by the introduction of *in vitro* binding techniques to receptor studies. The use of the radioligand-receptor binding assay provided the first quantitative characterization of the membrane-bound receptors in the isolated cells or particulate cellular preparations. This was followed by the development of efficient solubilization procedures which allow the conversion of the complex membrane system into the relatively simple state essential for further purification of the membrane receptor protein. Affinity chromatography or the covalent affinity or photoaffinity labelling technique (using irreversible or immobilized ligand or radioligand) could then be applied for the further purification and isolation of the receptor protein. More recent additions to the receptor-isolation oriented studies are immunochemical methods (immunoaffinity chromatography, use of receptor-specific monoclonal antibodies, or

anti-idiotypic antibodies) leading to the application of molecular genetics to this field of research.

CHARACTERIZATION OF THE MEMBRANE-BOUND RECEPTORS AND/OR RECEPTOR SUBTYPES

The major task in the membrane receptor studies using the radioligand binding technique which was accomplished in the last 10 years has been direct identification and characterization of the receptor-binding sites in various particulate subcellular preparations of the receptor-bearing tissues.

For most of the membrane receptors in the brain and peripheral tissues, summarized in *Table 2.2*, the basic binding parameters are known, and this in turn has brought more possibilities to focus on the biochemical properties and structure of the receptor and other signal-transducing membrane components.

Radioligand-binding studies have also become a powerful tool for classification, localization and regional distribution of the receptor subtypes.

For some of the membrane receptors, the existence of the distinct receptor subtypes was already postulated in the classical pharmacological studies (catecholamine receptors and histamine receptors). The classification was derived from the different order of potency of a series of drugs influencing the same receptor in different tissue preparations. The variations in the response led in 1967 to the subdivision of β-adrenergic receptors into β_1- and β_2-subtypes [63] and in the 1970s α-adrenergic receptors into α_1- and α_2-subtypes [64]. The initial subclassification of α-receptors was made on an anatomic basis, presuming that the α_1-subtype represents the postsynaptic and α_2, the presynaptic (auto)receptor. This subdivision is no longer valid, because α_2-receptors have been found also at postsynaptical locations [65]. Current subclassification of α-adrenergic receptors could only be based on the biochemical approach, where α_1-receptors mediate their effect through phospholipid turnover and Ca^{2+}-metabolism and α_2-subtypes mediate their effects through inhibition of adenylate cyclase activity (see previous section).

Further evidence for delineation and quantification of the multiple membrane receptor subtypes came from radioligand binding. Practically, two approaches for the receptor-subtype classification have been used: (a) labelling the receptors with the radioligands which possess high affinity and high selectivity for the receptor subtype under investigation. This approach can be used only when such a subtype-selective radioligand is already available (see *Figure 2.6*); (b) an alternative (indirect) approach to the differentiation of the receptor subtypes is based on the radioligand displacement studies: it involves labelling the entire receptor population by a nonsubtype selective radioligand

and then displacing the binding of the radioligand (present at fixed concentration in the assay) with the unlabelled subtype-selective or nonselective agonists or antagonists (varying concentration in the assay). Individual competition curves for each competitor are constructed and IC_{50}, K_i values and slope factor (Hill coefficient, n_H) are determined [66] (see also *Table 2.3* and previous section).

For better results, inhibition binding data could be subjected to the linearizing transformation by constructing a plot: % B (% bound ligand in the presence of competitor) (y axis) against B (concentration of competitor in the assay) (x axis) (Hofstee plot) [60,67]. Inhibition of binding of a nonselective radioligand by a nonselective competitor results in a linear Hofstee plot, whereas a subtype-selective competitor produces a curvilinear Hofstee plot, thus indicating multiple receptor sites. The mixed system can be resolved by computer-assisted analysis (curve fitting to a one- or two-site model) to determine the subtype ratio and affinity of the ligand for each site [68]. For catecholamine receptors, radioligand-binding studies have provided clear support for the earlier pharmacological β-receptor subclassification into β_1 or β_2 subtypes in spite of the fact that available β-receptor radioligands, such as [^{3}H]dihydroalprenolol, are not subtype-specific. By taking advantage of differences in the potency of various β-adrenergic drugs for one or another subtype, one can distinguish the portion of the ^{3}H-labelled ligand binding to β_1- as opposed to β_2-receptors (using the displacement approach) [69].

Radioligand-binding studies have helped to clarify the regional distribution of β-adrenergic subtypes. β_1- and β_2-subtypes (which are both adenylate cyclase-linked) are distributed throughout the brain (a marked variation in β_1- in contrast to a more homogeneous distribution of the β_2-subtype was observed) [70]. β_1-receptors in the periphery exist primarily in the heart and adipose tissue, whereas the β_2-subtype is found in vascular tissue such as smooth muscle, lung and airways [71]. Good correlation exists between the radioligand binding and the physiological function of the β_1–β_2-subtypes.

Binding studies of α adrenergic receptors have also provided evidence for heterogeneity of receptor sites in the CNS and periphery. Both subtypes (α_1, α_2) were identified in the brain and periphery (α_1-receptors exist throughout the brain, airways, cardiac muscle, vascular smooth muscle and liver) and α_2 receptors were found in the brain, blood platelets, adipocytes, and vascular smooth muscle). In contrast to β-adrenergic receptors, sufficient α_1- or α_2-selective radioligands are available for their identification. Agonist-ligands include the imidazolines ([^{3}H]clonidine and p-[^{3}H]aminoclonidine (PAC)) and catecholamines ([^{3}H]adrenaline and [^{3}H]noradrenaline), which have been successfully used to identify α_2-receptor sites in the brain.

Antagonists [^{3}H]WB4104 ([2-(2,6-dimethoxy)phenoxyethanolamino]methylbenzodioxan) and an ergot alkaloid, [^{3}H]dihydroergocryptine ([^{3}H]DHE), were the first ligands available for binding studies. [^{3}H]WB4104 (α_1-specific) was one of the first ligands used to label α_1-receptor sites in rat brain [72], whereas [^{3}H]DHE was later shown to be nonselective ligand, labelling α_1- and α_2-sites with equal affinity [73,74]. We have used [^{3}H]DHE to label α-adrenergic receptors in vascular smooth muscle membranes. Using the displacement approach with two unlabelled selective α_1- (prazosin) and α_2- (yohimbine) antagonists, we were able to show presence of α_1- and α_2-subtypes in this tissue [75,76].

More recently developed radioligands used for α_1-α_2 differentiation [76a] include [^{3}H]prazosin (α_1-specific), [^{3}H]yohimbine ([^{3}H]YOH), [^{3}H]rauwolscine ([^{3}H]RAUW) (α_2-selective). Rauwolscine shows higher selectivity for α_2-sites than does yohimbine [77]. Some newer ligands are still under investigation [78].

Binding studies of α_1- and α_2-receptors, in addition to differentiating α_1- from α_2-sites, have also shown some discrepancies (differences in the number of receptor sites) when investigators were using [^{3}H]agonist instead of [^{3}H]antagonist ligands for their identification. These findings were first interpreted as two different physically and functionally distinct sites, one labelled by the agonist and the other by the antagonist [79], but later it was concluded that agonist-ligands and antagonist-ligands label with different affinities different affinity states of the same receptor (α_2-receptors in bovine cortex) [78].

It is also apparent from the binding studies that α_2-sites in the CNS and periphery have similar characteristics and that pre- and postsynaptic α_2-sites are pharmacologically indistinguishable, and this made the differentiation of pre- and postsynaptic α_2-receptor populations technically problematical.

Chemical sympathectomy with 6-hydroxydopamine (6-OHDA) proved to be partly successful in differentiating pre- and postsynaptic α_2-receptors in the brain. Intraventricular 6-OHDA administration to 30-week-old rats provokes in the first week a 20% and 30% decrease in the number of α_2-receptor binding sites labelled with [^{3}H]PAC or [^{3}H]YOH, and this may indicate that about 20% of labelled α_2-sites are auto-receptors residing on noradrenaline (NA) terminals. However, the picture was clouded by the later (after 4 weeks) increase in α_2-receptor number, probably due to 'denervation supersensitivity' of the tissue caused by 6-OHDA [78].

In the third type of catecholamine receptors – the dopaminergic receptors – three distinct receptor subtypes, D_1, D_2, D_3 (nomenclature of Cresse) [80], have been identified, up to the present time, by radioligand-binding and cyclase assays. The general characteristics of the above subtypes are as follows. The

D_1 subtype has been shown to be adenylate cyclase-linked (stimulatory) and can be labelled with high affinity by [^{3}H]thioxantenes (nM range), but its pharmacological or physiological rôle in the CNS (where it occurs primarily in the dopaminergic brain areas) is as yet unknown.

D_2 sites (linked to adenylate cyclase in inhibitory fashion) whose occurrence is confined to dopaminergic brain areas, have been shown to mediate virtually all known pharmacological as well as physiological functions of dopamine (DA). They can be labelled with high affinity with dopaminergic antagonists: [^{3}H]haloperidol, [^{3}H]spiperon (spiroperidol) and [^{3}H]sulpiride and agonists: [^{3}H]bromocryptine and [^{3}H]apomorphine (K_D in the nM range).

D_3 sites can be labelled with dopaminergic agonists such as [^{3}H]dopamine and *N-n*-[^{3}H]propylnorapomorphine (K_D in the nM range) and show low affinity for antagonists (μM range). They were hypothesized as being putative dopamine-autoreceptors, but no adequate evidence has ever been provided.

Neither D_1 nor D_3 sites at present fulfil the criteria for being the receptor sites (considering the correlation with physiological function in the brain). For D_1 sites, the possibility exists that they may have a rôle in the periphery (mediating parathyroid hormone release?) [81,82].

Histamine receptors have also been differentiated on pharmacological grounds, previous to radioligand-binding assays, into H_1 and H_2 subtypes [83]. Whereas specific radioligands such as H_1-antagonists like [^{3}H]mepyramine [84] and an antidepressant like [^{3}H]doxepin [85] are available for labelling H_1 sites, difficulties in the attempts to label H_2 sites were observed. Specific H_2-antagonists such as cimetidine and ranitidine were tritiated and used in the binding assays, but were later shown to label recognition rather than receptor sites (see also previous sections) [48,52]. Currently under investigation is a tritiated H_2-antagonist, [^{3}H]tiotidine, which is showing some promising results [86]. We have used [^{3}H]mepyramine to map the H_1 receptor sites in the vascular smooth muscle membranes. The properties of the specific high-affinity binding component (shown in *Figure 2.6*) indicate that the H_1 receptor has been labelled in this tissue.

The results of the competition experiments (*Figure 2.7*) carried out with different histamine H_1- and H_2-agonists and antagonists showed that they inhibit [^{3}H]mepyramine binding in the same order as they stimulate or inhibit histamine receptors in the intact tissue. Good correlation exists between the inhibition properties of the agonists and antagonists for the ligand-binding sites (K_i) and their pharmacological characteristics (ED_{50}).

In this study, it was shown that the high-affinity [^{3}H]mepyramine binding component in vascular smooth muscle membranes satisfies the receptor criteria outlined in *Table 2.3* [87,87a,b].

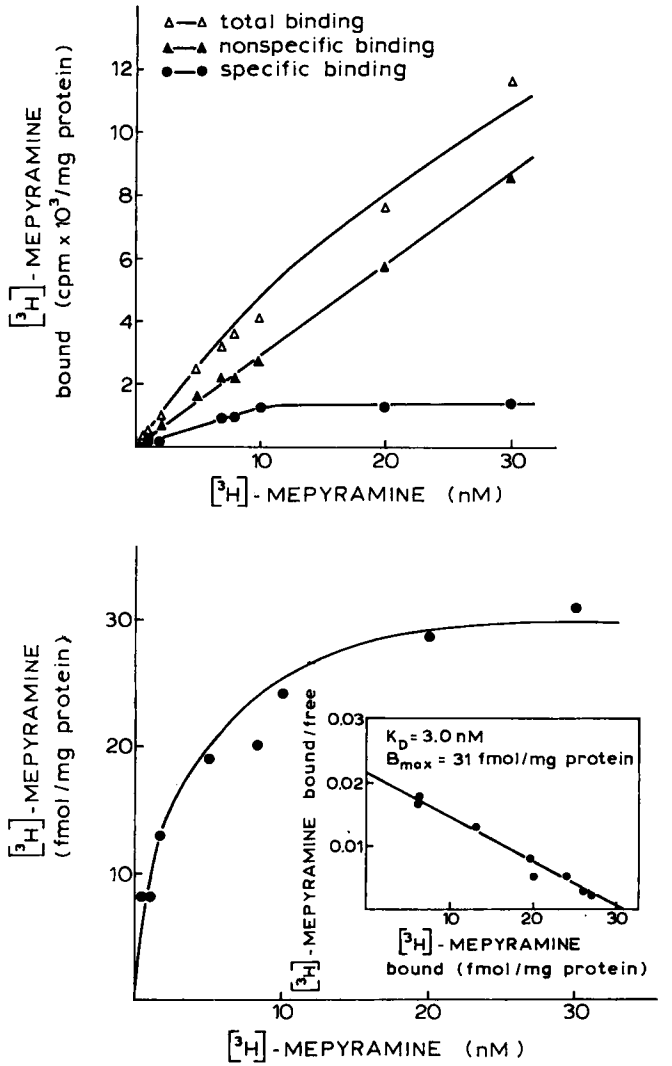

Figure 2.6. Upper: Binding of [^{3}H]mepyramine to bovine aortic membranes as a function of increasing concentration of [^{3}H]mepyramine in the presence (nonspecific binding) and absence (total binding) of 100 μM triprolidine in the assay. Specific binding was calculated as a difference between the total and nonspecific binding. Lower: Specific binding of [^{3}H]mepyramine to bovine aortic membranes as a function of increasing concentration of [^{3}H]mepyramine. Scatchard's analysis of specific [^{3}H]mepyramine binding. The ratio of bound-free radioligand (B/F) is plotted as a function of bound ligand (B). The slope of the line = $-1/K_D$ (r = 0.98) and the number of binding sites (B_{max}) = intercept of the plot with the abscissa.

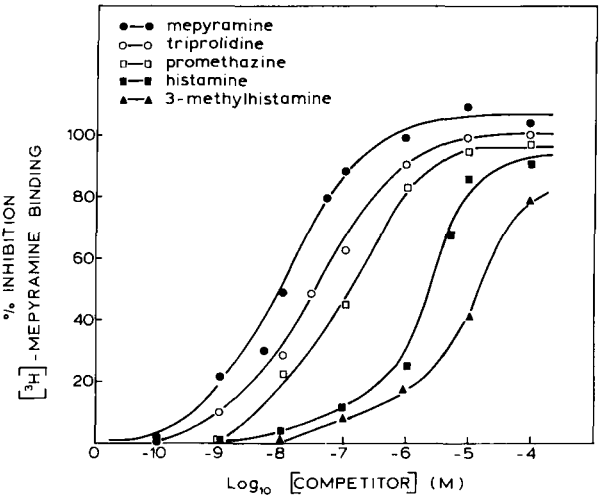

Figure 2.7. Drug (H_1-antagonists, agonists) displacement of [^{3}H]mepyramine binding to bovine aortic membranes. [^{3}H]Mepyramine (2 nM) was incubated with bovine aortic membranes for 15 min at 25° C with varying concentrations of the unlabelled drugs shown. IC_{50} values (concentration of unlabelled drug giving half-maximum displacement of radioligand) for each drug were determined and used to calculate K_i *values (see* Table 2.3*).*

Similar subclasses of receptor sites as for catecholamine and histamine receptors (used as an example in this section) have been shown for other biogenic amine (serotonin, [88]), neuropeptide (opioid receptors, [56]), neurotransmitter (GABA receptor), and hormone receptors.

Whereas for receptor subtypes discussed in this section (α-, β-adrenergic subtypes, partly dopaminergic receptors), good agreement between the radioligand binding parameters and pharmacological effects in most tissues exists, the physiological or pharmacological rôle of many multiple receptor subtypes identified by radioligand-binding assay (especially in the brain) is hard to determine and several problems are still outstanding: in the binding assay, the use of multiple radioligands and multiple competing ligands can provide evidence concerning the receptor type, but the binding assay cannot determine whether a ligand-receptor interaction leads to an agonist, antagonist or non-event response, and new pharmacological models are required. In several receptor systems, there is need for more highly selective agonists and antagonists for radioligand as well as for pharmacological test procedures to define the rôle of multiple receptor sites.

MEMBRANE RECEPTOR ISOLATION

Receptors exist in extremely low concentrations in the biological membranes and, as outlined in the introduction, require extensive purification in the isolation process. Different strategies are used in the attempt to isolate membrane receptor proteins: focussing on the radioligand-binding approach, two main procedures facilitated the progress in the molecular characterization of the receptors: (a) solubilization of the membrane receptor with mild (nonionic) detergent followed by purification on biospecific affinity chromatography and final extraction of the resin-attached receptor protein by elution, and (b) irreversible affinity labelling of the membrane-bound receptors with 'site-directed drugs' (irreversible ligand affinity or photoaffinity labelling reagents), followed by the subfractionation of receptor using ionic detergent sodium dodecyl sulphate (SDS) combined with polyacrylamide gel electrophoresis (SDS/PAGE).

Affinity chromatography purification

Membrane receptors belonging to the class of intrinsic membrane proteins cannot be extracted from the membrane by simple methods involving chelating agents, changes in ionic strength and pH, but require the use of detergents. For membrane protein solubilization, the detergents of choice are those which interact with hydrophilic and lipophilic regions in a disruptive fashion without causing protein denaturation. They include detergents such as digitonin cholate, deoxycholate, Tween-20, Triton X-100, lubrol PX, CHAPS, (3′-(3-cholamidopropyl)dimethylammonio-1-propane sulphonate) and lysolecithin.

Membrane receptor proteins vary in their response to surfactants, depending on their properties, and usually a wide range of detergents have to be evaluated for particular receptor solubilization to obtain maximal results. Many attempts to solubilize membrane receptors have led to substantial loss of specific (receptor) binding in the solubilized preparation. Two types of criterion must be considered in solubilizing the receptor protein with nonionic detergents. (a) Criteria of receptor specificity. Ideally, solubilized receptor should retain binding characteristics of a membrane-bound receptor. Binding parameters of the receptor in the membrane-bound form must be compared with those of the receptor in the solubilized form, identifying the solubilized protein as receptor protein (see criteria in *Table 2.3*). (b) Criteria to assess and optimize the receptor solubilization method should allow the true conversion of the membrane-bound receptor into the molecular dispersed system. The conditions for receptor solubilization have to be adapted to the animal species and the

receptor-bearing tissue. Detergent-solubilized receptors also require some adaption of the radio-ligand binding assay to properly separate bound from unbound ligand in the solubilized preparation. Bound radioactivity can be assayed by filtration after precipitation of protein (with ammonium sulphate or γ-globulin-poly(ethylene glycol), gel-filtration, adsorption on charcoal (the most often used procedure) [16].

The nicotinic acetylcholine receptor (AChR) was the first neurotransmitter receptor to be isolated and purified, successfully using detergent solubilization and affinity chromatography.

The purification procedure of AChR included nonionic detergent extraction of the AChR from the membrane followed by one-step affinity chromatography on the resin-attached α-neurotoxin, and final elution of the toxin-bound receptor protein from the resin by cholinergic ligand (carbamylcholine) [89]. Purified AChR from *Torpedo californica* was shown to be a five-subunit (α_2, β, γ, δ) glycoprotein with a molecular weight of 250,000 having the cholinergic binding site within the α-subunit [90–92].

This rapid advancement in the molecular studies of AChR receptor was possible thanks to availability of electric organs of electric fish (electric eel or electric rays) as an exceptionally rich source of receptor (containing 1000-fold more AChR per g of tissue than mammalian muscle) and discovery of specific ligands (snake α-neurotoxins such as α-bungarotoxin) which bind specifically and with high affinity to AChR [92a].

Attempts to isolate other membrane receptors were severely hampered by the usually low concentration of the receptors in the tissue. In the catecholamine receptor field, investigators were particularly successful in the purification of the β-adrenergic receptors using ~~avian~~, frog or turkey erythrocytes as a source of the receptor. β-Receptors were solubilized using digitonin and chromatographed using Sepharose 4B-bound β-antagonist, alprenolol [93]. Using this technique, a 100- to 500-fold purification of the frog erythrocyte β-receptor was obtained [94] and with additional purification using high-pressure liquid chromatography (HPLC), receptors of apparent homogeneity were secured. The purified preparation, as shown in the radioligand-binding assay using [^{3}H]dihydroalprenolol, retained 31% of receptor activity [95].

More recently, α_1- as well as α_2-adrenergic receptors have been purified. Using digitonin as solubilizing agent, followed by affinity chromatography on an agarose-immobilized benzazepin derivative of SKF 101253, the same group of investigators (see Ref. 96) were able to purify the human platelet α_2-adrenergic receptor which retained α_2-adrenergic binding specificity. Affinity chromatography for purification of digitonin-solubilized α_1-adrenergic

receptor from liver plasma membrane was also developed [97].

Dopaminergic receptors from different brain regions and species were solubilized using either CHAPS, digitonin or cholate, but best yields were obtained with digitonin and lysophosphatidylcholine (LPC) [98,99]. The receptor was partly purified using affinity chromatography [100].

Irreversible receptor-labelling ligands

Irreversible receptor-labelling reagents (active-site-directed labels) have been introduced into the receptor studies mainly to overcome two major problems in the isolation of the receptors by radioligand binding: first, the rapid dissociation kinetics of the conventional reversible receptor-ligands, which do not permit the isolation of the receptor in the form of a ligand-receptor-complex; and second, often rapid loss or modification of the characteristic binding properties of the receptor when a particulate receptor is solubilized during the purification procedure. For these reasons, stable radioactive ligands were sought which couple covalently to the membrane receptors in the crude membrane preparation and, once attached to the receptor, follow the subfractionation of the receptor-binding protein from the membrane during the purification procedure.

Irreversible ligands used for the isolation of the membrane-receptor proteins are either (a) chosen from the group of known irreversible antagonists prepared in the radioactive form, or (b) are newly synthesized derivatives of the reversible receptor ligands, which possess high affinity for the receptor site and also biological activity, but also contain a photosensitive functional group which, when photoactivated, is capable of forming a covalent bond at or near the binding site [101,102].

Irreversible antagonists have been successfully used in the isolation of α-adrenergic receptors, thanks to the group of selective irreversible α-antagonists, the 2-haloalkylamines (dibenamine, phenoxybenzamine). Using tritiated benzylphenoxybenzamine, [^{3}H]POB, the first isolation of the subunits of the α-adrenergic receptor protein of rat-liver plasma membrane was reported [103]. The α-adrenergic receptor in the crude membrane preparation was prelabelled with [^{3}H]POB, and this was followed by receptor solubilization with SDS and fractionation on SDS-gel electrophoresis. In the electrophoretic pattern, the label was attached, and identity with the α-adrenergic receptor was established for the M_r 44,800 (45 kDa) subunit.

Including proteinase inhibitors in the assay, a higher value, of M_r 85,000, for the specifically [^{3}H]POB-labelled SDS subunit of the liver plasma membrane α_1-receptor was obtained [104], two of which may form the membrane

receptor. The intact membrane α_1-receptor thus has an average molecular weight of 160,000.

The second group of irreversible α-adrenergic antagonists which should be mentioned as potential irreversible ligands for use in α-receptor isolation are the tetramine disulphides (benextramine and its derivatives) [105].

Photoaffinity labelling has been particularly successful in the isolation of β-adrenergic receptors, in spite of some initial pitfalls. Photoaffinity derivatives synthesized earlier generally showed low affinity for the receptor ($K_D > 10^{-7}$ M) or the specific interaction with the β-adrenergic receptor could not be demonstrated convincingly [106].

The newly synthesized radioactive photoaffinity labels such as the derivative of the β-antagonist pindolol, ^{125}I-labelled *p*-azidobenzylpindolol (IABP), or the derivatives of carazolol ^{125}I- or ^{3}H-labelled *p*-azidobenzylcarazolol (pABC) and ^{125}I-labelled aminobenzylcarazolol (pAMBC) have overcome the above difficulties by higher specific radioactivity ($\approx$ 2,000 Ci/mmol for iodinated products), more practical for labelling relatively few receptors in the crude membrane preparation, high affinity for the β-receptor-binding site and high yields of the specific photoincorporation in the binding site (about 10%). Using these ligands, the subunit structure of β_1- (duck, turkey erythrocyte membranes) and β_2- (frog erythrocyte membranes) receptor subtypes has been elucidated (based on the covalently labelled subcomponents of the above receptors) [106,107].

There is no equally suitable radioactive irreversible photoaffinity label, as for β-adrenergic receptors, available at the present time for the dopaminergic receptor subfractionation.

Further experiments concerning the subunit stoichiometry and properties of the essential intersubunit bonds [102] are under way for many membrane receptor systems and may soon provide a clue to relate the physicochemical differences in the subunit structure with the functional differences of the receptor subtypes [22,104] and generate enough knowledge to propose a minimum subunit-structure model for the native membrane receptors.

RADIOLIGAND BINDING IN RECEPTOR REGULATION

Cellular receptors as dynamic components of the cell membrane can be functionally altered by a variety of regulatory factors and conditions. Until recently, however, it was possible to draw only indirect conclusions about the receptor alterations under well-known phenomena such as tissue desensitization, supersensitivity, neuronal maturation, or hormonal modulation. With

the introduction of the radioligand-binding approach to the receptor studies, it is now possible to follow experimentally qualitative and/or quantitative changes in the receptor under the above circumstances. Qualitative changes in the receptor result in the alteration of the following binding parameters: changes in the affinity of the radioligand for the receptor (measured as K_D) and changes in the affinity of the drugs competing for the radioligand-binding sites (measured as K_i); quantitative changes result in the alteration of the receptor number (measured as B_{max}) [66].

Recent progress in the study of the molecular mechanisms involved in membrane receptor regulation has revealed that receptor regulation can be of homospecific (self-regulatory) or heterospecific nature: in homospecific regulation, the receptor ligand can regulate the function and/or number of its own receptor (the same ligand-receptor interaction that leads to cellular response can simultaneously lead to a change in receptor number, and/or ligand affinity for the binding site can either increase or decrease as a consequence of receptor activation). In heterospecific regulation, the activation of one receptor by ligand-receptor interaction leads to changes in a second distinct receptor system (crossover of single ligand at several different receptor sites) [103].

Of the various receptor regulatory factors most often studied is the long- and short-term desensitization phenomenon. Tissue desensitization involves several different molecular mechanisms, which also include changes in the receptor itself [109]. The short-term receptor desensitization – a widely observed phenomenon in pharmacological experiments which involves a rapid loss of receptor sensitivity to ligand following stimulation – is not well understood. The mechanism probably involves reversible changes in receptor conformation or loss of the cofactors (GTP, ions or possibly also the phosphorylation of the receptor) essential for the transduction of the signal [110].

Long-term changes in the receptor sensitivity have been observed following prolonged treatment with drugs or agonists. Not only negative (down-regulation) but also positive (up-regulation) changes in the receptor number and/or affinity have been described, in addition to other factors involved in the desensitization. Radioligand binding studies in adrenergic receptor systems (α, β) have resulted in the common finding that the receptor number is reduced in the desensitized tissue (down-regulation). After prolonged exposure of the cells to the β-adrenergic agonist, (−)-isoprenaline, the number of specific [^{3}H]dihydroalprenolol-binding sites in frog erythrocyte membranes is decreased (68%) [111]. These changes are not accompanied by the changes in the affinity of the ligand for the receptor. The fall in the β-adrenergic receptor

number in the desensitized state is probably due to the receptor translocation (internalization), since desensitization of the cells greatly increases binding of [^{3}H]dihydroalprenolol to the cytosol [112].

The recognition that the receptors, as other integral membrane proteins, are not fixed entities and are free to move in the plane of the membrane (which first came with the introduction of the fluid mosaic model for the membrane [113]) greatly influenced the knowledge on receptor internalization mechanisms.

The mechanism of receptor internalization which results in down-regulation of some of the receptors after chronic drug treatment, according to current knowledge [114], comprises several steps: (a) the receptor clusters over the coated pits on the membrane surface after ligand (agonist) binding; (b) clustered receptor is then internalized on smooth vesicles (receptosomes); (c) receptosomes are processed by the Golgi apparatus or lysosomes; (d) receptor is then recycled and/or resynthesized and returned to the cell surface. Prolonged treatment with the drug *in vivo* or exposure of the cells (*in vitro*) activates the internalization process but does not affect the replacement process, leading to the apparent lowering of the receptor number shown in radioligand-binding assays.

The process of ligand-triggered (agonists only; not antagonists) receptor internalization is usually a homospecific regulation, but can be also of heterospecific nature, since crossover of particular ligand with two or more receptor systems can also cause down-regulation of several receptors [108].

In the dopamine-receptor system, several authors have observed adaptive changes of dopamine receptors to the prolonged treatment of neuroleptic drugs. In striatal dopamine receptors, physiologically important D_2 receptor-binding sites labelled with [^{3}H]spiperone decrease after 1 ~~month~~ treatment with a neuroleptic (trifluoperazine) [115], whereas after 6–12 months the binding was increased, showing supersensitivity of the system. At the same time as the quantitative changes, some qualitative changes also occur in the receptor under the above conditions (K_D varies with time). The authors observed opposite effects of neuroleptic treatment on D_3-sites (DA agonist binding sites labelled with *N-n*-[^{3}H]propylnorapomorphine), whereas the D_1-sites were not affected.

The interruption of the innervation normally results in an increase in the effector organ sensitivity, known as organ supersensitivity. The influence of denervation on receptors has also been tested using radioligand binding. In the α- and β-adrenergic systems, an increase in the receptor number is observed following the chemical (6-OHDA or guanethidine) treatment or surgical denervation [116,117] and this could account for organ hypersensitivity.

A variety of examples of heterospecific receptor regulation caused by drugs, hormones and peptides are also now known. VIP, for instance, rapidly

enhances binding of muscarinic agonists to cat salivary gland membranes. Similar up-regulation of α- and β-adrenergic receptors was reported following reserpine treatment in rat brain and guanethidine treatment in rat hearts (β-receptors). Antidepressants such as imipramine influence the affinity of [^{3}H]5HT ligand for rat synaptic membrane (see Refs. 108,118). Hormones such as thyroid hormones and sex hormones also regulate the receptors: the effect of hyperthyroidism, for example, shows opposite results on α- and β-receptors (increase in β-receptor- and decrease in α-receptor-binding sites), whereas in the hypothyroid state the number of α- and β-receptors is decreased [119]. The heterospecific regulation described above was observed in intact cells, membranes and purified receptor preparations.

On the basis of our present knowledge, the main physiologically relevant changes playing a rôle in receptor regulation (resulting in the changes of receptor number and/or affinity for ligand) could be classified into following main groups: (a) changes in the rate of receptor biosynthesis and turnover (homospecific regulation); (b) changes in membrane-linked mechanisms involving ligand cross-over (heterospecific regulation); (c) various heterospecific biochemical reactions, leading to covalent (receptor phosphorylation, proteolysis, disulphide–sulphydryl group exchange) or noncovalent bond formation (changes in membrane potential, receptor-receptor or receptor-ion nucleotide interaction, changes in membrane lipids) etc. (for review see Refs. 108,122).

Comparatively fewer studies have been concerned with the changes in the receptors during ontogenetic development and ageing. There are reports on ontogenetic development of 5-hydroxytryptamine (5-HT) receptors, muscarinic receptors, and α- and β-adrenergic receptors in the brain [120]. Weiss, Greenberg and Cantor [121] extensively studied development of β-receptors and adenylate cyclase in different brain regions of rats (1–64 days old) using [^{3}H]dihydroalprenolol binding. They observed a rapid increase in β-receptor-binding sites in the pineal gland between the 2nd and the 16th day after birth (from 50 to 250 fmol/mg protein) which accompanies the development of adenylate cyclase. The density of β-receptors in other brain regions increases slowly or remains almost constant. Radioligand-binding studies also indicate that, with advanced age, the β-receptor number declines in several areas of the brain and periphery, resulting in the reduced ability of aged tissues to respond to adrenergic agonists. Receptor systems that are not readily developed early in life become deficient at advanced age [121].

RADIOLIGAND BINDING IN CLINICAL STUDIES OF MEMBRANE RECEPTORS

Radioligand-receptor-binding data from animal models clearly demonstrate that one of the molecular mechanisms underlying the disease states in man can be the alteration in the receptor, similar to the receptor alterations caused by physiological and pharmacological regulatory factors discussed in the previous section [108,122].

Unfortunately, the use of radioligand binding in human pharmacology receptor studies is hampered by the fact that the tissue from the appropriate target organ is usually difficult to obtain. Clinical radioligand-binding studies could thus only be conducted on the circulating cells, and biopsied and post-mortem material.

Of the circulating cells, human lymphocytes containing β-receptors are showing potential usefulness for clinical investigations. They have been used for studying receptor alterations in asthma, and atopic and thyroid diseases.

Testing the hypothesis that imbalance in α/β-adrenergically mediated control of bronchial reactivity is one of the important factors in asthma, workers [123] have studied the alteration in human lymphocyte β-adrenergic receptors. They found a decrease in the number of β-adrenergic receptors in asthmatic patients in comparison with that in the control group, whereas others found α-adrenergic hyperactivity.

The possibility of using blood platelets for studying α-receptor abnormalities in the diseased state has been studied. Using [^{3}H]DHE binding assay, a fall in the α-receptor number in thrombocytosis has been observed [119].

Most investigators are also concerned with the alterations of the central neurotransmitter receptors in neuropsychiatric disorders (DA, GABA, opiate, ACh receptors).

Several groups of investigators have studied the alteration of DA receptor abnormalities in schizophrenia. Earlier radioligand-binding studies of Lee and Seeman [124], using [^{3}H]haloperidol and [^{3}H]spiroperidol binding to dopamine receptor rich regions of post-mortem schizophrenic human brains, demonstrated an elevated number of D_2 receptors in schizophrenic patients which is compatible with the hypothesis that this disease is associated with the overactivity of the postsynaptic dopamine receptors [125]. At the same time, other groups [126] tested the possibility that opiate, in addition to dopamine, receptor alterations may occur in schizophrenia. Using [^{3}H]spiroperidol and [^{3}H]naloxone binding, they found changes in the receptor number and affinity in different brain regions. This and more recent studies where [^{3}H]spiperone (spiroperidol) of higher specific radioactivity was used as a ligand for labelling

dopamine-D_2 receptors in the brains of schizophrenics clearly indicated an increase in D_2 receptor number, considering that the majority of patients received neuroleptics during their lifetime. Thus, the relationship between D_2 receptors and schizophrenia and the extent to which the neuroleptics could influence the results have to be further clarified [127].

Another example of receptor involvement in human disorders relates to possible GABA receptor alteration in Huntington's disease, but no alteration of GABA binding in several brain regions has been found [128].

Myasthenia gravis has been described [129] as a receptor disease on the basis of investigations of the alteration in the nicotinic cholinergic receptor in this disorder. It is well established that myasthenia gravis is a disease in which muscular weakness is caused by an autoimmune response to AChR in muscle, whereas neither the cause nor a specific treatment is known. Experiments using purified AChR from fish electric organs provided evidence that the autoimmune response to AChR is mediated primarily by antibodies which reduce the amount of effective receptor. A significant part of this advance can be attributed to monoclonal antibodies raised against AChR, leading to discovery and localization of the main immunogenic and pathogenic region of the AChR α-subunit [130].

Immunological approaches to the receptor-linked autoimmune diseases are likely to become increasingly important clinically and perhaps further receptor-linked autoimmune diseases will be discovered. On the other hand, basic research leading to a new insight into the receptor regulatory mechanisms under physiological and pathophysiological conditions may aid future diagnosis of receptor-related disorders.

RADIOLIGAND BINDING IN DRUG SCREENING AND DRUG ACTION

Pharmacological as well as behavioural and electrophysiological studies have shown that, of the many possible molecular sites of drug action, many classes of drugs elicit their effect by binding to the neurotransmitter or hormone receptors either as agonists or antagonists. The radioligand-binding technique has thus extended the potential for the determination of the biochemical locus responsible for therapeutic drug action and so has become of interest as a method in several aspects of drug screening:

(a) Radioligand binding provides a rapid and efficient *in vitro* possibility to examine in detail structure-activity relationships of series of new compounds or synthetic derivatives of known compounds, in the isolated receptor systems,

without complications arising from differences in drug absorption and metabolism. These studies represent also an efficient way to determine the pharmacologically active part of the drug molecule and direct further synthesis and drug design.

(b) Screening a compound on several receptor systems can shed light on drug interaction with more than one receptor type and/or multiple receptor subtypes and on possible side-effects arising from this interaction. This type of screen could be of importance in the development of more specific drugs with fewer side-effects.

(c) Radioligand binding can be the method of choice for distinguishing agonists from antagonists, thus classifying a new drug in one or other group.

(d) Correlation of drug-binding parameters with drug's clinical potency may suggest or lead to better understanding of its molecular mechanism of action.

(e) The radioligand binding approach is a method of choice for drug screening in the receptor-systems where adequate pharmacological models are not available for drug testing or give empirical results.

Up to now, most of the drug screens using radioligand binding have been focussed on psychotropic drugs (neuroleptics, tricyclic antidepressants and benzodiazepines) which interact with the neurotransmitter receptors in the CNS. This development was possible due to the identification and quantitative characterization of various neurotransmitter receptor sites through which a particular drug class produces its pharmacological effect, and now provides a well-defined *in vitro* possibility to test novel compounds.

Binding studies demonstrate that the interaction of antipsychotic-neuroleptic drugs with central neurotransmitter receptors are responsible for the therapeutic effects as well as for the major side-effects of this class of tranquillizers. Groups of neuroleptics (phenothiazines, butyrophenones, thioxanthens) have been screened on multiple receptors (dopaminergic, α-adrenergic, muscarinic, opiate) using radioligand binding. It was shown that all the above structurally diverse classes of neuroleptics inhibit binding of [^{3}H]haloperidol and [^{3}H]spiroperidol to calf striatal DA receptors. Their affinities for the DA-binding sites correlate remarkably well with their pharmacological action (antischizophrenic activity) in animals and man, so that it is possible to predict clinical potencies of these drugs from *in vitro* experiments [131]. The side-effects of neuroleptics (sedation and hypotension) have been attributed to the blockade of central and peripheral α-adrenergic receptors. As revealed by binding studies on α-receptors (inhibition of [^{3}H]WB4101 binding) and DA receptors (inhibition of [^{3}H]haloperidol binding), the highest incidence of side-effects is indicated by the neuroleptic drugs with a low ratio of K_i(WB4101)/K_i(haloperidol) (<1). The

Parkinsonian-like extrapyramidal side-effect of neuroleptics is attributed to their lack of effect on central muscarinic cholinergic receptors. Neuroleptics almost devoid of extrapyramidal side-effects (closapine) show high affinity for [^{3}H]quinuclidinyl benzilate-([^{3}H]QNB-) binding sites, whereas drugs with low affinity (phenothiazines, butyrophenones) have a high incidence of the above side-effects [131].

For the characterization of novel groups of benzodiazepines, a specific binding screen has been developed, after the presence of specific benzodiazepine receptors had been demonstrated in mammalian CNS, the properties of which suggest that this is a pharmacological receptor through which these compounds exert their effect. New compounds were tested as competitors of [^{3}H]diazepam, [^{3}H]clonazepam and [^{3}H]flunitrazepam binding [45,133].

Although tricyclic antidepressants exert their primary effect as inhibitors of neuronal uptake for NA and 5-HT, they have been screened for potential side-effects on α-adrenergic receptors (inhibition of [^{3}H]WB4101 binding) and as inhibitors of [^{3}H]QNB binding to muscarinic cholinergic receptors. It was shown that drugs with low affinity for [^{3}H]QNR-binding sites are clinically preferable, whereas a high affinity for [^{3}H]WB4101-binding sites indicates a greater possibility of producing hypotensive and sedative effects [132].

Receptor-binding screens can be used to classify a novel compound as agonist or antagonist. A typical model useful for this type of screening has been the opiate receptors, which have been shown to be regulated by the monovalent cation, Na^+ [133]. In these binding studies, opiate agonist and antagonist can be differentiated on the basis of the inhibition of binding in the presence and absence of NaCl: affinities of opiate agonists for [^{3}H]naloxane-binding sites decrease when NaCl is present in the binding assay (sodium shift), whereas the affinities of antagonists remain constant. For other membrane receptors regulated by mono- and di-valent cations and nucleotides (such as receptors coupled to adenylate cyclase, through GTP-regulatory proteins N_s, N_i), it was observed that guanine nucleotides (GTP or its nonhydrolyzable analogues) reduce the affinities of agonists, but not antagonists, for the receptor-binding site [30] in the radioligand-binding assay. This may have significance for the development of a screening method to differentiate agonists from antagonists.

Although radioligand receptor screening methods provide a rapid, inexpensive screen using only milligram or smaller quantities of a new drug, they are not intended to replace the *in vivo* testing of new compounds, but more as methods for gaining a better understanding of the molecular mechanisms underlying the therapeutic effect.

FUTURE PERSPECTIVES IN RECEPTOR RESEARCH

Research on cell surface receptors after the introduction of radioligand binding has yielded exciting new results in such specialized fields as biochemical investigations of the molecular nature of receptors, differentiation of receptor subtypes, localization and regional distribution of the receptors used in drug screening and drug action. Radioligand binding has also been used as a method for quantitative drug assay, and as a possibility of discovering qualitative and quantitative alterations in the receptor under the influence of the physiological regulatory factors, in ageing and in the diseased state.

Undoubtedly in the near future radioligand binding will still remain the method of choice for many of the above areas, but with the introduction of immunological and molecular genetic techniques into receptor research, a more sensitive immuno assay for receptors might supersede the ligand-binding assay.

Molecular characterization of receptors will be aided by the monoclonal antibody technique and immunological approaches. Monoclonal antibodies will be increasingly used in receptor purification, to map the topography of the receptor subunits and ligand-binding regions, to determine structural and functional similarities or dissimilarities of receptor subtypes, and to clarify receptor evolution. Anti-idiotypic antibodies may replace conventional affinity reagents in receptor purification.

Areas of current and future interest will remain in the identification and molecular characterization of all components of signal-effector coupling beside the ligand-binding aspect.

Structural knowledge will undoubtedly have more practical consequences in enabling the more rational and selective drug design, whereas the immunological approach will be of importance in the clinical studies of receptor-related autoimmune diseases. New, rapidly accumulating information on receptor regulation mechanisms will provide new insight into the receptor biosynthesis and turnover.

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3 Common Structural Features of Drugs, Transmitters and Peptides in the Central Nervous System

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INTRODUCTION

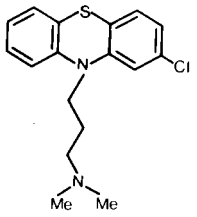
During the last 30 years, many attempts have been made to determine specific drug-receptor requirements within individual CNS drug classes [1]. Despite the availability of large numbers of drugs which could conceivably elucidate the patterns recognized by the various receptor proteins, no decisive results for any particular class have been forthcoming, although many thought-provoking theories have evolved. The dearth of drug-design successes based on modern techniques (NMR spectroscopy, MO calculations, X-ray crystallography, computer graphics, QSAR) prompts a questioning of these approaches. Are they any better than earlier, and still practised, drug-screening programmes? Is rational design of CNS-active drugs attainable at all with our present levels of understanding?

In this review, current theories of action for individual CNS-active drug classes are summarized. An alternative unifying theory is then presented for all CNS drug classes and receptor-transmitter types, and speculations about the evolutionary implications of this theory are advanced. It is postulated that sufficient information is now available for the rational design of new CNS drugs.

COMMON STRUCTURAL FEATURES WITHIN THERAPEUTIC CLASSES

ANTIPSYCHOTICS

Antipsychotic drugs such as chlorpromazine (1) and haloperidol (2) have been shown to act principally as antagonists at dopamine (3) receptors, at which



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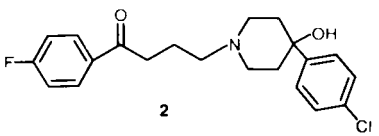

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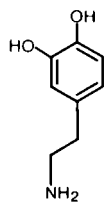

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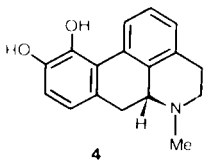

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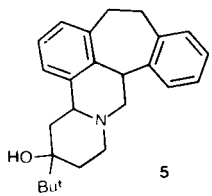

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binding and clinical activity are well correlated [2]. For this reason, conformation-activity studies have made use of various rigid and semi-rigid structures which contain the dopamine pharmacophore, the most important ones being the agonist, (−)-apomorphine (4), and the antagonist (+)-butaclamol (5). (+)-Butaclamol and its biologically active analogues have also been used to define the co-ordinates of a dopamine receptor map composed of an aromatic binding site, a hydrogen bond donor site, a nitrogen location site, and a lipophilic accessory binding site [3].

This proposal is an attractive hypothesis, partly because (+)-butaclamol is relatively rigid and enantiospecific, and partly because it also contains the phenylethylamine pharmacophore found in semi-rigid dopamine agonist and antagonist structures. As a result, several other studies relating various antipsychotics to (+)-butaclamol and its derivatives have since been made [4–7]. The results of these studies, summarized in *Table 3.1* and

Table 3.1. COMPARISON OF MODELS FOR ANTIPSYCHOTIC DRUG ACTIVITY
P, aromatic rings; L, lipophilic moiety; H, hydrophilic moiety; N, nitrogen.

Compounds used	*Methods used*	*Proposed binding features*	*Reference*
(+)-Butaclamol (−)-Apomorphine (+)-Octoclothepin	crystal structures and conformational analyses on Dreiding models	P1 (naphthalene site) N, L	[3]
(+)-Dexclamol (−)-Apomorphine Molindone	comparison of crystal structures	P1, P2 (carbonyl group) N, L	[4]
(+)-Dexclamol (+)-Octoclothepin Chlorpromazine Clozapine Benperidol Z-Piflutixol	crystal structures, MO calculations and computer superimpositions	P1, P2 N* (flexible anionic site)	[5]
(+)-Butaclamol Butyrophenones Tricyclics	crystal structures and binding data	P1, N, L, H	[6]
γ-Carbolines (+)-Dexclamol (−)-Apomorphine a piperidylidene xanthene Diphenylbutyl-piperidines	crystal structures and force field calculations	P1, P2, N	[7]

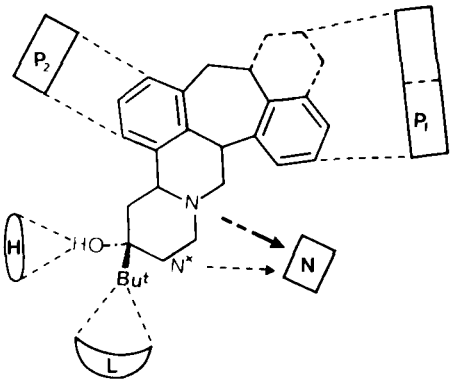


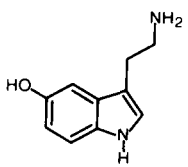
Figure 3.1. Diagram illustrating the various drug-receptor sites proposed for antipsychotic drug action (see Table 3.1*). The reference molecule illustrated is (+)-butaclamol. The symbols used in this and subsequent figures, representing receptor interactions, are as follows: P, hydrophobic interaction of aromatic groups; L, lipophilic group interaction; H, hydrophilic group interaction; N, hydrogen bonding or other generalized ionic interaction.*

Figure 3.1, clearly show that the presence of a pharmacophore comprising one or more aromatic rings and a nitrogen atom is a salient feature of all the proposed models. However, the topographic nature of the pharmacophore differs from one model to another.

ANTIDEPRESSANTS

The mode of action of this class of drugs is not fully understood, with activity appearing to be a composite of effects on catecholamine and serotonin (6) transmitter sites in the CNS [8]. However, it remains possible that there is a common mechanism of action amongst the recognized antidepressant types [9].

Most antidepressant conformation and structure-activity relationships are based on the tricyclic (e.g., imipramine, (7)) and atypical (e.g., mianserin (8))



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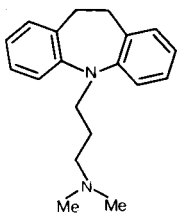

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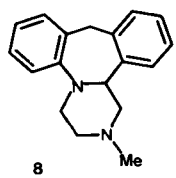

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classes, with imipramine having been studied in the greatest detail [10]. Thus the location of the aromatic rings compared with the side-chain nitrogen [8,11], the relative angle between the planes of these rings and with respect to the nitrogen [12,13], and the importance of aromatic versus saturated rings [14] have all been investigated. These and more recent studies [11,12,15–20], which use less flexible molecules such as mianserin to define precisely the biologically active conformational space of imipramine, are summarized in *Table 3.2* and *Figure 3.2*.

Table 3.2. COMPARISON OF MODELS FOR ANTIDEPRESSANT ACTIVITY

Compounds used	*Methods used*	*Proposed binding features*	*Reference*
Tricyclics Noradrenaline Serotonin	dynamic simulation of molecular framework, energy calculations	Uptake site specified by 3-D van der Waals volume, P1, N	[15]
Various rigid and flexible noradrenaline uptake inhibitors, e.g., Spiroamine A1866	geometric analysis of Dreiding models using four distance parameters	Two uptake sites: phenylalkylamine or antidepressant in a folded conformation P1, P2, N	[16]
Tricyclic and acyclic antidepressants Noradrenaline Serotonin	computer graphics using two distance parameters	P1, N	[17,18]
Imipramine (+)-Mianserin Isoquinolines	energy calculations and superimpositions	P1, P2, N	[19]
Cocaine Noradrenaline uptake inhibitors	Dreiding models	P1, N	[11,12]
Imipramine (+)-Mianserin Nomifensine	energy calculations and computer graphics	P1, P2, N	[20]

P, aromatic rings; N, nitrogen.

Despite the uncertainties of the nature of the drug-receptor interactions that lead to the control of depression, it is clear from these studies, and the novel structures derived from them, that a precise topographical alignment of at least one aromatic group and a nitrogen atom is an important aspect of antidepressant activity.

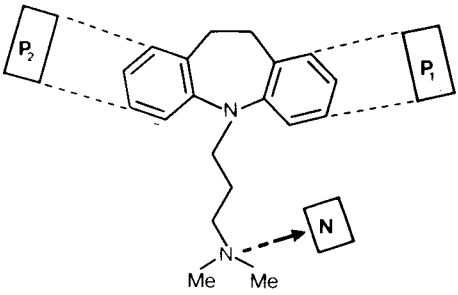
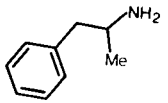


Figure 3.2. Diagram illustrating the drug-receptor interactions proposed in various models of antidepressant drug action (see Table 3.2*). The reference molecule illustrated is imipramine.*

STIMULANTS

The stimulant amines, represented by amphetamine (9), appear to act by enhancing the release and inhibiting the uptake of both noradrenaline (10) and



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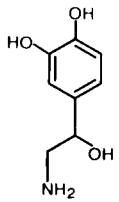

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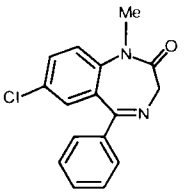
dopamine. NMR, X-ray crystallography and MO calculations [21] indicate that the side-chain is most probably directed away from the plane of the aromatic ring in both antiperiplanar and *gauche* conformations. Since there are only small energy differences between these most populated states, it has been necessary to use rigid analogues to help define the active conformations. Biological studies of benzobicyclo[2.2.2]octanes [22,23] support the antiperiplanar isomer of amphetamine as being the active conformation of the phenylethylamine pharmacophore required for stimulant activity.

ANTICONVULSANTS

Among clinically acting anticonvulsant drugs, most are thought to act at sites within the GABA-benzodiazepine receptor-ionophore complex, although

whether all of them bind at exactly the same site is still not certain [24–26]. The relatively weak activity of almost all anticonvulsants, together with their ability to bind at a host of different CNS receptors, makes the clear identification of the binding sites involved in anticonvulsant activity extremely difficult.



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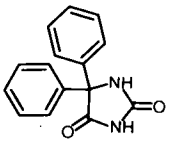

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The most successful approaches to date have related anticonvulsant activity back to the extensive structure-activity data available for the benzodiazepines. This approach was employed initially by Camerman and Camerman [27–29], who derived a four-point model (*Figure 3.3*) for anticonvulsant activity binding by comparison of diazepam (11) with diphenylhydantoin (12). Similar models have subsequently been proposed for a range of benzodiazepines [30] and other chemical classes [31]. The presence of an aromatic ring and a nitrogen or other heteroatom is a common feature of each of these proposed models. In other cases, notably the barbiturates related to pentobarbitone, no aromatic

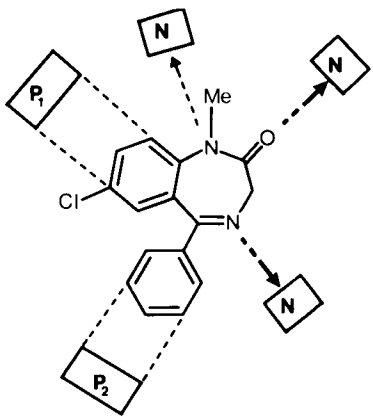
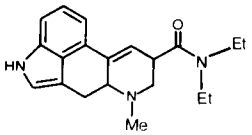


Figure 3.3. Proposed drug-receptor interactions in the Camerman and Camerman [27] model for anticonvulsant drug action. Other models include a rôle for the amide nitrogen [31] and the aryl ring substituents [30].

ring is necessary for activity, but comparison with other aryl-substituted barbiturates suggests that the larger alkyl groups are performing a function similar to that of the phenyl ring system in these series [32].

HALLUCINOGENS

Apart from some cannabinoids and phencyclidines, there are basically three structural classes of hallucinogens: indolethylamines, phenethylamines and β-carbolines [33,34]. The rigid structure of the classic hallucinogen (+)-LSD



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(13) is related to both the first two classes, and most of the models for hallucinogen receptor sites are therefore derived from (+)-LSD or the neurotransmitter serotonin (6) in a conformation which matches (+)-LSD. The most generally accepted hallucinogen model [35,36] is that shown in *Figure 3.4*, but the basic arrangement of aromatic group and nitrogen atom is common to this and all other models proposed for hallucinogen activity [37–40].

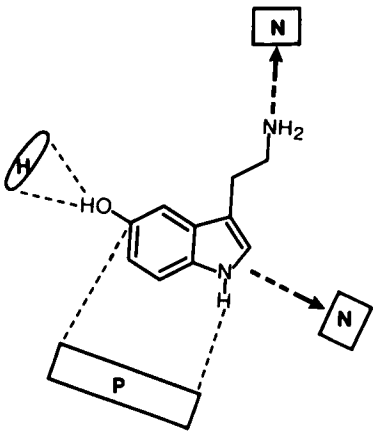
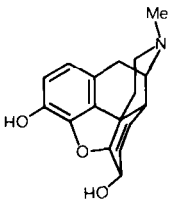


Figure 3.4. Diagram showing the functional groups involved in receptor binding by hallucinogenic drugs [35]. The reference molecule illustrated is serotonin in the conformation which matches (+)-LSD.

ANALGESICS

Opioid analgesics such as morphine (14), fentanyl (15) and methadone (16) play a major role in the clinical alleviation of pain. Many attempts have been



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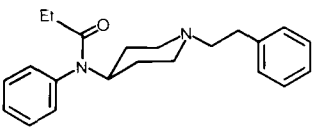

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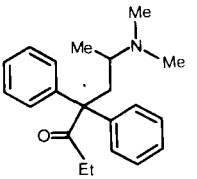

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made to establish reasons why these structurally diverse compounds bind to the same receptor (probably the μ-opioid binding site) to elicit a similar analgesic response, but as yet there is no clear-cut unifying explanation, despite the wealth of studies carried out.

Many structure-activity relationships for opiate analgesics have been presented since the original receptor model proposed by Beckett and Casy [41], but they all have in common at least one aromatic ring and a tertiary nitrogen atom. These studies [41–67] are summarized in *Table 3.3* and *Figure 3.5* which also includes extensions of the basic models to account for structural similarities between the opiate analgesics and enkephalins.

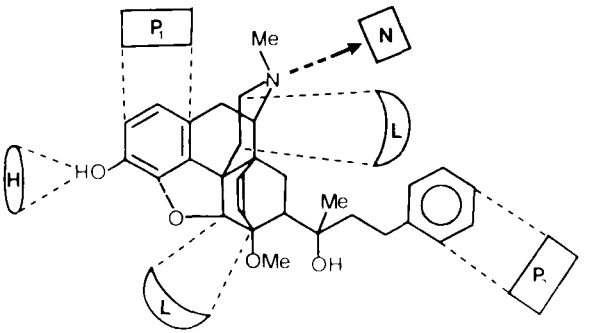


Figure 3.5. Diagram illustrating the drug-receptor interactions proposed in various models of analgesic drug action (see Table 3.3*). The reference molecule illustrated is the oripavine, 7-(1-hydroxy-1-methyl-3-phenylpropyl)endoetheneotetrahydrothebaine (PET).*

Table 3.3. PROPOSED STRUCTURAL REQUIREMENTS OF THE OPIOID RECEPTOR

SAR, structure-activity relationships; N, tertiary nitrogen atom; T, phenolic A-ring of morphine or tyrosine phenolic ring of enkephalins; P, non-phenolic aromatic ring (e.g., phenylalanine residue of enkephalins) or lipophilic equivalent; OH, C6 hydroxy of morphine or C6 methoxy of PET and corresponding enkephalin features (which vary between models); C5-C6, C5-C6 portion of morphine or *para* or *meta* carbon atoms of the phenylalanine residues of enkephalins; L, additional lipophilic moiety (e.g., C15-C16 ethylene bridge of morphine); H, additional hydrophilic moiety (e.g., C19 tertiary alcohol of etorphine); CPK, Corey-Pauling-Koltun.

Template	*Compounds fitted*	*Methods used*	*Proposed opioid binding features*	*Reference*
Morphine and morphinans	pethidine, methadone	stereochemical considerations	N, T, L	[41]
Axially or equatorially substituted 4-phenylpiperidine analgesics		SAR	N (pivotal), T or P	[42–44]
Etonitazene, phenazocine, fentanyl, PET	opioid peptides, antagonists and flexible analgesics	Dreiding and CPK molecular models	N, T, P	[45]
Morphine, PET	Met-enkephalin	CPK models	N, T, P, OH	[46]
Morphine	Met-enkephalin	NMR, framework models	N, T, L	[47]
Opiate agonists and antagonists	tyrosine residue of enkephalins	crystal structures	N, T	[48,49]
	anomalous analgesics	Stereochemical and SAR data	N, extended T or P binding site	[50]
Morphine, etorphine, GPA1657, naloxone, methadone	Tyr-D-Ala-Gly-Phe	conformational search program and computer graphics	N, T, C5-C6	[51]
Morphine	Met-enkephalin	conformational energy calculations	N, T, OH	[52]
PET	Met-enkephalin	fluorescence spectroscopy, CPK models	N, T, P	[53]
Benzomorphans	Met-enkephalin, morphine and flexible analgesics	crystal structures	N, T, P, H	[54]

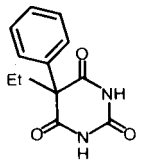
Table 3.3. continued

Template	*Compounds fitted*	*Methods used*	*Proposed opioid binding features*	*Reference*
Morphine, PET	Leu-enkephalin	crystal structures, superimposition	N, T, P, OH, L	[55]
PET	Met-enkephalin	energy minimization, overlap of PET and enkephalin	N, T, P, OH	[56,57]
PET	opioid peptides	cyclic enkephalin, CPK models	N, T, P	[58]
PET	Met-enkephalin	cyclic and isosteric enkephalin analogues, Dore-Smith Atomunit molecular models	N, T, P, L, H	[59]
Rigid and flexible opioid analgesics	phenylmorphans azabicyclanes	computer graphics	N, T or P, L	[60]
Morphine	Tyr-D-Ala-Gly-Phe	energy minimization	N, T, OH	[61]
Morphine	cyclic enkephalin	molecular models	N, T, C5-C6	[62]
Etorphine	Tyr-Gly-NH moiety of enkephalins	steric mapping procedure	N, T, L	[63]
Leucine enkephalin		rigid analogues, computer graphics	N, P, T, OH, L	[64]
General opioid ligand	μ and κ agonists and antagonists	molecular models, clay models	N, T, P2, OH, N substituents	[65]
Morphine	enkephalins, naloxone, pethidine	SAR	N, T, P2	[66]
Etorphine and analogues	Met-enkephalin	SAR	N, T, C5-C6, L, H	[67]

COMMON STRUCTURAL FEATURES OF DIFFERENT THERAPEUTIC CLASSES

The various structure-activity studies described in the previous section show the importance of an aromatic ring and a nitrogen atom in virtually all classes of CNS-active drugs. Most theories regarding their activities revolve around the existence of a common topographical arrangement of these groups within any biological class, and specific arrangements have been proposed for antipsychotics, antidepressants, stimulants, hallucinogens and analgesics [68]. The use of such topographical comparisons in all these studies led us to ask, what are the differences between the topographical arrangements of these groups in different drug classes?

In a first attempt to answer this question [69], we used computer graphic techniques to compare the crystal structures of the recognized representative compound from each of eight major CNS-active drug classes: chlorpromazine ((1), antipsychotic), imipramine ((7), antidepressant), amphetamine ((9), stimulant), diazepam ((11), anxiolytic), diphenylhydantoin ((12), anticonvulsant), LSD ((13), hallucinogen), morphine ((14), analgesic), and phenobarbitone ((17), hypnotic). This was done by superimposing the molecules in



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a way which maximized the overlap of the selected aromatic rings and nitrogen atoms. Perspective views of these eight compounds superimposed on each other showed that there was in fact a remarkable consistency in the topographical arrangement of the nitrogen and phenyl moieties throughout this series of molecules [69]. This simple experiment led us to make the following proposals.

(1) There is a common structural basis for the activity of many different CNS-active drug classes.

(2) The aromatic ring and nitrogen moieties are the primary binding groups whose topographical arrangement is fundamental to the activity of these drug classes.

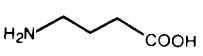
(3) It is the nature and placement of secondary binding groups that determine different classes of CNS drug activity [69].

Clearly, if these hypotheses could be verified, they would provide the basis

for an extremely useful common pharmacophore to use in drug design. However, while the existence of common structural features in the eight compounds above demonstrates that such a common pharmacophore could exist, it does not necessarily define the most probable common structure. We therefore undertook a further study [70] which incorporated three extensions aimed at precisely defining the common structural basis referred to in the first two hypotheses. The extensions were as follows.

First, although the same pharmacophore is apparent in the structure of associated neurotransmitters, it appears that both primary binding groups are not always required for neurotransmitter activity. For example, GABA (18), glycine (19) and acetylcholine (20) each lack an aromatic ring. However, the presence of a residual aromatic binding site in the receptors for these neurotransmitters is suggested by the structures of the antagonists bicuculline (21), strychnine (22) and procyclidine (23), respectively. The number of CNS drugs considered was therefore increased to include bicuculline, strychnine, procyclidine, and also the α-agonist, clonidine (24).

Second, since the conformation observed in the solid state of a given drug need not be the biologically active form, all possible conformations of the drugs in question were considered. Furthermore, in order to restrict the conformational space of the more flexible molecules such as chlorpromazine (1),



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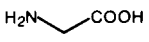

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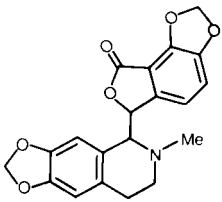

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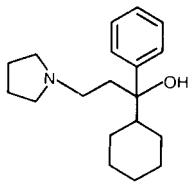

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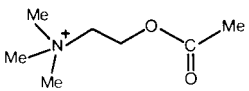

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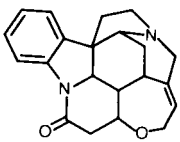

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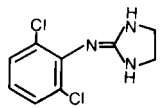

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imipramine (7), and amphetamine (9), three additional semi-rigid structures (apomorphine (4), mianserin (8), and strychnine (22)) were included in the study.

Third, it is not only the common topographical location of the aromatic group and nitrogen atom that is important, but also the location of the receptor

groups with which they interact. Since the geometry of these interactions restricts the number of possible orientations of a drug molecule at its receptor, the model was therefore extended to include these receptor interactions. The results of this study are described in the following section.

RECEPTOR MODELLING OF NITROGEN AND AROMATIC SITES

The receptor model was defined by assuming that the aromatic group of a drug molecule interacts via a planar hydrophobic bond with a group placed approx. 0.35 nm above or below the plane of the ring [71], and that the nitrogen atom forms a linear hydrogen bond with an electronegative atom of the receptor protein located tetrahedrally 0.28 nm away [72]. By way of an example, the extended structure for strychnine (22) with these three receptor points attached, is shown in *Figure 3.6a*. The initial receptor model was developed using the extended structures of the semi-rigid, chiral CNS drugs, apomorphine (4),

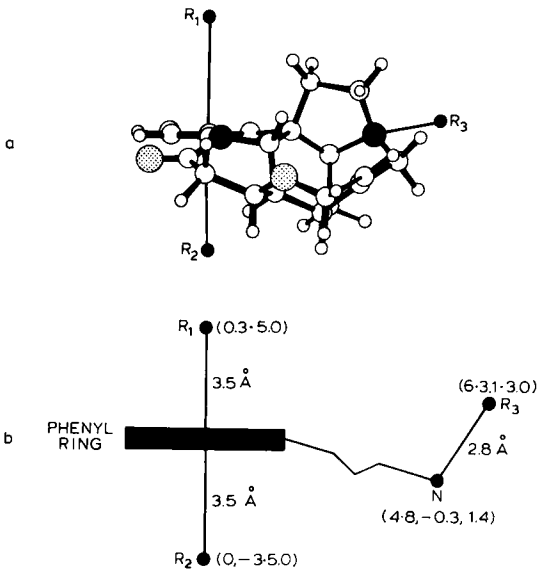


Figure 3.6. (a) Perspective drawing of strychnine, showing the construction of the hydrophobic receptor points R_1 and R_2 onto the phenyl ring, and of the hydrogen bond receptor point R_3 onto the relevant nitrogen. Dark and light shadings in this and subsequent figures represent nitrogen and oxygen atoms respectively. (b) Dimensions of the common model viewed from a point perpendicular to the XOY plane, showing the coordinates of the four points N, R_1, R_2 and R_3.

mianserin (8), LSD (13), morphine (14), and strychnine (22), which are known to be enantiospecific in their CNS action. From these were derived the co-ordinates of four points that were common to these least flexible drugs, on the assumption that if the original hypotheses were valid, then these rigid molecules – having the least degree of freedom – were likely to give the best approximation to any common model. The coordinates of these points were: R_1 (0.0, 3.5, 0.0), R_2 (0.0, −3.5, 0.0), R_3 (6.30, 1.30, 0.0) and N (4.80, −0.30, 1.40); where R_1 and R_2 define the position and orientation of the aromatic group, and R_3 locates the hydrogen bond receptor point (*Figure 3.6b*).

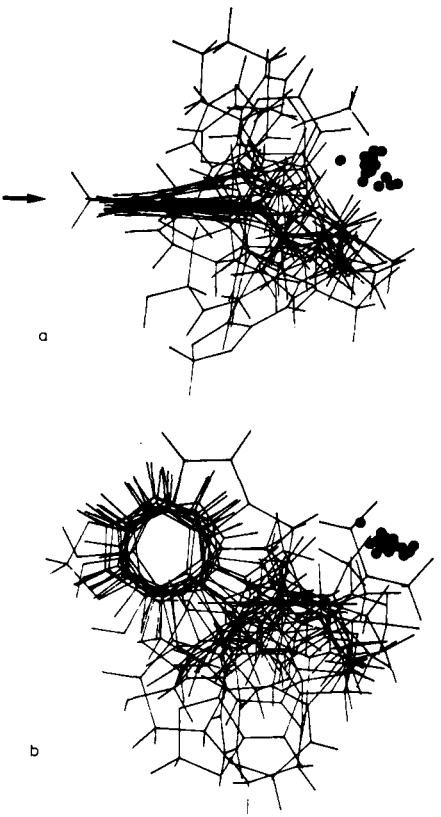


Figure 3.7. (a) Superimposition of all 14 molecules (1, 4, 7–9, 11–14, 17, 21–24) in their lowest-energy, best-fit conformation to the common model. The locations of the receptor point R_3 for each molecule are indicated (●) and the common aromatic ring plane is arrowed. (b) Figure 3.7(a) *viewed from above the common aromatic ring plane.*

The conformations of the more flexible molecules which could fit this model were then determined exhaustively using extended drug structures for which calculations gave low energy and minimum deviation from the corresponding points of the model. The number of these conformations was further reduced, using computer graphics, by requiring that there should be no overlap between any part of the drug molecules and their receptor essential volume. For this purpose, it was envisaged that the molecules bind at receptor sites whose approximate volume is complementary to that obtained by superimposing the five semi-rigid molecules on each other as in the common model. These three criteria provided a powerful method of sorting through the myriad conformational possibilities conceivably available to each drug when binding to its receptor. The resultant best 14 conformers are shown superimposed on each other in *Figure 3.7*, which shows both the common placement of the aromatic rings and nitrogen atoms, and the virtually identical positions of the common receptor points. That the model is valid in the present context is demonstrated by the fact that it was possible to find conformations of all nine flexible CNS molecules that, despite their quite different activities, could all be matched with the common model.

EXTENSION TO NEUROTRANSMITTERS AND NEUROPEPTIDES

In most cases the exact sites of CNS drug action are unknown, although it is likely that they will share sites of action with endogenous molecules, of which the most likely candidates are neurotransmitters and neuropeptides. If this is the case, one would expect similar structural requirements to those above to apply to the endogenous molecules.

NEUROTRANSMITTERS

The catecholamines dopamine (3) and noradrenaline (10) have phenylethylamine moieties similar to that of amphetamine (9), and it is therefore not surprising that they fit the proposed common model well in low-energy conformations. Serotonin (6) may also be matched with the common model using either the phenyl or pyrrole ring of the indole moiety, in either case with low-energy conformations of the ethylamine side-chain. This result corresponds to the same orientation as that obtained for LSD. The fit of these neurotransmitters to the common model in their low-energy conformations is shown in *Figure 3.8*. However, although these and all other known neurotransmitters incorporate a nitrogen atom, some neurotransmitters most

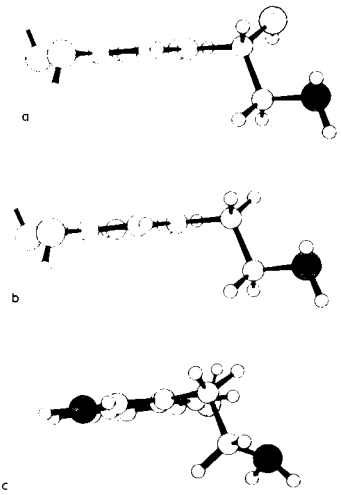
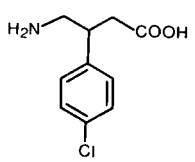


Figure 3.8. Superimpositions of the neutrotransmitters noradrenaline (a), dopamine (b) and serotonin (c) in low-energy conformations which match the common model.

closely related to the drug classes used to devise the common model lack an aromatic group. Key examples are GABA (18), glycine (19) and acetylcholine (20). What is their relationship to the common model?

We believe that there may well be a residual aromatic binding site in the receptor protein for each of these neurotransmitters. Evidence for this possibility comes partly from the fact that some drugs known to bind at these receptors also incorporate an aromatic ring. In the case of GABA (18), for example, the active conformation [73,74] derived for the antagonist bicuculline (21) from an analysis of GABA agonists and antagonists is identical with that derived from the common model (*Figure 3.9*). The combination of these two studies thus provides a means of identifying the relationship of GABA to the common characteristics of CNS receptors. This in turn helps to specify the position of such important binding groups as the carboxyl group of GABA for binding at bicuculline-sensitive sites.



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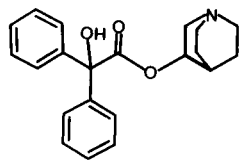

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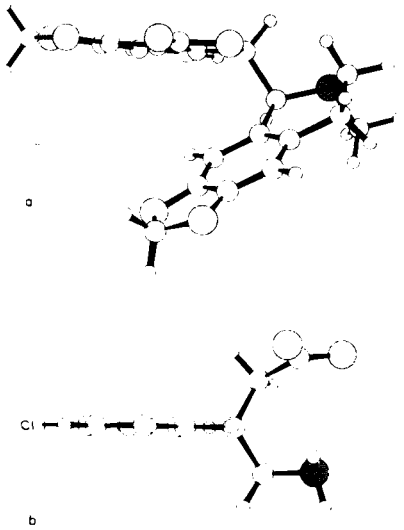


Figure 3.9. (a) The GABA receptor antagonist bicuculline in the low-energy conformation which fits the common model and the model proposed previously for bicuculline-sensitive GABA receptors [73,75]. (b) The bicuculline-insensitive GABA agonist (S)-baclofen in the low-energy conformation which matches the common model.

A similar analysis has been done for the GABA agonist (*S*)-(−)-baclofen (25), which binds at bicuculline-insensitive sites [75]. The best fit of (*S*)-(−)-baclofen to the common model in an energy-optimized conformation of the carboxyl side-chain, shown in *Figure 3.9*, has a GABA conformation different from the GABA moiety displayed by bicuculline in its best fit to the common model. The model thus provides a location of the carboxyl group of baclofen consistent with both the known stereospecificity of the agonist molecule, and with the extensive evidence for the fact that GABA adopts different conformations at bicuculline-sensitive and -insensitive sites [75].

Similar remarks apply to the relationship between glycine (19) and its rigid antagonist, strychnine (22), and to acetylcholine (20) and its constrained antagonist, procyclidine (23). In the latter case, the single consistent conformation of 24 different anticholinergics [76], as exemplified by quinuclidinyl benzilate (26), closely matches the topographical alignment of the aromatic group and nitrogen atom determined for procyclidine from the common model (*Figure 3.10*).

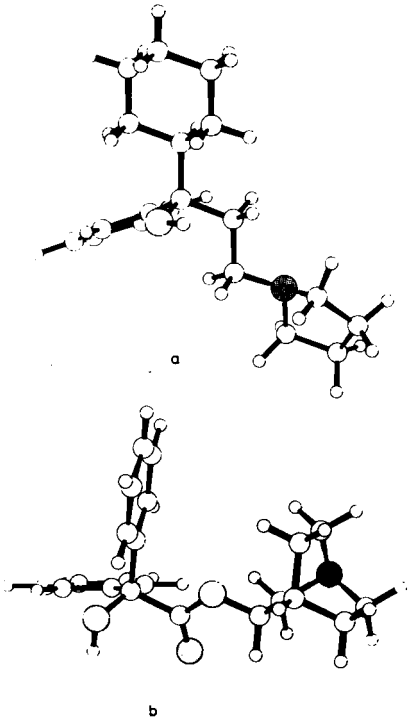
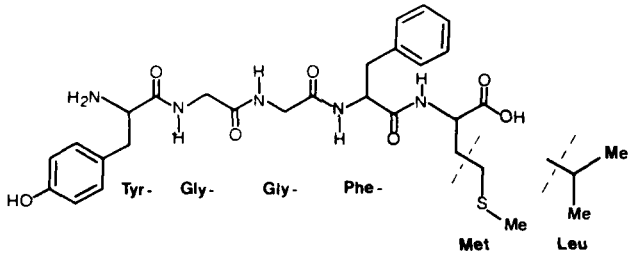


Figure 3.10. The anticholinergics procyclidine (a) and quinuclidinyl benzilate (b) in low-energy conformations which match the common model. The conformation illustrated for quinuclidinyl benzilate also closely matches that observed in a series of 24 related anticholinergics [76].

NEUROPEPTIDES

The last decade has seen the identification of a large and growing number of peptides with neurotransmitter-like functions within the central nervous system. Indeed, it now seems likely that brain peptides may account for more than 50% of CNS neurotransmission [77].

As in the smaller neurotransmitters, it is increasingly apparent that the aromatic ring-nitrogen pharmacophore also plays a major rôle in the peptide neurotransmitters, usually in the form of an N-terminal tyrosine or tryptophan. The classic example of this situation was provided by the discovery and characterization of the endogenous opioid peptides, methionine and leucine enkephalin (27) [78,79], which ushered in a new era in opioid receptor modelling, *viz.*, the study of the structural correspondence between the opioid peptides and non-peptides. An immediate conclusion was that the tyrosine



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residue of the enkephalins was probably the physiological analogue of morphine's A-ring and tertiary nitrogen, and virtually all subsequent studies (see *Table 3.3*) have assumed this to be the case. Indeed, the major remaining point of contention in the area of opioid peptide and non-peptide superimposition is whether the Phe^4 aromatic ring of the enkephalins binds to the same portion of the opioid receptor as the second aromatic ring of the potent oripavine (PET) (*Figure 3.5*). Several studies (see *Table 3.3*) support this possibility, which is illustrated in *Figure 3.11*. Other authors refute the claim that the two functional

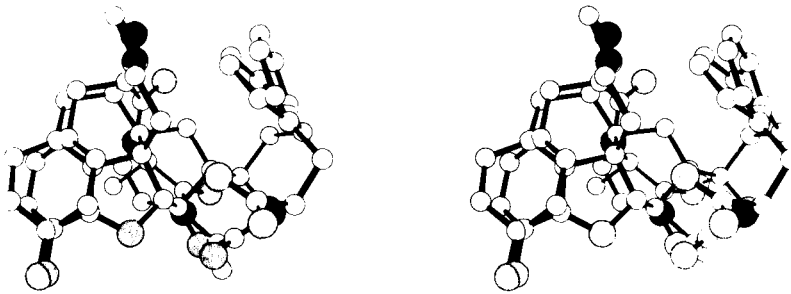
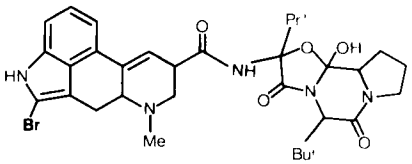


Figure 3.11. Stereoscopic view of the enkephalin analogue of Tyr-Gly-Gly-Phe superimposed on the structure of the potent oripavine, PET [61].

groups are equivalent, the evidence being that the Phe^4 residue is essential for opioid activity in the peptides (i.e., the tripeptide Tyr-Gly-Gly is inactive), whereas the phenyl ring of PET is not necessary for activity, though its presence is associated with a dramatic increase in potency. In all cases, however, the primary rôle of at least one aromatic ring and a tertiary nitrogen atom is assumed. This pharmacophore clearly matches the corresponding group in morphine, and hence is consistent with the common pharmacophore described above.

A similar situation may apply to another class of putative neuropeptides, the caeruleins, and particularly to cholecystokinin (CCK), which has been closely linked to dopaminergic and adrenergic systems in the CNS. Recent studies in our laboratory [80] have shown that the ergot alkaloids, such as bromocriptine (28) and ergotamine (29), which are also closely linked to catecholamine



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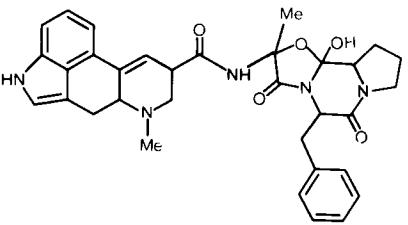

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systems [81], may represent nonpeptidic analogues of the biologically active C-terminal tetrapeptides of CCK, gastrin and related peptides. This analogy is illustrated in *Figure 3.12*, which shows the excellent correspondence between the Trp and Phe residues of the caerulein-like tetrapeptide (Trp-Gly-Gly-Phe) and a conformation previously identified [82] as a likely biologically active form of ergotamine.

EVOLUTIONARY IMPLICATIONS

The proposal that there is a common structural basis involving the same aromatic ring-nitrogen topography in most CNS-active drugs, neurotransmitters and peptides suggests the possibility of an evolutionary pathway from a single neurotransmitter molecule with its associated receptor to the whole class of CNS neurotransmitters with their specific binding sites. Three areas of evidence relating to the development of neurotransmitter systems that are in accord with this proposal are summarized below.

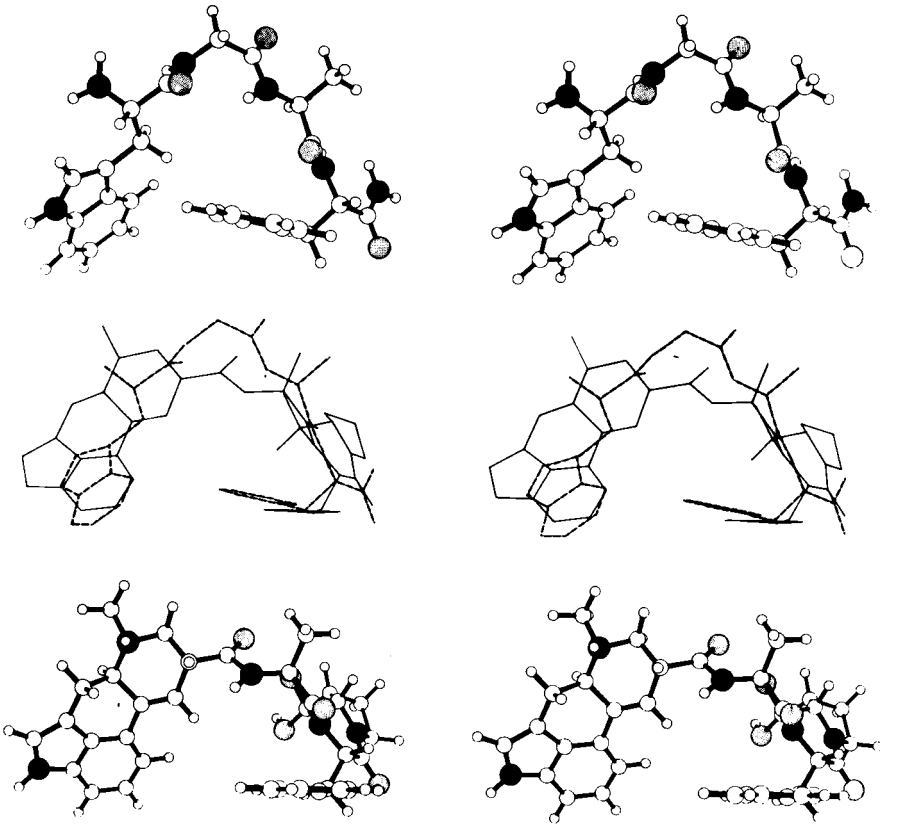

Figure 3.12. Stereoscopic view of CCK analogue Trp-Gly-Gly-Phe (top and centre, dashed lines) superimposed on a low-energy conformation of ergotamine (bottom and centre, solid line).

CHEMICAL EVOLUTION OF NEUROTRANSMITTER SUBSTANCES

The widely accepted scenario [83] for the evolution of living cells from non-living materials assumes that originally there was a reducing atmosphere composed mainly of the gases methane, carbon dioxide, carbon monoxide, ammonia, nitrogen and hydrogen sulphide, with oxygen existing only in a combined form. Experiments by Miller and Orgel [84] establish that these raw materials were convertible into the monomers (amino acids, sugars and bases) necessary to produce the polypeptides and polynucleotides essential to life as we know it. Non-nitrogenous substances such as lactic acid were also

produced, and it is from these that aromatic compounds like tyrosine and phenylethylamine could have been synthesized. Then followed several further stages of polymerization, droplet segregation, and the development of self-replication mechanisms.

Current theories of chemical evolution thus support the idea that the many primitive molecules contained an aromatic group and a nitrogen atom. Having been formed under favourable circumstances in the first place, the likelihood of these two components having a persistent rôle, whether as nutrients or as communicating molecules, was increased.

EVOLUTION OF PROTEINS

Proteins have been shown to have evolved to produce structurally and functionally diverse molecules [72]. For example, proteolytic enzymes have many physiological functions, ranging through protein digestion, activation of zymogens, blood coagulation, hormone release from peptide precursors and transport of secretory hormones across membranes [85]. In all these the basic function is the cleavage of peptide bonds, with the difference at the active site residing in the nature and disposition of the neighbouring amino-acid side-chains. Members of each of these families are generally believed to have evolved from a common ancestor, whose relatively simple original rôle has gradually been elaborated into more diverse and complex functions in higher organisms [85].

It seems reasonable to infer that a similar process of evolution, and hence diversification of structure and function, has also occurred with receptor proteins. This would account for the existence of groups of receptor proteins and sub-types [86], with each group having evolved from a common ancestor. Associated with each ancestral receptor protein there was probably a specific neurotransmitter, and with evolution of the proteins there was specialization of the messenger molecules as well. In addition to intercellular communication, such receptor multiplicity probably allows neurotransmitters to influence cells in subtle ways via a coding network [87,88].

From a consideration of the total number of enzymes known (approximately 3,000 [89]), it is conceivable that a similar order of magnitude of receptor proteins might exist, which suggests that there may be many more neurotransmitters than have until now been characterized. For example, it has been estimated that there could be 200 peptide neurotransmitters alone [77].

EVOLUTION OF NEUROTRANSMITTER FUNCTIONS

Based on phylogenetic evidence, the neurotransmitters dopamine, noradrenaline, serotonin, GABA and acetylcholine all fulfil this rôle in both vertebrates and those invertebrates that have a well-defined central nervous system [90–92]. A similar widespread distribution of peptides, of varying complexity and function, has been found [93].

The mere presence of these substances in the lower orders of invertebrates and in prokaryotic cells is not conclusive evidence that they are neurotransmitters, since in only a few cases are the various criteria for qualification as a neurotransmitter fulfilled. It has been suggested that their rôle could originally have been as nutrients or as metabolites of nutritional processes, and that the opportunism of evolutionary processes gradually resulted in their becoming communicating chemicals at that level [94]. Thus initially these molecules may have been pheromones used for signalling between organisms. Then, with the formation of ordered functioning structures, the rôle of the chemicals diversified into signalling devices between organs and organelles, that is, an endocrine function. Ultimately, with the development of more and more complex nervous systems, some of these chemicals may also have achieved neurotransmitter status. The presence of some peptides having neurohormone functions in the CNS may indicate a transition in evolution from hormone to neurotransmitter [82].

These ideas are supported by recent experiments [95], which show that the biochemical agents of the vertebrate endocrine and nervous systems as well as other intercellular messengers probably originated in unicellular organisms and that, with evolution of the anatomical components of communication, there has been specialization and increased complexity. However, the biochemistry has been conserved, and in most higher organisms the whole gamut of functions of these molecules thus persists [95].

The present proposal of a common structural basis for CNS drug action draws together these three main lines of evidence. The original components of the simplest molecules, consisting of an aromatic group and nitrogen atom, have been retained in most of the modern neurotransmitters, with different secondary binding groups producing subtle variations in the communication network through specificity of binding at receptor protein sub-types. Along the way, some systems probably evolved neurotransmitters lacking aromatic groups or nitrogen atoms (e.g., GABA). However, the available evidence suggests that even in those cases, complementary binding sites still remain for the deleted components.

CONCLUSIONS

Although the presence of an aromatic ring-nitrogen atom pharmacophore has long been recognized within a number of individual CNS drug classes, the concept that there may be a common pharmacophoric pattern across virtually the whole range of CNS active drugs and their associated neurotransmitter systems is a novel one. There are, however, four independent lines of evidence that tend to support this possibility.

First, there is the finding, detailed here and elsewhere, that low-energy conformations of all the molecules selected as key representatives of different CNS drug classes, together with their best known rigid analogues, share a common topographic arrangement of drug receptor binding groups.

Second, as outlined in the previous section, the concept makes sound evolutionary sense, not only in terms of the chemical evolution of precursor molecules and the subsequent evolution of proteins, but also in the development of networks for intercellular communication.

Third, it is increasingly apparent that none of the commonly used CNS drugs binds solely to the receptors where it exerts its primary activity. Indeed, studies of cross-binding between CNS-active drugs have shown promiscuous interactions with virtually all CNS receptors studied [96].

Fourth, this biochemical finding has its clinical equivalent in the increasingly frequent observation of drug classes with hybrid activities, including notably the recent observation of analgesic benzodiazepines [97] and benazepine dopamine agonists [98].

What are the implications of this common model and the general receptor site that it maps out? In its broadest sense for drug design, the common model of the primary binding groups, made up of the aromatic ring and nitrogen atom, provides a framework against which new drugs may be tested for potential CNS activity. In a specific sense, the topographical location of secondary or accessory binding sites relative to the primary sites could be used to categorize drugs as belonging to individual CNS classes. A corollary of this aspect is the ability to define precisely which secondary binding sites amongst a set of drugs determine their common attribute as distinct from those sites which determine side-effects through the ability to bind also at alternative neurotransmitter receptors. Drugs having a hybrid of structure and activity, such as the benzodiazepines, and the various structure-activity studies reviewed above, should also prove very useful in defining these secondary requirements.

The past few years have also seen the development of many novel drug design methodologies, notably in the area of receptor-based [99] and mechanism-based [100] design, but as yet these methods are only partially

applicable to the design of new CNS drugs, for which the techniques of receptor synthesis and characterization are still being developed. In the meantime, the discovery of a *common pharmacophore* among CNS drugs provides us with the opportunity to design new drugs incorporating specific vectors for binding at selected receptors, and thus reduce the level of nonspecific binding (i.e., side-effects), which plagues many otherwise valuable CNS active drugs.

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4 Recent Progress in the Development of New Antidepressant Drugs

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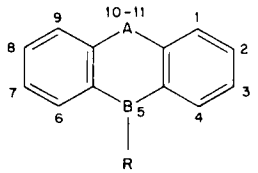
INTRODUCTION

Sadness is a normal short-term response to a stress or some loss. Sometimes this mood is outside (in intensity, duration or both) the limits accepted as 'normal' and constitutes a syndrome involving psychic and psychosomatic symptoms. The depression may be classified as 'reactive' (or neurotic or situational), which is precipitated by an acute and stressful event, or 'endogenous' (sometimes termed 'psychotic' depression when more severe symptoms are present), which may be genetically determined and associated with biochemical abnormalities. Most 'endogenously' depressed patients suffer only from depression (unipolar), while some may experience depression with intermittent episodes of mania (bipolar or manic-depressive illness). Patients with endogenous depression may experience a life-disturbing event so that their endogenous features may become complicated with neurotic symptoms leading to so called 'mixed' depression [1,2]. The possible neurochemical basis of depression has been reviewed recently by Ankier and Leonard [3].

Affective disorders (which include depression and mania) are a worldwide problem recognized in all cultures. In the U.S.A. alone, it has been estimated that 10 to 14 million people suffer from depression or mania at any one time. Since self-destructive behaviour forms an integral part of depressive symptomatology, it is recognized to be a serious disease. Indeed, it has been estimated that 15% of patients suffering from depression will die by suicide [4].

Kuhn [5] first reported the beneficial effect of an iminodibenzyl derivative, imipramine (1.1), in patients with depressive symptomatology when investigating the sedative properties of this tricyclic compound in patients suffering from a variety of chronic psychoses. Subsequently, many controlled studies on the effect of imipramine against depression have been undertaken, although few of the early trials were conducted properly. Nevertheless, there is little doubt of the effectiveness of imipramine in acute 'endogenous' depression [6].

Since the introduction of imipramine into psychiatric practice, several other structural analogues (known as 'tricyclic' or 'typical' antidepressants) have been developed and assessed clinically. These substances include desipramine (1.2), trimipramine (1.3), clomipramine (1.4), amitriptyline (1.5), nortriptyline (1.6), protriptyline (1.7) and doxepin (1.8).



(1)

	A	B	R
(1.1)	CH_2-CH_2	N	$(CH_2)_3 \cdot NMe_2$
(1.2)	CH_2-CH_2	N	$(CH_2)_3 \cdot NHMe$
(1.3)	CH_2-CH_2	N	$CH_2 \cdot CH \cdot Me \cdot CH_2 \cdot NMe_2$
(1.4)	CH_2-CH_2 (3-chloro)	N	$(CH_2)_3 \cdot NMe_2$
(1.5)	CH_2-CH_2	C	$=CH \cdot CH_2 CH_2 \cdot NMe_2$
(1.6)	CH_2-CH_2	C	$=CH \cdot CH_2 \cdot CH_2 \cdot NHMe$
(1.7)	$CH=CH$	C	$(CH_2)_3 \cdot NHMe$
(1.8)	CH_2-O	C	$=CH \cdot CH_2 \cdot CH_2 \cdot NMe_2$

The tricyclic antidepressants resemble the phenothiazines chemically, although the presence in some of an ethylene group in the middle ring imparts different stereochemical properties and prevents the conjugation among the rings present in phenothiazines. Nevertheless, the tricyclic antidepressants share pharmacological properties with the phenothiazines [7]; for example, anticholinergic activity, antihistaminic effects, local anaesthetic actions and cardiovascular effects including hypotension and changes in cardiac conduction. Although not explaining their therapeutic mechanism of action, some of these activities account for the unwanted effects in the normal clinical setting [8]. Indeed, the cardiovascular toxicity of tricyclic antidepressants may compromise their normal use in the elderly, children and in cardiac patients [9–11]. A particular problem with depressed patients is the risk of attempted suicide by overdose. The cardiac effects of the tricyclic antidepressants can lead to death due to asystole [12,13].

The clinical observation that iproniazid, used to treat tuberculosis, produced behavioural activation [14,15] led to the synthesis of several other monoamine oxidase (MAO) inhibitors which have been found to be clinically active as antidepressants [16]. Inhibition of monoamine oxidase in the gastrointestinal tract and liver, however, exposes patients to the potentially

toxic effects of dietary amines (i.e., tyramine and β-phenylethylamine) which would not normally enter the general circulation. This is known as the 'wine and cheese syndrome' or 'cheese reaction'. Recent evidence suggests that human small intestine and liver contain substantial quantities of MAO type A, while human brain contains predominantly MAO type B enzyme. The topic of MAO inhibitors as antidepressants will not be discussed in this review, since this has been done elsewhere [17]. However, some potentially interesting MAO inhibitors will be highlighted.

Since an initial report that lithium controlled and prevented manic affective episodes [18], this ion has been shown to be of therapeutic value in the treatment of the manic phase of manic-depressive (bipolar) illness and in the prophylaxis of both the manic and depressive phases of the illness. Lithium appears to 'dampen' the extreme swings of mood but periodic monitoring of plasma concentrations is necessary since the drug has a narrow margin of safety. The mechanism of action of lithium is unknown, although recent work suggests that it can inhibit presynaptic α-adrenergic and postsynaptic β-adrenergic mechanisms in the central nervous system [19,20]. The use of lithium as an antidepressant in man has been discussed elsewhere [21,21a].

Although the clinical efficacy of tricyclic antidepressants is generally accepted, it should be remembered that 'reactive' depression is a self-limiting disorder with a high response to placebo [22,23] and only 70–80% of patients treated with active drug improve compared with about 30% of patients receiving placebo treatment. Moreover, all antidepressants appear to have a lag period of between 1 to 3 weeks before any clinical effect is detectable [24,25]. These difficulties, coupled with known side-effects and potential toxicity of tricyclic antidepressants, MAO inhibitors and lithium, have led to the search for safer and more effective antidepressants. The success of this endeavour depends on the use of clinically relevant animal models of depression as well as adequate test procedures for assessing new compounds in the clinic.

The purpose of the present review is to describe briefly those preclinical procedures used to identify, evaluate and characterize putative new antidepressants. Some of the problems then encountered when designing and performing a clinical study on a putative antidepressant are then discussed briefly. The major part of the review is devoted to a summary of some chemical, pharmacological, biochemical and clinical aspects of those molecules currently known to be undergoing investigation as potential antidepressants. Also incorporated is information on those drugs which have recently become registered and established for the treatment of depression. The structural classification used in these latter sections is arbitrary, being based on the presence of characteristic nuclei or functional groups. Compounds are illustrated

throughout in the non-salt form. Wherever possible, analogous structures have been grouped together and structure activity relationships have been discussed. There remain a large number of miscellaneous structures which are discussed in a section of their own.

TEST METHODS IN ANIMALS

The discovery that the tricyclic agent imipramine [5] and the MAO inhibitor iproniazid [14,15] are effective as antidepressants arose through chance observations in the clinic. Since the clinical efficacy of these drugs could not have been predicted from pharmacological studies, animal models of depression had to be derived empirically, based on the acute interaction of these antidepressants and other chemical agents. However, such acute studies seem inappropriate, since antidepressants may take 1 to 3 weeks to produce benefit in the clinic. Consequently, attention has focused recently on behavioural models of depression and on adaptive changes of receptors caused by repeat administration of test molecules. Some of the more important models used in the laboratory are now discussed.

ACUTE PHARMACOLOGICAL INTERACTIONS

The observation that a depressive reaction occurs in some patients receiving reserpine for the treatment of hypertension [26–28] provided the rationale for using acute reversal of the effects of reserpine, and related drugs such as tetrabenazine, in animals as an experimental model of depression [29]. Pretreatment with many antidepressants, including tricyclic agents, reverses the effects of reserpine, such as hypothermia, salivation, ptosis, hypoactivity and miosis [7,30–32]; however, this model is also sensitive to drugs with a peripheral action [33,34]. The test's failure to detect the newer clinically effective antidepressants [35,36] and the ability of the tests to detect a wide range of non-antidepressant agents [37,38] have led to the development of alternative screening procedures.

The central nervous and autonomic effects of L-DOPA, the precursor of dopamine (DA) and noradrenaline (NA), are potentiated by tricyclic antidepressants in mice pretreated with a MAO inhibitor [39,40]. However, several other classes of drugs, such as central stimulants, anticholinergic and antihistaminic agents, also potentiate L-DOPA [40] and newer non-adrenergic antidepressants such as mianserin [41] and trazodone [42] give a negative result. Behavioural depression induced by 5-hydroxytryptophan (5-HTP), the

precursor of 5-hydroxytryptamine (5-HT), in rats working for milk reinforcement is reversed by antidepressant agents as well as by methysergide, which is not an antidepressant [43].

A model of depression based on the cardiovascular effects of the α_2-adrenoceptor blocker, yohimbine, in dogs has been proposed [44], while the lethality of yohimbine in mice is potentiated by tricyclic antidepressants, MAO inhibitors and some of the newer 'atypical' antidepressants. However, false positives occur with stimulants, anticholinergic and antihistamine agents [45]. Most antidepressants potentiate the effects of amphetamine [46–49], but the mechanism involved may be the impairment of the hepatic metabolism of amphetamine, so increasing the amount reaching the central nervous system [50,51].

Since antidepressants generally are antagonists at α-adrenoceptors, and since α_2 subsensitivity occurs following chronic treatment with many antidepressants, models of depression based on the α_2-agonist, clonidine, have been developed. Thus, chronic treatment with antidepressants antagonizes clonidine-induced sedation [52], enhances clonidine-induced aggression [53], attenuates clonidine inhibition of the acoustic startle reflex [54], and facilitates shock-induced aggression [55]. The α_2-adrenoceptor activity of antidepressants is also assessed by their ability to attenuate the clonidine-induced decrease in rat brain NA turnover [56].

BEHAVIOURAL MODELS

In the olfactory bulbectomy tests, rats are subjected to bilateral lesions of the olfactory bulbs which produce behavioural changes such as irritability, hyperactivity, an increase of plasma 11-hydroxycorticosteroids, and a deficiency in passive avoidance learning. These effects are reversed by tricyclic and also by antidepressants [57–59], although only the passive avoidance deficit appears to be reversed specifically by antidepressants. The mechanism involved may be the blockade of 5-HT uptake, although the effect is also blocked by several GABAergic drugs [60,61]. Tricyclic antidepressants block mouse-killing behaviour in rats [41,62–65], but this test is also not specific [66,67].

Exposure to uncontrollable stress produces performance deficits in subsequent learning tasks which do not occur in subjects exposed to an identical but controllable stress [68]. The 'learned helplessness' is reversed by subchronic treatment with antidepressants but not by acute imipramine or by chronic treatment with neuroleptics, stimulants, sedatives or anxiolytics [69,70]. The specificity of this model is still unclear, despite extensive testing [71].

In the 'behavioural despair' model, mice or rats are forced to swim in a narrow cylinder. After an initially frenzied attempt to escape, they adopt an immobile posture, the onset of which becomes more rapid on subsequent immersion. The onset of immobility in the second test is delayed by several clinically active antidepressants, such as tricyclic agents but not clomipramine, trazodone or salbutamol [72,73]. The model is sensitive to agents which activate NA and/or DA mechanisms but is insensitive to drugs which activate or reduce 5-HT mechanisms [74]. Moreover, a large number of non-antidepressants are active in the test [71,75] although some of these effects are nonspecific.

In a similar model, rats are subjected to a variety of different stresses, such as foot shock, food deprivation or a cold swim over a 3 week period. At the end of this period of chronic and unpredictable stress, the rats are exposed to loud noises and bright lights followed immediately by an open field test. An increase in activity occurs in unstressed animals which is not observed in the stressed animals. The latter effect is reversed by daily treatment with antidepressant agents during the period of chronic stress [76,77]. However, a causal rather than a statistical relationship between stress and depression has yet to be demonstrated conclusively [78].

Rats reared in social isolation from an early age show a marked hyperactivity as compared with controls. This effect is abolished by acute treatment with tricyclic antidepressants, MAO inhibitors and atypical antidepressants. Unfortunately, the effect of chronic antidepressant treatment cannot be determined in this model, since the induced hyperactivity is abolished by repeated daily handling [79,80]. In another separation model, animals undergo social isolation from either their mother on their peers and this results in an initial stage of 'protest' characterized by agitation, sleeplessness and vocalization followed by a stage of 'despair' characterized by decreased sociability, submissiveness, decreased environmental exploration and retardation [71,81]. There is a lack of information concerning the activity of non-antidepressant agents in this test. Chronic treatment with imipramine is reported to decrease self-clasping in peer-separated infant rhesus monkeys [82] and the chronic administration of desipramine increases social contact and decreases distress vocalization and self-orientated behaviour in maternally separated infant macques [83].

Most antidepressant agents reduce rapid eye movement (REM) sleep in man [84,85] and an animal model has been developed to detect such activity [86]. At the lowest doses that effect the sleep-wakefulness cycle, clinically used antidepressants selectively depress REM sleep. Other psychotropic drugs reduce REM sleep but also affect non-REM sleep at minimum effective sleep.

Since depression may be related to a lack of synchrony in diurnal physiological rhythms, a model of depression based on the adjustment of activity patterns to phase shifts in daily light-dark cycles has been proposed [87]. Imipramine selectively facilitates adjustment to phase shift.

RE-UPTAKE INHIBITION

Many antidepressants influence the presynaptic neuronal re-uptake of 5-HT, NA and/or DA, which leads to increased concentrations of these neurotransmitters in the synaptic cleft. No antidepressant is a 'pure' inhibitor of NA or 5-HT re-uptake; their selectivity in only relative. These activities are detected by a variety of laboratory test procedures [88,89] such as inhibition of the uptake of NA and 5-HT into brain synaptosomes and inhibition of the uptake of labelled NA and/or 5-HT into the rat heart, rat thrombocytes or human blood platelets.

MONOAMINE OXIDASE INHIBITION

MAO inhibitors are used therapeutically as antidepressants and may be distinguished biochemically and pharmacologically by their substrate specificity and inhibitor sensitivity. Thus the MAO type A enzyme preferentially deaminates NA and 5-HT, while MAO type B enzyme deaminates nonpolar amines such as benzylamine and 2-phenylethylamine. Examples of reversible MAO A inhibitors are amiflamine (FLA 336) and moclobemide, of irreversible MAO A inhibitors are clorgiline and LY 51641 and of irreversible MAO B inhibitors are selegiline ((−)-deprenyl) and pargyline. Whereas the classical tricyclic antidepressants are weak reversible MAO B inhibitors, there are non-selective irreversible inhibitors such as tranylcypromine and iproniazid [90–92].

RECEPTOR ADAPTION

Since a characteristic feature of antidepressant therapy is the lag phase of 1 to 3 weeks before any beneficial effect is observed, attention has been focused on adaptive changes following long-term administration to animals. Thus repeated administration of clinically effective antidepressants attenuates the NA-sensitive adenylate cyclase system, which is linked to a decrease of the density and functioning of central β-adrenoceptors [93–95] and the functional sensitivity of central presynaptic α_2-adrenoceptors [96–98] while increasing responsiveness of α_1-adrenoceptors, DA and 5-HT receptors in brain

[99–101]. These observations have been used as the basis of laboratory screening procedures.

CLINICAL ASSESSMENT

The number of putative antidepressants emerging from animal tests is now large. There is thus a need for well-designed and carefully controlled clinical studies to evaluate and compare the clinical efficacy and side-effects of these drugs. Unfortunately, available clinical methodology is subjective and therefore lacking in scientific precision [3]. To assist in an interpretation of the results of antidepressant studies, some current problems of methodology are now discussed briefly.

CLASSIFICATION OF DEPRESSION

No single classification of depressive illness is ideal, although several useful sets of criteria have been developed. For research purposes, depressive symptomatology may be classified as 'reactive' or 'endogenous' using the Newcastle Rating Scale [102]. The criteria used in a study organized by the Medical Research Council [103] have also proved to be a useful diagnostic tool for research purposes. The Schedule for Affective Disorders and Schizophrenia (sADS) is a structured interviewing procedure with rating scales designed to elicit information to enable a Research Diagnostic Criteria (RDC) diagnosis [104] to be made [105]. More recently, the Diagnostic and Statistical Manual (DSM 111) of the American Psychiatric Association has become established internationally as a classification of depression. Its strength is that it takes into account the personality, precipitating stress and physical condition of the patient and also provides clear-cut categories which define all types of affective disorder, without splitting them into psychoses, neuroses and personality disorders.

HOSPITAL *VS.* GENERAL PRACTICE PATIENTS

Although antidepressants may be prescribed for hospital in- or out-patients or for general practice patients, most cases of depression are managed in general practice [106]. Recruitment in general practice may yield large numbers for a study, but the hospital psychiatrist is normally better trained in the accurate diagnosis and careful rating of depression. Problems also exist with the recruitment of suitable cases in the hospital setting, since the patients are often already

on psychotropic medication [107], will probably be antidepressant treatment failures from general practice and may even be suffering from social, physical or personality problems. There is thus a need for more trained clinical trial investigators in the general practice setting.

USE OF A PLACEBO CONTROL

The use of placebo (negative control) as a parallel treatment to an established medication (positive control) and a test medication is an acknowledged problem [108]. It can be argued that it is unethical to perform an antidepressant study without a placebo control group, since such a design cannot adequately assess the pharmacological efficacy of a test medication. On the other hand, it may be considered improper to deny patients with severe depression an established treatment since they are potentially suicidal. However, where doubt exists about the efficacy of the established medication then the use of placebo is justified. One possible solution to this dilemma is the use of an 'active' placebo such as diazepam [109], which is known to be effective in reducing the symptoms of anxiety associated with depression but with only limited activity on the core symptoms of depression.

RATING THE SEVERITY OF DEPRESSION

Another problem in assessing a putative antidepressant is the impossibility of collecting objective laboratory-based data in depressed patients. Rating scales have been developed to quantify subjective information with the depressive syndrome being categorized into items to which numbers may be attributed so as to derive a total score. The apparent simplicity of some rating scales makes them vulnerable, since they appear easy to administer and appear to yield objective data. Visual analogue scales have been developed to circumvent the limitations in the use of language for measurement of a feeling [110,111]. Scales assessing the overall current global severity, based on discrete categories to grade continuous phenomena, are used to represent the investigator's general impression of the severity of the patient's illness. Assessment of therapeutic or global improvement compared with the start of active treatment, also using categories, is used to monitor the progress of a patient in a clinical study.

Most rating scales are prepared in a written form and should be used only if of a length that will maintain the concentration and secure the co-operation of the depressed patient under assessment. These scales may be inappropriate for the elderly, those of low intelligence, the very severely depressed or the illiterate. An automated system for the self-rating of depression utilizing a

microcomputer [112] facilitates the collection of 'delicate' information about topics such as suicide or sex. This technique encourages patients to admit to symptoms and feelings that would, perhaps, otherwise be denied and also enhances the speed of data collection and processing.

A problem exists in choosing a rating instrument to quantify depression in view of the large number available; since each has its own individual sources of error, well validated and familiar scales should be employed. Adequate training sessions should precede a study. It is also sensible that both investigator- and patient-rated scales be used to assess the severity of depression, with at least two investigator-rated scales being employed.

Studies on psychological, physiological, biochemical and/or pharmacological changes during depression may help to identify a test, or group of tests, which will provide specific and objective measurements of the patient's condition. Recent progress on this approach has been reviewed by Checkley [113], who concluded that an increased activity of the hypothalamic pituitary-adrenal axis, with a failure of the suppression of cortisol secretion by dexamethasone, is probably the best established biological change in depression. The growth hormone response to clonidine is also abnormal in depression and this may be a potential marker of α-adrenergic function. The prolactin response to fenfluramine or tryptophan may also be a useful marker of central 5-HT activity, while melatonin plasma concentration may be an index of central β-adrenergic receptor function. However, it is still not clear whether these changes are biological markers of depression or are nonspecific effects of stress, weight loss or other artefacts.

ASSESSING SIDE-EFFECTS

Assessing side-effects of antidepressants is notoriously difficult, since many pre-exist a study as symptoms of the depression itself or emerge during treatment but are not drug-related [2]. The use of a placebo-treated control group is, therefore, important, although symptomatic complaints may be reported even in healthy volunteers taking placebo [114,115]. Nevertheless, the use of a placebo allows assessment of the extent to which possible side-effects are drug-induced.

THE TYPE II SAMPLING ERROR

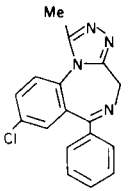
The finding that the efficacy of a test medication is not statistically different from a standard medication does not mean that the two treatments are equivalent clinically. Such an erroneous conclusion may occur as the result of

a statistical Type II sampling error (i.e., the treatments differ while the clinical study fails to demonstrate the difference) which occurs when the sample size is too small or the variability of the measurements too large [116]. Before a clinical study is initiated, it is important to make a value judgement on the required difference to be regarded as clinically relevant. This will help to determine the number of patients needed to yield a valid result.

ANTIDEPRESSANT STRUCTURES

BENZODIAZEPINES AND FUSED BENZODIAZEPINES [116a]

Alprazolam (2) differs from the 'classic' 1,4-benzodiazepines, diazepam (3.1), lorazepam (3.2) and chlordiazepoxide (4), by the presence of a triazolo ring in the basic structure.



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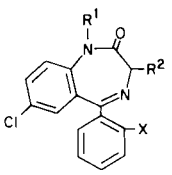

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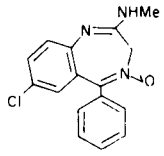


(4)

	R^1	R^2	X
(3.1)	Me	H	H
(3.2)	H	OH	Cl

This triazolobenzodiazepine was originally developed as an anxiolytic [117,118] and clinical experience indicates that 1.5 to 2.0 mg daily is comparable in efficacy with that of diazepam 15 to 20 mg daily in outpatients with anxiety [119]. It was Fabre [120] who first reported that alprazolam improved neurotic depression and subsequent reports suggested comparable efficacy to imipramine [121] and amitriptyline [122] in reactive depression. Alprazolam and imipramine have been reported [123] to be superior to placebo as assessed by overall global improvement; however, no statistically significant difference was found between the active medications and placebo on the Hamilton Depression Rating Scale, possibly due to a high placebo response. In a multicentre study involving 723 patients suffering from unipolar depression [124,125], alprazolam and imipramine were significantly more effective than placebo. Moreover, alprazolam was at least as effective as

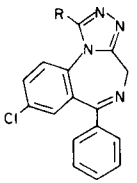
imipramine in relieving symptoms of depression and was superior in relieving somatic symptoms. Anticholinergic side-effects were reported most often by patients receiving imipramine, while drowsiness was the only side-effect reported often in the alprazolam group. Recently it has been reported [126] that alprazolam has a faster onset of antidepressant efficacy than has imipramine and with fewer side-effects.

There is a considerable overlap between anxiety and depressive states [127,128] and it is possible that alprazolam may improve depressed mood indirectly by relieving anxiety or by improving sleep. Although the mechanism of antidepressant action is unknown, sleep studies have shown that alprazolam has effects on REM sleep similar to those of tricyclic antidepressants, but is unlike the 'classic' benzodiazepines [129,130]. Recent work indicates that alprazolam has an effect similar to that of desipramine on a reserpine-induced model of depression in rats [131]. However, evidence suggests a useful effect of alprazolam in patients with mixed anxiety-depression, although its general acceptance as an antidepressant awaits further studies [119,132].

The ratio of anxiolytic to antidepressant properties of the triazolobenzodiazepines may be a function of the amino alkyl side-chain in the 1 position. Thus, introduction of a dimethylaminomethyl side-chain yields adinazolam (5.1), which results in clear antidepressant activity in classic animal tests [133,134]. Using the bilaterally bulbectomized rat model, it is reported [135] that both alprazolam and adinazolam reduce the hyperactivity occurring when the rat is placed in a stressful and novel environment. The effect was qualitatively similar to but weaker than that observed following the antidepressant agents, mianserin or nomifensine, and differed from that of the anxiolytic agent, diazepam, which facilitated the hyperactivity of the bulbectomized rat after chronic administration. Following 14 days' treatment with adinazolam, the responsiveness of rat hippocampal pyramidal neurons to the microiontophoretic application of 5-HT, but not to NA, was enhanced. Turmel and de Montigny [136] proposed that the antidepressant activity of adinazolam might be mediated by a heightened neurotransmission of 5-HT. Preliminary clinical studies of adinazolam indicated an antidepressant effect [137,138] with side-effects such as sedation, euphoria, blurred vision and dry mouth, which were reported as being mild [139]. In a 6 week double-blind study involving 80 patients, adinazolam was superior to placebo in relieving depression, with mild to moderate drowsiness being reported as the most frequent side-effect [140]. Further lengthening the aminoalkyl side-chain by one carbon, U-43,465F (5.2), reduces anxiolytic-sedative potency, while maintaining properties in common with classic antidepressants such as antagonism of oxotremorine, potentiation of yohimbine toxicity, antagonism of reserpine effects and hypothermia induced

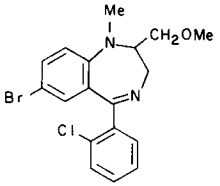
by high doses of apomorphine [134,141,142]. The results of controlled clinical trials with U-43,465F are awaited.

Metaclazepam (6) is a newly-developed benzodiazepine derivative with an alkoxymethyl group in position 2 and which is rapidly metabolized to *N*-1-desmethyl- and *O*-desmethylbenzodiazepines. The pharmacological



(5)

(5.1) R = CH_2NMe_2
(5.2) R = $CH_2CH_2NMe_2$



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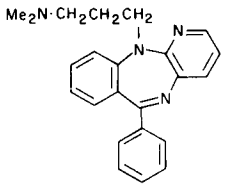

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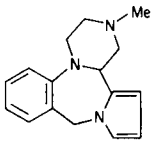

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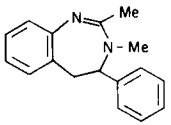


(9)

profile of metaclazepam, like its active metabolites, is that of an anxiolytic [143,144]. An anxiolytic action has been confirmed in man and a 'mood brightening' effect noted [145–147]. Metaclazepam at doses known to be clinically effective in treating anxiety is reported to cause little objective or subjective evidence of sedation or of any significant muscle relaxant effect [148,149].

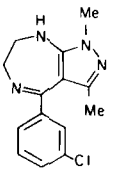
AHR 9377 (7) has been reported to be active in conventional preclinical tests for antidepressant activity, while being devoid of antihistaminic and anticholinergic side-effects [150,151]. It is reported to be a potent, selective, non-competitive inhibitor of NA re-uptake to down-regulate β-adrenergic receptors [152] and not to possess any sedative potential as assessed by behavioural or electroencephalographic parameters [153,154]. Autonomic effects of AHR 9377 suggest that this compound may possess a lower cardiotoxic liability than does amitriptyline or imipramine [155]. These properties indicate that AHR 9377 might have useful antidepressant activity in the clinic and results of studies are awaited.

Aptazepine (8), 1,3,4,14b-tetrahydro-2-methyl-2*H*,10*H*-pyrazino[1,2-*a*]pyrrolo[2,1-*c*][1,4]benzodiazepine, is a tetracyclic benzodiazepine which has been suggested to be a potential antidepressant without sedative activity [156]. Like mianserin, which it resembles structurally, aptazepine is a potent inhibitor of the binding of the α_2-adrenoceptor ligand, [^{3}H]clonidine, but only weakly active in displacing the α_1-adrenoceptor ligand [^{3}H]prazosin from its binding site. The potent α_2-adrenoceptor antagonist activity of aptazepine has been confirmed *in vivo* by its prevention of clonidine-induced antiwrithing activity and a marked antagonism of the ability of clondine to suppress locus coeruleus neuronal firing rate [157]. The clinical investigation of aptazepine as a potential antidepressant would be of interest to assess further the involvement of α_2-adrenoceptors in depression.

HRP 534 (9), a 1,3-benzodiazepine derivative, has been reported to have marked antitetrabenazine activity, to inhibit NA and 5-HT uptake into rat brain synaptosomes and to lack anticholinergic activity as evidenced by negligible displacement of [^{3}H]quinuclidinyl benzylate from rat brain muscarinic receptors, and by insignificant antagonism of the cholinergic stimulation produced by physostigmine or oxotremorine [158]. This suggests that HRP 534 might be clinically useful as a novel nontricyclic antidepressant which is devoid of anticholinergic side-effects.

BENZODIAZEPINE-LIKE STRUCTURES

Zometapine (10) is a pyrazolodiazepine similar to imipramine in its action of potentiating methylamphetamine-induced increases in self-stimulation in the rat but without an inhibitory action on aromatic amine uptake at neural



(10)

membranes [159,160]. Two preliminary uncontrolled studies have reported antidepressant activity in patients [161,162]. Mild nausea was the most frequently observed side-effect.

DIBENZOXAZEPINES

The tricyclic dibenzoxazepine amoxapine (11.1), which is the demethylated metabolite of the neuroleptic agent, loxapine (11.2), is a potent inhibitor of NA uptake with a pharmacological profile similar to that of imipramine in animal models [163]. Initial uncontrolled studies showed that amoxapine has antidepressant activity [164–166], while controlled studies have subsequently indicated that amoxapine relieves core symptoms of depression more rapidly than does imipramine [167], amitriptyline [168] or doxepin [169]. In a double-blind placebo-controlled study involving 158 depressed patients [170], amoxapine was reported to be superior to placebo on physician ratings but not as judged by the patient ratings. In general, the controlled studies with amoxapine have involved relatively small numbers of patients [171] and further studies are indicated to confirm the exact rôle of this compound in the treatment of depression.

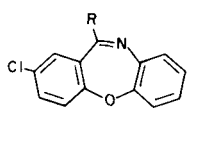
Nitroxazepine (12) has similar pharmacological properties to those of imipramine in animal models by reversing reserpine- and tetrabenazine-induced effects, in exacerbating yohimbine-induced effects, and in potentiating the behavioural response to L-DOPA and those of small dose of amphetamine. However, unlike imipramine, nitroxazepine induced anti-aggressive effects in vicious septal-lesioned rats and fighting mice and was less active in tests for anticholinergic activity [172]. A number of controlled studies performed a few years ago suggest that nitroxazepine is an effective and well-tolerated antidepressant [173–176].

SQ-10,996 (13) is 7-chloro-5,11-dihydrodibenz[*b*,*e*][1,4]oxazepine-5-carboxamide with antiepileptic, antidepressant and anxiolytic activity in animal models [177,178]. Clinical trials in depressed patients are awaited.

DIBENZAZEPINES (IMIPRAMINE-LIKE)

Lofepramine (14.1) is an analogue of imipramine with higher lipophilicity, due to the substitution of the aliphatic amino group by a 4-chlorophenacyl group, which is partially metabolized to desipramine. It is unlikely, however, that repeat-dose administration of lofepramine results in an accumulation of clinically significant concentrations of tissue desipramine, since plasma concentrations in patients are below therapeutic values [179].

Recent interest in this 'tricyclic' antidepressant centres around the possibility that it has little anticholinergic activity and low cardiotoxicity. Pharmacologically, lofepramine is similar to imipramine and desipramine in tests such as the antagonism of reserpine-induced hypothermia and ptosis,



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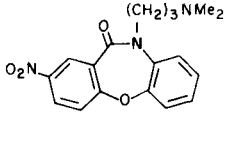

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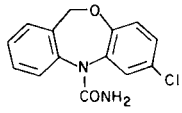
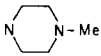


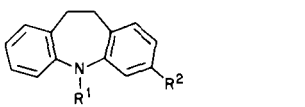
(13)

(11.1) R= 

(11.2) R= 

reversal of the hypotensive effect of reserpine, and potentiation of L-DOPA in mice and NA in pithed rats. However, lofepramine showed little or no peripheral anticholinergic activity [180]. In man, lofepramine has antidepressant activity comparable with that of imipramine, amitriptyline or clomipramine [181–185] but with significantly less anticholinergic side-effects [186–188]. A recent open study involving 223 patients who received lofepramine for periods of up to 61 days revealed an antidepressant and an anxiolytic effect, with an incidence and severity of anticholinergic side-effects which was low [189].

Cianopramine (14.2), which is structurally related to clomipramine, is a potent and relatively specific inhibitor of the uptake of 5-HT in animal models [190,191]. *In vitro*, cianopramine was reported to have 6-times the potency of clomipramine and 14-times the potency of imipramine in inhibiting [^{3}H]5-HT uptake by human platelets [192]. Using a single-blind crossover design, the effect of cianopramine and placebo on cardiovascular function was compared in nine normotensive subjects. The results suggested a positive chronotropic and inotropic effect, probably due to the stimulant effect of 5-HT [193]. In an open study involving 21 depressed patients, cianopramine was found to possess antidepressant activity with tolerance being 'good' or 'very good' in 80% of the subjects [194]. The results of controlled studies in depressed patients are awaited.



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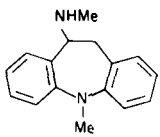


(15)

	R^1	R^2
(14.1)	$(CH_2)_3$ N. Me $\cdot CH_2 \cdot CO$– 	H
(14.2)	$(CH_2)_3$ N. Me_2	CN

Metapramine (15) differs from imipramine by the incorporation of a methylamino group into position 10 and replacement of the aminoalkyl group with a methyl. Like desipramine, it reduces the number of available β-adrenoceptors in the rat cerebral cortex, an action probably due to the enhancement of the availability of NA [195].

DIBENZOCYCLOHEPTANES (AMITRIPTYLINE-LIKE) AND SOME OTHER 6-7-6 TRICYCLIC SYSTEMS

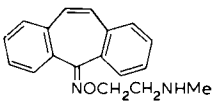
Amitriptyline, which is metabolized to nortriptyline, is the prototype of a group of aminoalkylated dibenzocycloheptenes with antidepressant activity. Demexiptiline (16) is a modification of the nortriptyline structure in which has been introduced a 10,11 double bond and a 5 double bond (C = N) which gives a rigid steric structure. In animal models, demexiptiline has antidepressant activity but with mild anticholinergic effects [196]. The results of controlled clinical studies are awaited.

Amineptine (17) has a long aliphatic side-chain attached to the tricyclic ring and the presence of one acid and one amine group which differentiates it from similar tricyclic structures. It is an antagonist of reserpine-induced hypothermia and ptosis, and the central depressant effects of tetrabenazine and is also active in other classic animal tests for antidepressants. Of particular interest, studies with *in vitro* synaptosomal preparations have shown that amineptine increases the release of DA from presynaptic storage sites and also inhibits DA re-uptake [197,198]. Its activity on the dopaminergic system appears to be specific, since amineptine has no effect on NA release or on the re-uptake of NA and 5-HT. Clinical studies have shown efficacy equivalent to amitriptyline in neurotic depression [199,200]. Similar results have been reported against imipramine [201] and clomipramine [202]. Activity superior to maprotiline [203] in patients with neurotic depression has been reported. These studies also suggest that anticholinergic side-effects are not a problem with amineptine, but hepatic disturbances with cholestasis occur in about 1 : 10,000 patients. The latter effect is probably due to an immune-type reaction which is reported to be reversible on treatment withdrawal [204].

Amitriptylinoxide (18) is one of the urinary metabolites of amitriptyline. It has a 'classic' antidepressant profile in animal models, but its sedative and anticholinergic activities are weaker than those of amitriptyline [205,206]. These results suggest that amitriptylinoxide should be of potential use in the treatment of depression associated with anxiety and agitation and should have a low incidence of anticholinergic side-effects. Indeed, clinical trials in man seem to confirm this prediction [207–210] and also show a more rapid onset

of therapeutic effect than amitriptyline [211]. Further results are awaited.

Ro 8-1998 (19) has been compared with imipramine in a double-blind study involving 30 patients with endogeneous depression. Overall efficacy was similar when assessed using several ratings, including the Hamilton Depression Rating Scale. Side-effects, including anticholinergic events, were similar, although Ro 8-1998 caused a decrease in systolic blood pressure and an increase in heart rate [212]. R-806 (20) has an antidepressant profile similar to that of imipramine but with less anticholinergic activity [213]. In open uncontrolled studies, R-806 showed some improvement of depressive symptoms, although adverse events were not uncommon [214,215].



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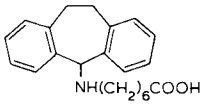

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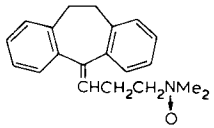

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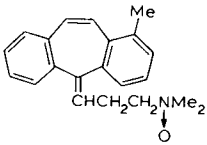

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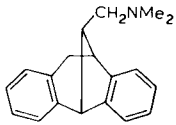

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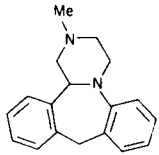
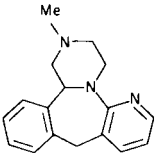


(21)

Mianserin (21) is a tetracyclic piperazinoazepine originally developed as an antihistamine and anti-5-HT compound [216] with some structural resemblance to aptazepine. Although not showing an antidepressant profile in standard pharmacological screens [36,217,218], some activity similar to that of the tricyclic agents has been shown with mianserin in more recently developed procedures [219,220]. It is a potent inhibitor of H_2-receptor-mediated adenylate cyclase formation in mammalian brain [221], has central anti-5-HT actions in mice, rats and rabbits [222,223] and has presynaptic α-adrenoceptor blocking activity [56,224]. The latter property might be responsible for the induction of subsensitivity of the NA receptor-coupled adenylate cyclase system reported following chronic administration to rats [225]. The mechanism of action of mianserin appears to involve release of noradrenaline mediated via cortical α_2-adrenergic autoreceptor blockade and this property resides stereoselectively in the (*S*)-(+)-enantiomer of mianserin. It is likely, however, that desmethylmianserin and 8-hydroxymianserin metabolites contribute to the overall facilitating effect of mianserin on

noradrenergic transmission *in vivo*. It still remains unclear whether this effect is exclusively responsible for the antidepressant activity of mianserin or whether the stereoselectivity of mianserin enantiomers toward 5-HT also plays a complementary rôle [226].

Earlier clinical studies on mianserin have been reviewed [227]. It was concluded that, although they indicate that it has antidepressant activity, in general the trials involved relatively small numbers of patients and were of



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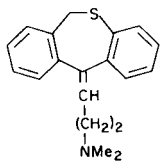

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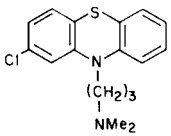

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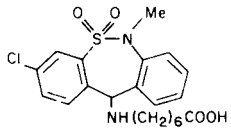


(25)

short duration, and that comparability of treatment groups was less than ideal. Drowsiness occurred during the first weeks of treatment and appeared to be related to its potent antihistaminic effect. Mianserin has the advantage of being less likely than the tricyclic antidepressants to cause serious cardiotoxicity following overdose. Like nomifensine [228] and trazodone [229], mianserin [227] has few anticholinergic effects. The results of more recent studies in volunteers [230] and depressed patients [231] favour the use of mianserin in the elderly and in patients with cardiac disease. Moreover, recent experience in controlled studies against the tricyclic antidepressants, nomifensine [232–234] and trazodone [235–237], confirms that mianserin is an effective antidepressant.

6-Azamianserin (22) is a racemic mixture and an analogue of mianserin which is active in pharmacological studies. Like mianserin, the enantiomers are stereoselective in some preclinical tests, particularly those related to the

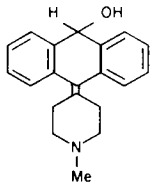
blockade of presynaptic α-adrenoceptors, where the activity resides in the (+)-enantiomer [238]. The results of computerized EEC analyses on the effects of the (+)- and the (−)-enantiomers of 6-azamianserin in volunteers suggest that both enantiomers will be clinically active [239]. Clinical studies are awaited.

Dothiepin (23) has a chemical structure resembling chlorpromazine (24), but its pharmacological effects are similar to those of other tricyclic antidepressants [240]. Clinical studies in several thousand patients indicate that dothiepin has antidepressant activity equivalent to those of amitriptyline and imipramine but with a lower incidence of anticholinergic effects [240]. It may, therefore, be a useful antidepressant for use in the elderly.

Tianeptine (25) is an antidepressant which increases the release of and inhibits the uptake of 5-HT. It is devoid of stimulant, sedative or anticholinergic activity but possesses an anxiolytic action [241,242]. The results of controlled clinical studies are awaited.

ANTHRACENES WITH OR WITHOUT BRIDGING GROUPS

Danitracen (26) is a 9-substituted dihydroanthracene which has anti-5-HT and antihistamine activity as well as central anticholinergic activity [243]. It antagonizes reserpine- and tremorine-induced effects, potentiates the actions



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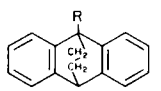


(27)

(27.1) R = $CH_2CH_2CH_2NHMe$
(27.2) R = $CH_2CHOH{\cdot}CH_2NHMe$

of amphetamine, and in EEG experiments exerts a potent depressant activity on arousal reactions. These results suggest that danitracen may be an antidepressant with sedative properties [244]. In rats, danitracen produced a decrease in 5-HT concentrations in cerebrum, cerebellum, medulla and whole brain. Apomorphine- and amphetamine-treated rats pretreated with danitracen showed a decrease in 5-HT concentrations in brain regions, with no effect on 5-hydroxyindole acetic acid (5-HIAA) concentrations in whole brain, as well as a decrease in NA concentrations. It is, therefore, suggested that increased 5-HT metabolism, as well as NA neurones, is implicated in the mechanism of action of danitracen [245].

Maprotiline (27.1) is a dibenzotricyclooctadiene compound with an aliphatic side-chain terminating in a secondary amine. It is an antidepressant agent which differs structurally from tricyclics by the presence of an alicyclic bridge across the central ring, thus forming a tetracyclic compound, although it possesses some resemblance to nortriptyline. In animal models, maprotiline has a sedative and tranquillizing effect and it has potent activity in blocking the re-uptake of NA without effects on 5-HT re-uptake [246]. Clinical studies have demonstrated that maprotiline has equivalent antidepressant activity to the tricyclic antidepressants [247–249]. Maprotiline has been reported to cause a more rapid onset of action than imipramine or amitriptyline [250] but it has been concluded that maprotiline has no clinical features that distinguish it from the older tricyclics [251]. Maprotiline has been reported to have no cardiotoxic effects in physically healthy depressed patients [252], depressed cardiac patients [253], and control cardiac patients [254].

Oxaprotiline (27.2) is the hydroxy analogue of maprotiline currently being developed as an antidepressant. The (+)-enantiomer and oxaprotiline, but not the (−)-enantiomer, reverse reserpine-induced hypothermia and ptosis, tetrabenazine-induced catalepsy in rats and NA uptake in rat heart and brain. In eight healthy subjects, oxaprotiline, the (*R*)-(−)-enantiomer and amitriptyline all caused a decrease in salivation, while supine heart rate and mean arterial blood pressure were increased by oxaprotiline but not by (*R*)-(−)-oxaprotiline. The (+)-enantiomer, therefore, has the greater anticholinergic and sympathomimetic properties [255]. In a 4 week double-blind clinical study involving 278 patients, oxaprotiline possessed antidepressant activity similar to that of amitriptyline but was reported to be better tolerated [256]. More recently, Roffman and Gould [257] reported that oxaprotiline was more effective than amitriptyline. In a double-blind multicentre study comparing oxaprotiline with amitriptyline and placebo in 308 patients with moderate depression, both active treatments, but not placebo, induced a reduction in salivary flow. The reduction of salivary flow by week 5 was greater in the amitriptyline than in the oxaprotiline group. More patients receiving amitriptyline than oxaprotiline had subjective complaints such as dry mouth, blurred vision and constipation [258].

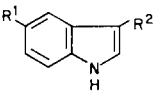
INDOLES

The possible involvement of 5-HT in the etiology of mood disorders [3] has given impetus to the development of new compounds with highly selective and potent 5-HT uptake-inhibiting properties. Indalpine (28.1), a 4-alkyl piperidine derivative, is a selective inhibitor of the re-uptake of 5-HT [259] which lacks

antagonist activity on reserpine or amphetamine effects in classical tests. In a volunteer study, it did not affect pulse rate, heart rate, vision or reduce salivary volume, although an increased pupil diameter occurred [260]. The latter effect may be due to a central mechanism. In a double-blind study involving 100 patients suffering from severe depression, indalpine had antidepressant efficacy similar to that of clomipramine [261]. In another double-blind study, indalpine was found to have antidepressant activity similar to that of imipramine but with fewer adverse reactions [262]. Recently, a 1 month open pilot study [263] suggested that indalpine possessed antidepressant and anxiolytic activity in patients with endogeneous depression. Anticholinergic side-effects and weight gain were absent. Indalpine has just been withdrawn following a number of reports of agranulocytosis in patients receiving this drug.

RU 24969 (28.2) is a potential new antidepressant which was reported to be the most potent 5-HT agonist of a series of piperidinyl indoles and is suggested to act at 5-HT receptors which are not coupled to adenylate cyclase [264]. Controlled clinical data are awaited.

IH-3 (29) is an MAO A inhibitor whose activity may be related to a structural similarity to 5-HT (indole nucleus, lateral chain in 3 and oxygen atom in 5). It is proposed that isopropyl hydrazide is liberated from this compound *in vivo* [265].



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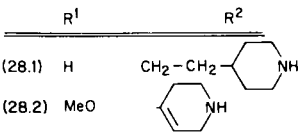
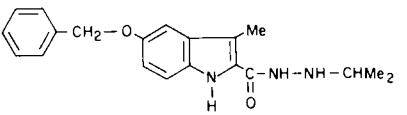


(28.1) H

(28.2) MeO



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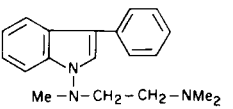


(30)

Binedaline (30) is an *N*-alkylated derivative of 1-amino-3-phenylindole reported to antagonize reserpine-induced and tetrabenazine-induced ptosis in rats and hypothermia in mice. It potentiates the efect of yohimbine in rats and antagonizes the apomorphine-induced hypothermia in mice. In contrast to tricyclic antidepressants, binedaline has no antihistaminic activity and does not antagonize central or peripheral effects of acetylcholine or 5-HT. Binedaline

inhibits NA uptake into rat brain synaptosomes and following chronic administration a down-regulation of β-adrenoceptors and 5-HT receptors is reported to occur [266–270]. In healthy volunteers, the lack of anticholinergic activity and an α-adrenoceptor blocking action has been shown [271]. Results from patient studies on this new potential antidepressant are awaited.

CARBAZOLES AND CARBAZOLE-LIKE DERIVATIVES

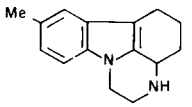
Pirlindole (31) is a piperazino[1,2-*a*]indole derivative with pharmacological activity similar to that of the tricyclic antidepressants but with the advantage of a lack of anticholinergic activity. Studies on its mode of action indicate that it possesses both NA re-uptake and a short duration MAO inhibitory activity [272–274].

In the anesthetized dog, pirlindole has minimal cardiovascular effects [275]. In volunteers, a weak potentiation of the effects of tyramine on blood pressure has been reported. The so-called 'cheese reaction' is therefore unlikely to occur. Open studies also suggest an antidepressant effect for pirlindole with no signs of the 'cheese-reaction' being observed [276–279]. In a double-blind placebo-controlled study in patients with endogenous depression, pirlindole caused a significant improvement in symptoms, although hypotension emerged as a side-effect [280]. Preliminary results of other double-blind studies suggest that pirlindol is of similar efficacy to amitriptyline and imipramine [281,282], although some studies report pirlindole to be inferior to amitriptyline [283,284]. The results of further studies involving a large number of patients and using placebo as control are awaited.

Ciclindole (32) potentiates catecholamines, antagonizes reserpine-induced ptosis in mice and causes effects similar to imipramine on electro-encephalographic recordings of cats with chronically implanted electrodes. A preliminary uncontrolled study in chronic alcoholic inpatients with depression suggest that ciclindole possesses antidepressant activity [285]. Subsequently, other studies have supported the antidepressant effect of ciclindole with dry mouth, sweating, drowsiness and insomnia being reported as common but mild side-effects [286,287].

Tienocarbine (33.1) is a tetrahydrothienopyridoindole with potent and specific DA agonist activity. It is virtually free of NA potentiating, MAO inhibiting and anticholinergic effects, but potentiates the stereotypy induced by amphetamine and the stimulant effect of methylphenidate. Tienocarbine also antagonizes the cataleptogenic effect of reserpine in mice, and immobility produced by tetrabenazine, and has an antiaggressive effect in mice and rats [288]. Tienocarbine displaces [^{3}H]dopamine and [^{3}H]spiperone from their

specific binding sites in the nucleus caudatus of calf brain [289]. This pharmacological profile suggests anxiolytic activity combined with neuroleptic activity with some characteristics of an antidepressant. Tiflucarbine (33.2) is a fluorinated derivative of tienocarbine, which is effective in some animal antidepressant tests. A fluorine atom was introduced to prevent metabolic hydroxylation of the benzene ring of the indole moiety. Complementary binding studies with the β-adrenergic antagonist dihydroalprenolol as ligand indicated a reduction of the density of binding sites [290]. Further studies with this potential antidepressant are awaited.



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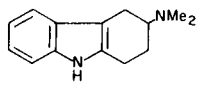

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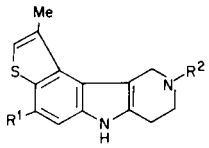
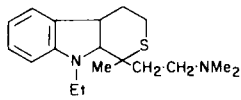


(33)

	R^1	R^2
(33.1)	H	Me
(33.2)	F	Et



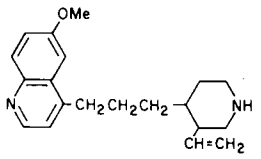
(34)

Tandamine (34) is a tetrahydrothiopyranoindole which antagonizes reserpine-induced effects, prevents tetrabenazine-induced ptosis, and inhibits noradrenaline uptake in animal experiments. Its activity resides predominantly in the (−)-isomer. The *N*-demethyl analogue of tandamine, which is a major metabolite, is less potent than the parent as a reserpine antagonist [291]. Tandamine is a blocker of the uptake of NA but has no effect on the uptake of 5-HT [292]. Using the cat nictitating membrane preparation and the isolated guinea-pig vas deferens, tandamine was found to have a pharmacological profile similar to that of imipramine [293]. In a pilot study, tandamine was well-tolerated and reported to be effective in treating retarded, but not agitated, depression [294]. The results of further studies are awaited.

QUINOLINES AND ISOQUINOLINES

Viqualine (35) differs from clomipramine by possessing a marked 5-HT releasing effect with no effect on (5-HIAA) concentrations and is 20-times more potent and has a longer duration of effect than does clomipramine in inhibiting 5-HT uptake by rat synaptosomes. In contrast to clomipramine, viqualine does not antagonize cardiac NA depletion induced by 6-hydroxydopamine, which confirms its selectivity for 5-HT [263]. In healthy volunteers, a single dose of viqualine inhibited [^{3}H]5-HT uptake by platelets but had no significant effect on platelet 5-HT content [295]. Pharmacologically, viqualine resembles other selective 5-HT uptake inhibitors such as indalpine and zimelidine. Results of controlled clinical trials are awaited.

Nomifensine (36) is a tetrahydroisoquinoline compound with a β-phenylethylamine structure characteristic of amphetamine-like compounds; it produces stereotyped behaviour and has stimulant properties in animals [296,297]. It resembles imipramine in antagonizing reserpine-induced hypothermia in mice and in reversing catalepsy caused by tetrabenazine and reserpine in mice and rats. Nomifensine has no significant anticholinergic or



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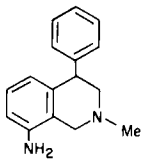

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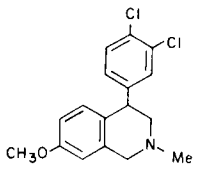

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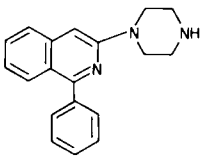


(38)

antihistaminic effects but interestingly, while being a potent inhibitor of NA re-uptake into rat brain synaptosomes, it induces the release of DA and inhibits its neuronal re-uptake [228,298,299]. It has a weak effect on 5-HT [300], although the 4-hydroxy metabolite has been reported to have significant inhibitory actions on 5-HT uptake [301]. Hindmarch and Parrott [302] reported that nomifensine may cause deterioration in the ability of volunteers

to fall asleep. In patients, several studies have compared nomifensine with either placebo or a standard reference antidepressant. Overall, the findings on this established antidepressant demonstrate superiority over placebo and show that, in divided or single doses of up to 150 mg daily, it is at least as effective as imipramine or amitriptyline [228,303,304], with the most commonly observed side-effects being sleep disturbance, restlessness, nausea and dry mouth [305]. The drug* does not have any significant adverse effects on the cardiovascular system and appears to be well tolerated by patients with pre-existing cardiovascular disease [306].

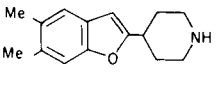
Diclofensine (37) is also an isoquinoline derivative but differs from nomifensine in its peripheral and central pharmacological activity. Thus it is a potent inhibitor of DA, NA and 5-HT uptake [307,308]. Recent results *in vitro* suggest that amine uptake-inhibiting is stereospecific and resides in the (*S*)-(+)-enantiomer of diclofensine [309]. Unlike nomifensine, diclofensine has little if any amphetamine-like central stimulant properties [310]. In a controlled volunteer study [311], diclofensine was found not to influence salivary flow or consistently affect pupil diameter and had no significant effect on subjective measurements of sedation, mood, heart rate, blood pressure, systolic time intervals or high-speed electrocardiogram. Diclofensine has been found not to alter prolactin secretion, thyroid function or vigilance, but stimulates hypothalamic-pituitary-growth hormone and the adrenal cortex axis [312]. Early clinical work suggested an antidepressant effect in man but with tremor, agitation, insomnia and atropine-like side-effects occurring [313,314]. In a placebo-controlled double-blind study, diclofensine was particularly effective in depressed patients with symptoms of severe psychomotor retardation. In depressed patients with features of severe anxiety, agitation and excitation, an initial rapid response was not sustained [315]. A double-blind placebo-controlled study involving 40 depressed patients showed an antidepressant effect of diclofensine, with dizziness being the most frequently reported side-effect [316]. In the elderly, diclofensine improved depressive states more quickly and effectively than did placebo without pronounced anticholinergic side-effects [317]. In another double-blind placebo-controlled study involving 60 elderly depressed patients, diclofensine was superior to placebo. Psychomotor tests revealed a lack of sedation [318].

Perafensine (38) is a new isoquinoline derivative, with substituents in the 3 position, shown to be active in tests predictive of antidepressant activity [319]. The results of further studies are awaited.

*Nomifensine has recently been withdrawn worldwide, since it has been associated with acute haemolytic anaemia.

BENZOFURANS AND ISOBENZOFURANS

CGP-6085A (39), a tricyclic piperidine structure, is a potent and selective inhibitor of 5-HT uptake. This benzofuran inhibited the uptake of 5-HT into the midbrain synaptosomes at 4 mg/kg, whereas the uptake of NA was not inhibited by doses as high as 300 mg/kg. Similarly, the depletion of whole rat brain 5-HT was prevented by low doses of CGP-6085A while the uptake of NA by the rat heart was not affected at high doses [320,321]. In spontaneously hypertensive and normotensive rats, CGP-6085A had a hypotensive response which correlated with the inhibition of 5-HT [322]. Further results are awaited.



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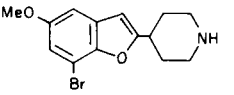

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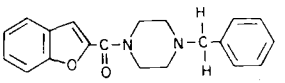

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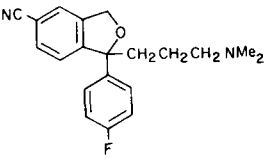


(42)

CGP-11305A (40) is a selective, reversible and short-acting MAO A inhibitor which causes a dose-dependent increase of NA, DA and 5-HT in the rat brain. It also exhibits some inhibitory properties on the uptake of 5-HT and, to a lesser extent, that of NA both *in vitro* and *in vivo*; however, compared with its MAO inhibitory activity, uptake inhibition occurs at doses 30–100-times higher [323,324]. An open study involving 16 patients has revealed some antidepressant activity [325]; however, the results of controlled clinical trials are awaited.

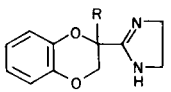
Befuraline (41) reverses the effects of reserpine and tetrabenazine and has an aggression-inhibiting activity, without sedation, in animal models. The central anticholinergic effects of befuraline are negligible while the effects of apomorphine or tryptamine are not potentiated [326]. In an open study, the therapeutic efficacy of befuraline was tested in 64 patients with various depressive syndromes. Improvement was found in 33 cases using a daily dose of 150 mg within the first 8 days of treatment [327].

Citalopram (42) is a potent and highly selective inhibitor of the neuronal uptake of 5-HT with no antagonistic activity against 5-HT, NA, DA, histamine,

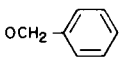
acetylcholine or GABA [328–331] whose structure resembles that of the tricyclic agents only by containing a 3-carbon side-chain with a tertiary amino group. The mono- and didesmethyl and *N*-oxide metabolites of citalopram are weaker antagonists of 5-HT uptake, although selectivity of action is retained. In animals, citalopram possesses weak α-adrenoceptor blocking activity but has negligible anticholinergic properties and low cardiotoxicity [331,332]. An initial clinical study performed in 10 endogenously depressed inpatients showed a good or moderate response in 7 patients with few, mild side-effects [333]. In another study [334], 11 of 16 endogenously depressed and 2 of 3 non-endogenously depressed patients responded to citalopram. Hypersomnia (3 patients) and a transient elevation of hepatic enzymes (2 patients) were observed. Plasma concentrations of citalopram did not correlate with therapeutic outcome. One patient took an overdose causing a plasma concentration about 6-times higher than the average therapeutic concentration without any signs of severe toxicity. Øfsti [335] reported on the results of an open study in 26 patients with a long history of severe affective psychosis who completed 4 weeks treatment with citalopram. Fifteen patients showed a marked or moderate response within the first 2 weeks of treatment, with only transient nausea and increased sweating occurring. Further data are awaited so that the rôle of this specific 5-HT uptake inhibitor in the treatment of depression may be assessed.

IMIDAZOLES, PYRAZOLES AND BENZIMIDAZOLES

Idazoxan (43.1) is an α_1-adrenoceptor agonist at doses similar to those at which it exhibits α_2-adrenoceptor antagonist activity in the pithed rat preparation



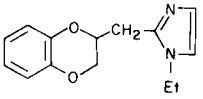
(43)

(43.1)	R =	H
(43.2)	R =	Me_2
(43.3)	R =	Et
(43.4)	R =	Pr
(43.5)	R =	
(43.6)	R =	OMe_2
(43.7)	R =	OEt
(43.8)	R =	

[336]. *In vitro*, it is a highly specific α-adrenoceptor antagonist with some selectivity for the α_2-adrenoceptor when tested in the human platelet system. It is equally potent as an inhibitor of the platelet aggregatory responses induced either by a non-selective agonist (adrenaline) or by UK-14304, an agonist which acts selectively at α_2-adrenoceptors, and exhibits properties of a competitive agonist acting preferentially at the platelet α_2-adrenoceptors [337]. At central and peripheral adrenoceptors in the rabbit, idazoxan was found to be a selective α_2-adrenoceptor antagonist [338]. In healthy volunteers, idazoxan antagonized the central effects of the α_2-agonist clonidine [339]. In a double-blind study involving 8 normotensive volunteers, intravenous idazoxan caused a modest antagonism of the pressor response to the α_1-agonist, phenylephrine, as well as the mixed α_1- and α_2-agonist, NA, but a substantial antagonism of the pressor response to α-methylnoradrenaline, which is predominantly an α_2-adrenoceptor agonist [340,341].

Several 2-alkyl analogues of idazoxan have been synthesized. Thus 2-methyl-RX801079 (43.2), 2-ethyl-RX811033 (43.3), 2-*n*-propyl-RX811054 (43.4), and 2-isopropenyl-RX811005 (43.5) have peripheral and central α_2- to α_1-adrenoceptor antagonist selectivity equal to or greater than those of idazoxan. RX811033 and RX811054 are both more potent α_2-adrenoceptor antagonists than idazoxan [342–347]. The 2-methoxy-RX821002 (43.6), 2-ethoxy-RX811059 (43.7) and 2-methoxyphenyl-RX821004 (43.8) analogues all have α_2-adrenoceptor potency similar to that of idazoxan, while RX821002 and RX811059 are both more potent than idazoxan; however, unlike idazoxan, RX821002, RX8211059 and RX821004 are reported not to affect significantly diastolic blood pressure in the pithed rat [342,346].

RS-21361 (44) is also a selective α_2-adrenoceptor antagonist reported to be more selective than rauwolscine, with an α_2/α_1 ratio greater than 616 [348]. In



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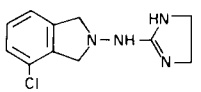

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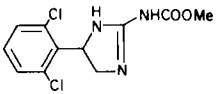

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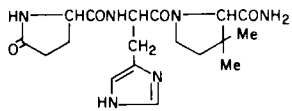


(47)

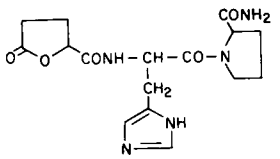
the pithed rat, it was found to be more potent as an α_2-adrenoceptor than as an α_1-adrenoceptor antagonist, but was less potent than mianserin as an α_2-adrenoceptor antagonist against the presynaptic action of clonidine in the rat vas deferens and as an antagonist of guanoxabenzamine-induced mydriasis [344].

BE-6143 (45) is a selective α_2-adrenoceptor antagonist which may have antidepressant activity [349]. *In vitro*, BE-6143 acts as a partial agonist at post-synaptic α_1-adrenoceptors and as an antagonist at presynaptic α_2-adrenoceptors in rabbit aorta and pulmonary artery. It has higher affinity for the presynaptic than for the postsynaptic receptors and its affinity for the presynaptic α_2-adrenoceptors exceeds that of yohimbine and rauwolscine; however, BE-6143 inhibits NE release by a non-α-adrenergic mechanism [350].

RS-51,324 (46) is more potent than imipramine in enhancing L-DOPA-induced excitement in mice, does not inhibit MAO and is less active than imipramine or amitriptyline in blocking [^{3}H]NA uptake in mouse heart. Potentiation of 5-HT-induced cardiovascular depression and a reduction of left ventricular blood pressure, myocardial contractility, heart rate and cardiac output were reported. RS-51,324 has no anticholinergic activity and only a weak antihistaminic effect [351]. In summary, RS-51,324 appears to act via NA uptake inhibition and lacks both sedative and anticholinergic potential. Further results are awaited.

It has been reported that thyrotropin-releasing hormone (TRH) produces an antidepressant effect [352,353]. RX77,368 (47) and DN1417 (48) are both TRH analogues which may have potential antidepressant activity. Both drugs share the neuropharmacological property of the tricyclic antidepressants in reversing reserpine-induced effects [354,355].

Pyrazolepropanamine (49) reduces locomotor activity and antagonizes reserpine-induced ptosis in mice. It inhibits NA uptake in rat synaptosomes



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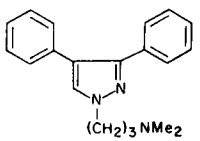

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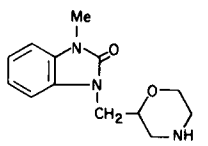


(50)

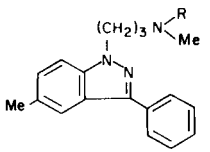
and is up to 50-times less active than imipramine in blocking 5-HT uptake *in vitro*. This compound has no significant anticholinergic or antihistaminic effects *in vivo* and is reported to be less cardiotoxic than is imipramine [356].

UP-61404 (50) is a benzimidazole analogue of viloxazine which reduces locomotor activity in mice, and antagonizes reserpine, oxotremorine or tetrabenazine-induced hypothermia and ptosis [357]. These actions indicate a possible antidepressant action. UP-61404 inhibits cerebral MAO A and B activities in rats and mice. The inhibition was more sustained than that of viloxazine, but much less potent or sustained than that of pargyline or isocarboxazide, which suggests that the therapeutic activity may involve moderate and more reversible MAO inhibition. UP-61404 possesses less anticholinergic activity than the tricyclic antidepressants and is likely to be less cardiotoxic [358,359].

INDAZOLES AND DERIVATIVES

FS-32 (51.1), and its desmethyl derivative FS-97 (51.2), show a dose-dependent linear antireserpine action in the mouse, whilst the tricyclic antidepressant, imipramine, demonstrated a bell-shaped dose-response curve. FS-32 was slightly less active than FS-97 in this test [360]. Neither FS-32 nor FS-97 has any effect on the release of DA and NA from striatal and hypothalamic synaptosomes and both have very weak activity in displacing the specific binding of [^{3}H]quinuclidinyl benzilate to striatal synaptic membranes. Thus, FS-32 and FS-97 appear to differ from the tricyclic antidepressants particularly in lacking central antimuscarinic activity [361]. Although no difference in the specific binding to β-adrenergic, serotonergic or dopaminergic receptors occurred after a single dose of FS-32, FS-97, imipramine or desipramine in rats, 4 weeks treatment resulted in a decrease in the specific binding to β-adrenergic and dopaminergic receptors. Serotonergic receptor sensitivity was not altered. It is postulated that this adaptation at postsynaptic receptors, rather than the almost immediate presynaptic uptake inhibition of amines, is the main mechanism of action, since clinical improvement with antidepressants is observed after several weeks [362].

Cartazolate (52) has been studied in normal volunteers and patients and it has been suggested that this compound has a combination of antidepressant



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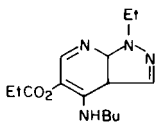


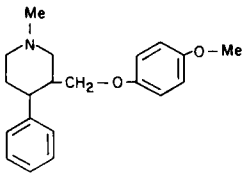
(52)

(51.1) R = Me
(51.2) R = H

and anxiolytic activity [363–365]. Chromatographic analysis of the urine following ingestion of cartazolate has indicated the presence of seven urinary metabolites. These result from hydroxylation of the 5-carboxy acid ethyl ester, *N*-demethylation of the pyrazole ring, γ-hydroxylation of the *n*-butyl side chain, removal of the *n*-butyl group, and conjugation with β-glucuronic acid [366].

ANISOLE DERIVATIVES

Femoxetine (53) is an inhibitor of 5-HT uptake by synaptosomes from rat brain, with a potency comparable with that of clomipramine, and a weak inhibitor of peripheral NA uptake [367,368]. In volunteers, femoxetine increased cardiac contractility in comparison with placebo and had less effect on intracardiac conduction than amitriptyline. Generally, anticholinergic side-effects were less frequent with femoxetine [369]. Femoxetine was significantly inferior to amitriptyline in the treatment of endogenously depressed patients in a 6 week study. However, it produced a smaller pressor response to NA or to tyramine than amitriptyline and also had fewer anticholinergic effects [370]. An antidepressant effect and a low incidence of anticholinergic



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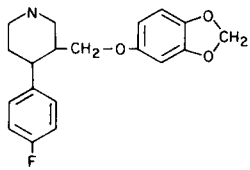
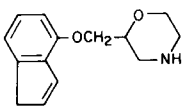


(54)

effects with no correlation between plasma concentration and clinical efficacy have been reported [371]. Femoxetine caused a reduction of circulating 5-HT in 12 depressed patients, although this effect did not correlate with therapeutic effect [372]. The antidepressant effect of femoxetine has been confirmed in a double-blind study involving 42 outpatients with depressive illness using desipramine as comparator [373]. The treatments were not significantly different for efficacy, but patients treated with femoxetine reported significantly less severe anticholinergic effects. There was no significant correlation found between therapeutic effect and the plasma concentration of either active agent. Recently, femoxetine was reported to be as effective as amitriptyline in a double-blind 6 week general practice study involving 81 depressed patients. Nausea was the most commonly observed event in the femoxetine group, while a greater frequency of anticholinergic side-effects was recorded after treatment with femoxetine [374].

Paroxetine (54) is a close structural analogue of femoxetine with potent and selective 5-HT uptake inhibitory activity [375–377]. The *in vitro* rank order of potency for inhibition of 5-HT uptake into mouse forebrain synaptosomes is reported to be greater than for citalopram, femoxetine, fluoxetine, alaproclate, imipramine or zimelidine [378]. Using tritiated paroxetine, maximal binding to human platelet membrane was similar to that of imipramine, whereas affinity was much higher for paroxetine [379]. In normal volunteers, the EEG profile following paroxetine is similar to that reported for other antidepressants, such as fluvoxamine or zimelidine, for 5-HT uptake inhibitory properties, but dissimilar from those of the 'classical' sedative antidepressants [380]. In an open evaluation involving 19 depressed patients, a marked improvement was noted in 11 patients, but there was no correlation between therapeutic efficacy and the plasma concentration of paroxetine. Two patients complained of dry mouth at the start of treatment and a further patient experienced burning sensation together with periodic light headache [381]. The results of controlled studies are awaited.

YM-08054-1 (55) inhibits the uptake of NA and 5-HT into rat brain synaptosomes. It is more potent than classical tricyclic agents in potentiating hind-limb abduction and tremor induced by 5-HTP. It is only a weak inhibitor of the deamination of NA and β-phenylethylamine and is, therefore, not an MAO inhibitor. YM-08054-1 is a more potent inhibitor of reserpine-induced hypothermia in the mouse than amitriptyline, clomipramine, imipramine, desipramine, iprindole or viloxazine. YM-08054-1 is also devoid of anticholinergic activity [382].



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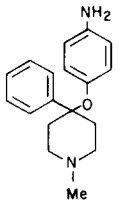
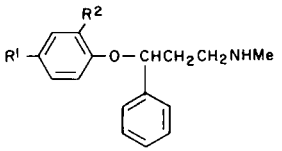


(56)

B777-81 (56) reverses reserpine- and tetrabenazine-induced ptosis in a similar manner to that of imipramine, but with a shorter duration of action. It also antagonizes reserpine-induced hypothermia but it is without effect on the decrease in locomotor activity or catalepsy following reserpine. *In vitro*, B777-81 inhibited 5-HT uptake in platelets, potentiated the effect of NA on isolated vas deferens and had less anticholinergic effect than imipramine in the isolated ileum [383].

Tomoxetine (57.1) is a racemic mixture, and is a competitive inhibitor of NA uptake in synaptosomes of rat hypothalamus. The optical (–)-isomer, is 2- and 9-times more potent than the racemate and the (+)-isomer, respectively. All



(57)

	R^1	R^2
(57.1)	H	Me
(57.2)	H	O Me
(57.3)	CF_3	H

three compounds are relatively weak in the inhibition of DA and 5-HT uptake. The optical (–)-isomer is a relatively weak ligand for α_1-, α_2- and β-adrenoceptors, acetylcholine-muscarinic receptors, histaminergic H_1-receptors and the receptors of γ-aminobutyric acid and benzodiazepines [384]. In depressed patients, tomoxetine is reported to improve depression, as assessed using the Hamilton Depression Rating Scale, without any apparent sedative effect [385]. The results of controlled studies are awaited.

Nisoxetine (57.2), the 2-methoxy analogue of tomoxetine, is also an NA uptake inhibitor with a potency about twice that of the racemate of tomoxetine [384]. It also possesses some DA uptake inhibitory activity. Fluoxetine (57.3), in which the 2-methoxy group of nisoxetine is replaced by a *p*-trifluoromethyl group, is a specific and potent inhibitor of 5-HT uptake into synaptosomes of rat brain without significant effect on NA uptake [386,387]. Within this analogous series, a trifluoromethyl group in the phenoxy ring at the *para*-position confers optimal effect on 5-HT uptake inhibition. Substitution with fluoro, chloro, methyl or methoxy groups did not result in greater activity. The *N*-demethylated (primary amine) and the *N,N*-dimethylated (tertiary amine) derivatives inhibit the uptake of monoamines with effectiveness similar to that of fluoxetine (a secondary amine). Even after chronic administration, fluoxetine does not reduce the density of either postsynaptic β-adrenoceptors or postsynaptic 5-HT_2 receptors [388]. In rats treated for 6 weeks with fluoxetine, a subsensitivity of 5-HT receptors in the frontal cortex of rat brain has been reported [389]. In a study on the inhibition of 5-HT uptake by fluoxetine in man, a reduction of endogenous 5-HT in platelets with no effect on NA as measured by responses to the pressor effect of tyramine and NA has been

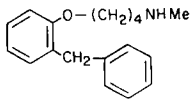
reported [390]. An antidepressant effect in an initial open assessment was also described.

The full pharmacology of fluoxetine has recently been reviewed [391]. It was noted that, unlike many antidepressants, fluoxetine has little affinity for muscarinic, histaminic H_1, serotoninergic 5-HT_1 or 5-HT_2 or noradrenergic α_1- or α_2-receptors in rat brain membranes. It has been reported that 1 of 9 depressed patients receiving fluoxetine developed a dystonic reaction, Parkinsonian rigidity and increased serum prolactin concentrations. These are all signs of a decreased dopaminergic activity which might be mediated via the increase in 5-HT activity, resulting from the blockade of 5-HT uptake, causing an inhibition of both nigrostriatal and tubero-infundibular dopaminergic neurons [392]. Fisch [393] reported that no intracardiac conduction defects were observed in 312 patients who had received fluoxetine. In a double-blind randomized parallel group study, fluoxetine was significantly superior to imipramine and also to placebo [394,395]. In another controlled study, fluoxetine was reported [396,397] to have an efficacy similar to that of doxepin in the elderly. The most frequently reported adverse experiences were nervousness-anxiety, drowsiness-sedation, dry mouth and nausea in the fluoxetine group and dry mouth, drowsiness-sedation, constipation, dizziness and vision disturbances in those patients receiving doxepin.

In a further controlled study, fluoxetine and imipramine were superior to placebo, with fluoxetine showing some evidence of an earlier onset of action [398]. Fluoxetine has been reported to have a better 'Efficacy Index-Side Effects' rating than amitriptyline and a lower incidence of anticholinergic autonomic side-effects, while having therapeutic efficacy comparable with that of amitriptyline [399]. The collective experience of over 1000 patients with depression who participated in double-blind controlled clinical studies has been summarized by Stark and Hardison [400,401]. Fluoxetine was superior to placebo and at least as active as imipramine, doxepin or amitriptyline. Fluoxetine showed less anticholinergic activity than did the tricyclic agents, while nausea and insomnia occurred more frequently in the fluoxetine-treated patients. Generally, however, side-effects were mild and interestingly did not include the weight gain associated with many standard antidepressants. Moreover, fluoxetine does not appear to produce the 'flue-like' syndrome or neuropathy, reported with the 5-HT uptake inhibitor, zimelidine, or cause phospholipidosis in humans, which has been reported in animals receiving cationic drugs [402].

DIPHENYLMETHANE DERIVATIVES

MCI-2016 (58), an (ω-aminoalkoxy)benzene derivative, markedly antagonizes the hypothermia and depressive-symptoms induced by reserpine. It also antagonizes tetrabenazine and has relatively weak sedative and anticholinergic activity [403,404]. MCI-2016 has also been shown to be an inhibitor of the neuronal uptake process for NA [405], with no direct effect on the presynaptic α_2-adrenoceptor. In contrast to imipramine, amitriptyline and maprotiline, MCI-2016 did not inhibit REM sleep. The result of clinical studies in depressed patients are awaited.



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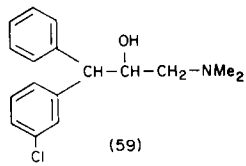

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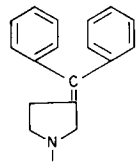


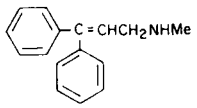
(60)

Climeprol (59) possesses antireserpine activity and potentiates the central effects of NA. It also possesses some activity in inhibiting the uptake of 5-HT and DA, but is virtually devoid of anticholinergic activity [406,407]. The results of an open study on 15 depressed patients suggest that clemeprol may be a useful antidepressant agent [408]. The results of controlled studies are awaited.

AHR-1118 (60), also known as pridefine, is a relatively specific non-competitive inhibitor of the neuronal uptake of catecholamines [409]. In rats pretreated with saline or reserpine, it significantly increased NA concentrations in the hypothalamus [410]. In a 4 week open clinical study involving 7 depressed patients, plasma 3-methoxy-4-hydroxyphenylglycol, a metabolite of noradrenaline, increased as a rapid improvement in symptoms occurred [411,412]. In a previous comparative study with amitriptyline, AHR-1118 was reported to possess an earlier onset of action, although insomnia was reported [413]. Further controlled studies are needed to assess the usefulness of AHR-1118.

BW-247 (61) is a secondary amine, closely related in structure to the tricyclic antidepressants, which inhibits NA uptake in aortic strips [414] and rat cerebral cortex slices [415]. Unlike the tricyclic antidepressants, it is reported not to possess anticholinergic activity [416].

The use of a GABA receptor agonist in depression has been discussed [417]. The suggestion that progabide (62), a gabaminergic agonist [418,419], had potential antidepressant properties has been confirmed in a double-blind study



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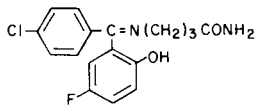

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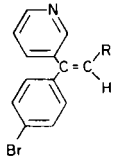


(63)

(63.1) R = CH_2NHMe
(63.2) R = CH_2NMe_2

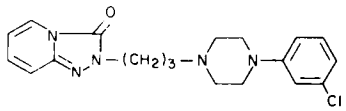
[420]. More recently, the results of a double-blind study involving 27 depressed patients suggested that progabide had an efficacy similar to that of imipramine. Tolerance was good [421]. With this interesting compound, the results of further clinical work involving placebo studies in larger numbers of patients are awaited.

Nomelidine (63.1), the active *N*-demethylated metabolite of zimelidine (63.2), is a potent and specific inhibitor of [^{14}C]5-HT uptake in synaptosome-rich homogenates of rat hypothalamus [422]. A similar inhibition of radiolabelled 5-HT uptake following oral administration has been reported [423]. Since whole-brain concentrations of nomelidine were higher than those of the parent, zimelidine, the effects of zimelidine on 5-HT uptake were postulated to be due primarily to the formation of nomelidine. The therapeutic efficacy of zimelidine is well established [424], but its recent withdrawal due to a small number of cases of potentially serious adverse events, including the neurological disorder known as the Guillain-Barré syndrome, may well discourage the further clinical development of nomelidine.

MISCELLANEOUS

Trazodone (64) is a triazolopyridine derivative which is not active in acute conventional animal models for antidepressants, such as reserpine antagonism or L-DOPA potentiation. It has no significant effect on DA or NA, no atropine-like effects and is not an MAO inhibitor [425,426]. It is a weak and specific inhibitor of the synaptosomal uptake of [^{3}H]5-HT [427], but it is also a potent central 5-HT antagonist [428]. Evidence is accumulating that chronic treatment with antidepressants, including trazodone, may cause a down-regulation or subsensitivity of rat central β-adrenergic receptor function [429,430]. This type of mechanism may explain why antidepressants take several days or weeks to exhibit an antidepressant activity in patients.

Several clinical studies have established the efficacy and side-effect profile of trazodone using placebo, conventional tricyclic agents and non-tricyclic



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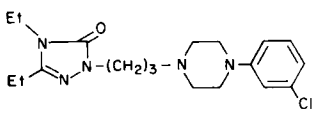
agents [229,236,431–439]. Trazodone may represent an advance over the established tricyclic agents in terms of fewer side-effects and a wider margin of safety. Thus, an analysis of the occurrence of anticholinergic side-effects in 15 multicentre studies revealed an incidence for trazodone which was significantly lower than that for the tricyclic agent, imipramine, but not significantly different from placebo [440]. Recently, Van de Merwe, Silverstone and Ankier [441] found that trazodone did not share the potential cardiotoxicity of amitriptyline. If reproducible, this is an important safety factor for depressed patients who are generally at risk of suicide due to overdosage, as well as for the elderly who are a special group at particular risk. In a controlled study, Ather, Ankier and Middleton [109] confirmed the efficacy and tolerability of trazodone in the elderly, although drowsiness emerged as a common side-effect. Bayer, Pathy and Ankier [442] reported that the sedative effect of trazodone is more prolonged in the elderly than the young, but drowsiness may be used to advantage when trazodone is given in a single night-time dosage regimen for patients where sleep disturbance is a particular problem [443].

Etoperidone (65), a phenylpiperazine-substituted triazole derivative, is an analogue of trazodone with a similar pharmacological profile [444,445]. Thus etoperidone has a biphasic effect on central 5-HT transmission owing to its 5-HT agonist and antagonist effects which appear to be dependent on dose [446]. The cardiovascular effects of etoperidone are mainly due to its α-adrenolytic activity, while those of imipramine were attributable to the potentiation of catecholamines and to its anticholinergic effects [447]. Double-blind studies using imipramine [448] and amitriptyline [449] as comparators suggest an antidepressant action but with the advantage of a lower incidence of anticholinergic side-effects. Further information is awaited.



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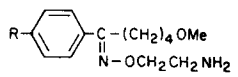


(66)

(66.1) R = Cl
(66.2) R = CF_3

Clovoxamine (66.1) is a potential antidepressant in the series of 2-aminoethyloxime ethers of aralkyl ketones. It is a potent inhibitor of 5-HT and NA

uptake, but has virtually no anticholinergic activity. Atrioventricular block has been reported in dogs at high dosages [450]. Clovoxamine is a strong antagonist of reserpine-like effects such as ptosis and hypothermia in mice and rats, inhibits 5-HT and NA uptake in rat hypothalamus synaptosomes, and is effective in potentiating 5-HTP in mice [451]. A preliminary open study in 12 patients with major depressive disorder resulted in an amelioration of depression in most patients, although one patient became hypomanic [452]. Other workers [453] have reported similar therapeutic effects. Preliminary results of double-blind controlled studies have shown that clovoxamine has therapeutic efficacy similar to that of amitriptyline [454,455], clomipramine [456] or imipramine [457], with a lower incidence of anticholinergic side-effects. Further details are awaited.

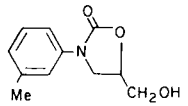
Fluvoxamine (66.2) is an analogue of clovoxamine which possesses a relatively specific inhibitory activity on 5-HT uptake on rat synaptosomes with a potency similar to imipramine. Pharmacological studies in animals show that it does not possess significant antihistamine or anticholinergic activity, is neither sedating nor stimulating and has a low cardiotoxic effect [458,459]. Although fluvoxamine has no affinity for β-adrenergic receptors when given acutely, after 8 days of treatment there is a significant decrease in the number of β-adrenoceptors in rat cortical membranes [460]. Early clinical trials involving relatively low numbers of patients indicate antidepressant activity with a low side-effect profile [461–468]. In a 1-year study involving 31 depressed patients, fluvoxamine was reported to improve mood within 4–7 days and suicidal ideation disappeared within 5–6 days [469,470]. In a recent multicentre placebo-controlled study conducted in 8 centres from 4 countries and involving 464 depressed patients [471], fluvoxamine- and imipramine-treated patients revealed a significant amelioration of depression after 4 weeks of treatment when compared with placebo. When compared with placebo and imipramine, the most commonly reported side-effect in the fluvoxamine group was nausea and/or vomiting in the first 2 treatment weeks. This compared with a significantly greater incidence of anticholinergic symptoms in the imipramine group; fluvoxamine did not differ significantly from placebo. In another study involving 93 investigators from 61 centres, 505 depressed patients were entered into an open study comparing fluvoxamine using three different dosage regimens. Preliminary results [472] suggest that the greatest efficacy is obtained with a single evening dose, while a twice-daily dosage regimen may be better tolerated.

In a double-blind, crossover, placebo-controlled study comparing fluvoxamine and clovoxamine in 27 healthy volunteers, no effect on ECG was observed except for a slight decrease in heart rate [473]. In a multicentre study

performed in Europe and the U.S.A., which included 230 depressed patients without cardiac disease, the electrocardiographic profile of fluvoxamine was compared with those of clomipramine, imipramine and placebo [474]. A small but statistically significant decrease in heart rate was found with fluvoxamine. Fluvoxamine did not differ from placebo in any other respect.

Toloxatone (67) is an oxazolone with the neuropharmacological profile of an antidepressant; for example, it antagonizes the effects of reserpine and reserpine-like compounds [475,476], potentiates 5-HTP and tryptamine effects in rodents, but is without anticholinergic activity [477]. Biochemical studies have shown that toloxatone is a selective, reversible and competitive inhibitor of type A MAO *in vitro* and *in vivo* [478–481]. The MAO-inhibitory effect of toloxatone has been demonstrated in healthy volunteers and depressed patients [482] and preliminary studies suggest that toloxatone has antidepressant properties [483–486].

Cimoxatone (68) is also a potent reversible MAO inhibitor shown to be selective for type A MAO *in vitro* and *in vivo* [487,488], to be active in standard tests for antidepressants and to be devoid of anticholinergic activity [489]. In



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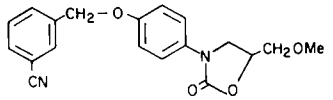

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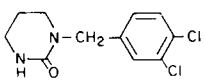
open studies in depressed patients, cimoxatone was effective against depression [490–493]. The results of controlled studies with both toloxatone and cimoxatone are awaited.

EU-2841 (69), 1-(3,4-dichlorobenzyl)-3,4,5,6-tetrahydro-2(1*H*)-pyrimidone, possesses antitetrabenazine activity and inhibits NA and 5-HT uptake in synaptosome preparations of rat forebrain with a potency similar to that of tricyclic agents. It is devoid of anticholinergic or antihistaminic activity [494].

DL-262 (70), 3-(1,2-diphenylethyl)-1,4,5,6-tetrahydro-*asym*-triazine, competitively inhibits DA and NA accumulation into rat synaptosomes, non-



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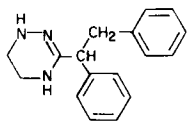
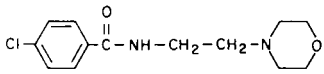


(70)

competitively inhibits 5-HT uptake [495] and reverses reserpine-induced ptosis and hypothermia [496].

Moclobemide (71) is a benzamide derivative, devoid of the hydrazine moiety, which is a short-acting reversible MAO inhibitor in rats with selectivity for the type A enzyme. Since MAO inhibition is weak when tested *in vitro* and when injected into the cerebral ventricles, the drug is likely to act mainly *via* metabolites [497,498]. In rat studies, moclobemide did not produce liver necrosis or abnormalities of liver function and may, therefore, not share the propensity of classical hydrazine MAO inhibitors to cause liver toxicity [499]. Moclobemide inhibits MAO in the human platelet [500]. Inhibition at sympathetic neurons was less pronounced and of shorter duration, from which it might be expected that moclobemide is less likely to cause side-effects such as the 'cheese reaction', a hypertensive crisis following the ingestion of tyramine-containing foods, or an adverse interaction with tricyclic therapy [501].



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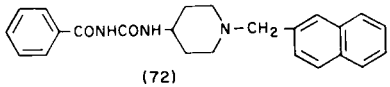
A sleep study in ten manic-depressed patients revealed that moclobemide improved sleep quality [502]. In an open 6 week study, the antidepressant action of moclobemide was evident on the 3rd day of treatment, with further increased activity subsequently. No serious side-effects were noted, except for transient mild agitation and insomnia [503]. Another preliminary open study involving 34 depressed patients suggested that moclobemide was effective and well tolerated [504]. In a randomized clinical study of 38 patients, moclobemide showed antidepressant activity similar to that of clomipramine but with less frequent, shorter lasting and less severe complaints of anti-cholinergic events, tremor and dizziness. Orthostatic hypotension was more pronounced on clomipramine [505,506]. In a double-blind parallel group study involving 30 depressed patients, moclobemide was reported to have clinical efficacy similar to that of desipramine. Anticholinergic side-effects associated with a significant increase in heart rate and a decrease in systolic blood pressure were seen in the desipramine group [507]. In a recent open study of ten geriatric depressed outpatients, Postma and Vranesic [508] reported that moclobemide diminished depressive symptoms, with only one patient complaining of dizziness. In a double-blind study in 25 depressed patients, moclobemide and amitriptyline had similar efficacy with no significant difference for side-effects [509]. The results of long term double-blind controlled studies involving a larger number of patients are awaited.

Panuramine (72) is a potent inhibitor of 5-HT uptake *in vitro* (rat brain synaptosome preparation) and *in vivo* (intraventricular uptake) with little or no effect on NA uptake. Selectivity for 5-HT uptake was greater than that of fluoxetine or zimelidine [510]. Clinical results are awaited.



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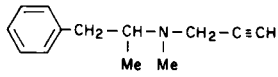

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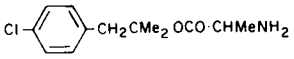
Selegiline, the laevorotatory isomer of deprenyl (73), is more potent than the dextrorotatory form as a specific, irreversible MAO B inhibitor [511–513a] which does not cause the cheese reaction [514]. Waldmeier and Felner [515] reported that, after repeated dosing of rats for 2–14 days, the inhibition of MAO by selegiline was less selective for the B enzyme. Selegiline may have other actions in addition to MAO B inhibition. For example, in rats it possesses indirect sympathomimetic activity [516], while Dzoljic, Bruinvels and Bonta [517] found that selegiline increased brain concentrations of DA without modifying concentrations of NA. A balanced crossover study in six volunteers showed that selegiline treatment was associated with a significant increase in the frequency of periods of wakefulness and Stage 2 sleep and a significant decrease in REM sleep and sleep Stages 3 and 4 [518]. In an open study, selegiline in combination with 5-HTP showed a significantly greater and faster onset of antidepressant action than placebo alone [519]. A positive relationship between mood improvement and the degree of platelet MAO inhibition in patients treated with selegiline was found. In an open clinical evaluation, selegiline was effective as an antidepressant and no postural hypotension was reported [520], although other workers [521] have failed to detect any antidepressant activity in patients. Preliminary results have suggested that selegiline may be superior to placebo as an antidepressant only in doses that inhibit MAO A [522]. Selegiline may be most effective in patients with neurotic depression and relatively ineffective in those with panic attacks and phobic symptoms [523]. Further clinical data are awaited.

The alanine ester, alaproclate (74), is a competitive 5-HT uptake inhibitor in murine midbrain slices which is less potent than clomipramine *in vitro* and *in vivo*. It is 100-times more potent in blocking 5-HT than NA uptake [524]. The structure-activity relationships of alaproclate analogues suggest that the presence of the geminal dimethyl moiety is critical for activity. Alaproclate potentiates the 5-HTP syndrome in animals and is reported not to have anticholinergic activity. In a 4 week open dose-ranging study involving 16

depressed patients, a 50% or greater improvement was reported in 14 patients. The effect was not dose-dependent with single daily oral doses between 100 and 400 mg. Except for one patient who withdrew after 3 weeks because of a lack of efficacy and possible adverse reactions, including inner tension, drowsiness, diarrhoea, headache and abdominal pain, no other patient reported side-effects [525].



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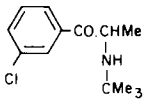

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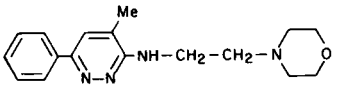
Bupropion (75), a chloropropiophenone, is the first of the monocyclic aminoketone class of drugs to be used clinically as an antidepressant. Pharmacologically it has no significant effect on MAO, NA or 5-HT, is more potent than imipramine and amitriptyline in inhibiting DA, but has only slight antihistaminic and anticholinergic activity. In 'classic' tests for antidepressant activity, bupropion is only active in reversing tetrabenazine sedation and in potentiating the effects of L-DOPA. In contrast to amitriptyline, bupropion reduces the intense motor activity and mortality induced by amphetamine [356,526,527].

Studies in healthy volunteers have shown that bupropion is devoid of central stimulant or sedative effects. Moreover, bupropion shows no cardiovascular or anticholinergic side-effects [528]. Several clinical investigations have revealed significant antidepressant activity compared with placebo and with efficacy and onset of action comparable with that of amitriptyline [529–538]. Its non-sedating activity makes bupropion particularly useful for patients with features of retardation as well as in the elderly. In addition to a lack of anticholinergic side-effects, long-term treatment does not appear to be associated with a change in body weight. Although rare, the most common side-effects associated with bupropion appear to be skin rash, agitation and nausea-vomiting.

Minaprine (76) is a 3-aminopyridazine reported to be as active or slightly less active than is imipramine against reserpine-induced ptosis, akinesia and hypothermia. It potentiates 5-HTP induced tremor and head twitches in mice. Unlike the 'classic' tricyclic antidepressants, minaprine does not potentiate yohimbine toxicity, antagonize apomorphine hypothermia or antagonize the effects of oxotremorine [539,540]. In low doses, minaprine stimulates central dopaminergic transmission by an unknown mechanism [541,542]. Thus it induces stereotypy and antagonizes neuroleptic-induced catalepsy in rats

without affecting locomotion, and blocks spontaneous turning in mice with a unilateral lesion of the striatum.

Minaprine is reported to antagonize the so-called inhibitory syndrome in neurotic and psychotic patients which is characterized by decreased spontaneous activity, reduction in basic drives, slowed thoughts, feelings of



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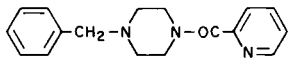
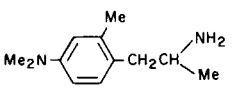


(77)

tiredness and social withdrawal [543]. Comparison of minaprine with placebo in a study involving 94 outpatients suffering from symptoms of 'inhibition', but without the presence of marked depression, revealed a favourable effect for minaprine as early as day 14 [544]. In patients suffering from masked depression, minaprine and nomifensine were reported to possess equivalent efficacy [545]. No difference in efficacy between minaprine and maprotiline was found by Radmayr [546] in a double-blind study involving 40 outpatients suffering from endogenous depression. The usefulness of minaprine in depression awaits results from placebo-controlled studies in which the number of patients allow a full statistical and clinical evaluation.

Piberaline (77), 1-benzyl-4-(2′-pyridylcarbonyl)piperazine, shows pharmacological activity in tests for antidepressants. In the Porsolt behavioural despair tests, its antidepressant activity was distinguishable from psychostimulant activity [547,548], while it agonizes the reserpine-induced DA depletion in certain brain areas, similarly to that by amitriptyline [549]. In contrast to tricyclic antidepressants, piberaline improves the acquisition of avoidance conditioning and delays the extinction of learned behaviour [550]. The results of clinical studies are awaited.

Amiflamine of FLA 336 (78) is a 4-dimethylamino-α-methylphenethylamine



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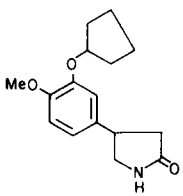

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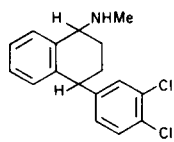


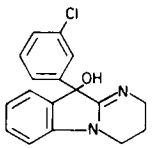
(80)

derivative which is a potent, selective and reversible MAO A inhibitor [551,552]. Further results with this potential antidepressant are awaited.

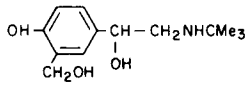
Rolipram (79) is a cyclic adenosine monophosphate- (AMP-) selective phosphodiesterase inhibitor which enhances NA transmission by direct stimulation of tyrosine hydroxylase and by an increase in neuronal activity *via* an undefined mechanism. DA transmission appears to be attenuated, possibly via GABA-mediated inhibition of DA neurons [553,554]. In an open study involving patients with endogenous depression, rolipram revealed antidepressive activity associated with an 'activating' action. The side-effects recorded such as sleeplessness and dry mouth were slight [555,556].

Sertraline (80), (+)-*cis*-(1*S*,4*S*)-*N*-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthylamine, is a selective competitive 5-HT uptake inhibitor active in the behavioural despair model which on chronic administration diminishes NA stimulated cyclic AMP formation in rat limbic forebrain [557]. In a study on cardiovascular function in nine healthy volunteers, neither sertraline nor mianserin differed from placebo with respect to systolic time intervals, vital signs or 24 h ECG [558]. The results of clinical studies are awaited.

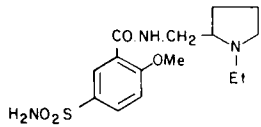
Ciclazindol (81) is a pyrimido[1,2-α]indole derivative with selective NA-uptake inhibitory activity [559]. In volunteers, ciclazindol antagonized pupillary and pressor responses to methoxamine and tyramine, suggesting a blocking activity on α_1-adrenoceptors as well as NA uptake inhibition [560,561]. Controlled studies have shown that ciclazindol has antidepressant activity similar to that of amitriptyline but with fewer anticholinergic side-effects and no cardiotoxicity [562,563]. The results of placebo-controlled studies involving a sufficient number of patients to show significance are awaited.



(81)



(82)



(83)

Salbutamol (82) is a phenylethylamine β_2-adrenoceptor agonist, used for the treatment of asthma, which has been reported to possess antidepressant activity in man [564–566]. Tachycardia and tremor occurred as side-effects but they diminished in intensity after 2–3 days. However, its penetration of the blood brain barrier is poor [567], so that the evaluation of a β-adrenergic

agonist for the treatment of depression awaits the development of a more lipid-soluble agent.

Sulpiride (83) is an orthopramide or *o*-onisamide which may exert a disinhibitory effect in depression. This action may be due to a preferential presynaptic agonist action on DA neurones causing an overactivation of cerebral DA postsynaptic receptors resulting in behavioural arousal and motor facilitation [568]. The results of controlled clinical studies are awaited.

Other recent interesting developments include the report that *S*-adenosyl-L-methionine, a methyl donor occurring naturally in the central nervous system, may be effective in patients with major depressive disorders [569], and the reports that ACTH 4-10 caused a reduction in 'depression' and 'confusion' as well as an increase in 'vigor' in patients with mild senile organic brain syndrome [570]. Further developments are awaited.

DISCUSSION AND CONCLUSIONS

Since the etiology of depression is still poorly understood, the experimental preclinical pharmacologist must rely on a battery of animal screening procedures to evaluate a new antidepressant. For many years these animal models were capable only of selecting molecules with amine re-uptake or MAO inhibitory properties. However, it is now clear that several compounds which are active in these tests are therapeutically inactive [571] while some clinically active 'atypical' antidepressants (i.e., trazodone and mianserin) are inactive in such 'classical' animal models. These screening tests still have an important rôle at the primary screening stage, since it is preferable to identify a 'false' positive than to miss a potentially useful compound.

The 'classical' animal models of depression fail to recognize that depression in man may be induced by environmental factors, that depression is a complex clinical entity and also that chronic treatment with antidepressants induces biochemical changes which are not observed after acute administration [572–575]. Moreover, animal models of psychiatric illness will always be limited by the assumption that human cognitive dysfunction may be mimicked in lower animals. Furthermore, although antidepressants show considerable affinity for central neurotransmitter binding sites, in no instance does affinity correlate with clinical efficacy. Indeed, the observation of binding to these sites may allow prediction of side-effects rather than clinical efficacy.

New and useful animal models have been developed which can detect in the laboratory those drugs already known to be clinically active. Coupled with this effort, attempts have been made to study mechanism(s) of action so as to help to elucidate fundamental therapeutic action and also the molecular pathology

of depression. The newer tests are based on observation of behaviour, psychoendocrine changes, sleep patterns and biochemical effects. Many of these new procedures recognize that chtonic, rather than acute, treatment is more likely to be relevant to therapeutic efficacy. Such models will, of course, eventually suffer from the disadvantage of tending to identify chemical analogues rather than novel classes of antidepressants. While the fundamental basis of clinical depression remains unknown, the validation of existing laboratory models and the development of more specific and selective animal tests must depend not only on scrupulous observation and methodological rigour but also on good communication between workers in laboratory and clinical research. Too often in the past, preclinical and clinical activities have been treated as separate entities.

Having expended much time and money on the preclinical development of a potentially useful antidepressant, an examination of its efficacy and side-effects profile is undertaken in humans. The establishment of internationally approved criteria for the diagnosis of depression has been an important recent technical advance helping to define the population under study. However, it must, unfortunately, be recognized that the clinical testing of new antidepressants is not a simple or a precise activity. For example, the techniques used currently to quantify depression are based entirely on subjective assessment by the physician and/or the patient. With such potentially important sources of imprecision, it may not be surprising that so many chemically diverse structures have been reported to possess antidepressant activity similar to that of those conventional 'tricyclic' agents, such as imipramine, which are used as the yardstick of clinical efficacy. Indeed, closer examination of many clinical reports reveals that the number of patients being investigated is relatively small and, hence, it seems likely that a statistical Type II sampling error has occurred. Although the availability of an adequate number of patients suitable for a carefully controlled study in a reasonable time-frame must be acknowledged as a significant logistic problem, the scientist at the bench may well be receiving misleading feedback, in many instances, from clinical colleagues. In addition to these methodological problems, it may be that some fundamental fault exists in the assessment procedures currently being used by clinical investigators. There is thus a need to identify and develop reliable and accurate objective procedures for the determination of antidepressant activity in the clinic based on pharmacological, physiological, behavioural or biochemical 'markers'. Not only would these provide more reliable data on which preclinical developments could be based, but also, from an ethical point of view, fewer patients would need to be exposed to therapeutically inactive compounds which may be as toxic as the effective antidepressants.

Unfortunately, none of the newly available compounds represents an advance in terms of overall antidepressant efficacy or possesses a quicker onset of therapeutic activity. These objectives remain major challenges for the future. Meanwhile, it is important to establish whether any of the effective newer antidepressants will help patients in whom standard treatments have failed. The finding that some of these newer antidepressants show anxiolytic activity within a few days should be exploited in general practice where it is often not practical to separate co-existing symptoms of depression and anxiety. In addition, an early anxiolytic effect may enhance compliance in those depressed patients who lack motivation. Compliance may also be enhanced by sedative antidepressants which are rapidly effective in improving insomnia, one of the cardinal symptoms of depression. The physician's current choice of effective antidepressants with either a 'sedating' or an 'alerting' profile means that patients presenting with 'agitated' or 'retarded' symptoms, respectively, may be treated more effectively. It might even be possible to use antidepressants in combination, with an 'alerting' drug being given in the morning and a 'sedating' drug being given in the evening to enhance sleep.

It is concluded that there have been useful advances in the drug treatment of depression in the last few years. Drugs are now available which are generally effective in most patients and which have less anticholinergic activity and are less cardiotoxic than the 'tricyclic' agents and MAO inhibitors. These new compounds may, therefore, be more suitable for use in the elderly, for treating depressed patients with glaucoma, and for those patients with pre-existing cardiac disease, but they also provide a greater margin of safety in overdose for a clinical condition where attempted suicide is a real possibility.

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5 Chemotherapeutic Agents for Herpesvirus Infections

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INTRODUCTION

Antiviral chemotherapy has come of age [1,2]. More than 25 years have elapsed since the first antiviral agent to be marketed (idoxuridine, IDU, 5-iodo-2′-deoxyuridine) was described. Subsequently, several other nucleoside ana-

logues, namely, cytarabine (Ara-C, cytosine arabinoside, 1-β-D-*arabino*-furanosylcytosine), trifluridine (TFT, trifluorothymidine, 5-trifluoromethyl-2′-deoxyuridine), vidarabine (Ara-A, adenine arabinoside, 9-β-D-*arabino*-furanosyladenine), thymine arabinoside (Ara-T, 1-β-D-*arabino*-furanosylthymine), 5-iodo-2′-deoxycytidine (IDC) and 5-ethyl-2′-deoxyuridine (EDU), made their appearance as antiviral agents. IDU, TFT, Ara-A, IDC and EDU have been licensed for clinical use (the latter two only in France and W. Germany, respectively): IDU for the topical treatment of herpetic keratitis (as 0.1% eyedrops) and herpetic skin lesions (at 10% in DMSO); TFT for the topical treatment of herpetic keratitis (as 1% eyedrops); Ara-A for the topical treatment of herpetic keratitis (as a 3% eye ointment) and for the systemic treatment of herpetic encephalitis (at 15 mg/kg per day for 10 days, intravenously); and IDC and EDU for the topical treatment of herpetic keratitis and mucocutaneous herpes lesions.

However, the field of antiherpes chemotherapy has been revolutionized by the advent of the second generation of antiherpes drugs, which, in contrast with the aforementioned 'first generation' antiherpes drugs, are much more selective in their antiherpetic activity. This second generation of antiherpes drugs includes such compounds as acyclovir [ACV, acycloguanosine, 9-(2-hydroxyethoxymethyl)guanine], bromovinyldeoxyuridine [BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine], fluoroiodoaracytosine [FIAC, 1-(2-fluoro-2-deoxy-β-D-*arabino*-furanosyl)-5-iodocytosine] and foscarnet (PFA, phosphonoformate). These compounds owe their selective antiherpes activity to a specific interaction with one or more virus-encoded enzymes, i.e., 2′-deoxythymidine (dThd) kinase and DNA polymerase; and, as a consequence of this interaction, compounds such as BVDU may easily reach therapeutic indexes of up to 10,000.

Foremost of the second generation of antiherpes drugs is acyclovir (aciclovir) [3,4], which has now been licensed and marketed for the treatment of a variety of herpesvirus infections, including herpetic keratitis (as a 3% eye ointment), primary genital herpes (5 × 200 mg/day for 5–10 days, perorally), and mucocutaneous herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections in immunosuppressed patients (up to 30 mg/kg per day for 7 days, intravenously). Furthermore, acyclovir has been found efficacious when given intravenously to patients with herpetic encephalitis or neonatal herpes, and it has also proven effective in the prophylaxis of genital herpes recurrences and recurrent HSV infections in bone-marrow and kidney transplant recipients. Its usefulness in the topical treatment of recurrent herpes labialis or genitalis has not been established unequivocally.

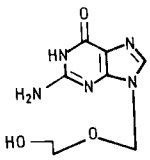
Independently of acyclovir (ACV), several other selective antiherpes agents

have been developed, namely, BVDU, FIAC and PFA and, in turn, ACV, BVDU, FIAC and PFA have served as the prototype or model compounds for the synthesis of a whole array of new congeners, namely DHPG (2′-NDG, BW-759, BIOLF-62), (*R*)-DHBG (buciclovir), 2′-nor-cGMP, 6-deoxy-ACV, 2,6-diamino-2′-NDG, BV*ara*U, carbocyclic BVDU, FMAU, FEAU and many others. Several of these new compounds appear to have advantages over acyclovir in that they are either more potent or selective *in vitro*, exhibit a broader spectrum of activity, appear more efficacious *in vivo*, or are better absorbed when administered perorally. In the present review article we will address (i) the general pathways for the synthesis of these compounds, (ii) their antiviral activity *in vitro*, (iii) their mechanism of antiviral action, (iv) their antiviral activity *in vivo*, and, (v), when applicable, the results from clinical studies.

SYNTHETIC PATHWAYS

ACYCLOVIR ANALOGUES

Acyclovir [9-(2-hydroxyethoxymethyl)guanine (1)] heralded a new generation of antiviral compounds, as it had a much higher efficacy and much lower toxicity than compounds previously available [5,6]. Following the discovery of this compound, many other acyclic nucleosides have been synthesized,



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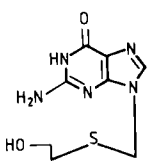

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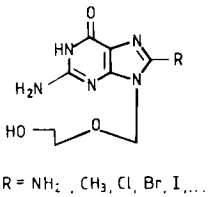

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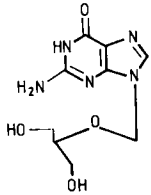
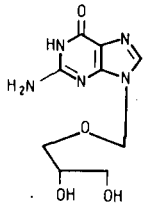


(4)

including 5-substituted 1-[(2-aminoethoxy)methyl]uracils [7], 1-[(2-hydroxyethoxy)methyl]pyrimidines [8,9], 9-[(2-aminoethoxy)methyl]guanine and other nitrogen isosteres of acyclovir [10] as well as a variety of mono-, bi- and tricyclic derivatives [11]. These compounds exhibited little or no activity against herpes simplex virus or other DNA and RNA viruses. Considering the great variety of acyclovir analogues which have been synthesized, we will discuss mainly those compounds that at least initially showed some promise. Thus, we are restricted almost entirely to guanine derivatives (or compounds which can be converted enzymatically into guanine derivatives). Apart from

acyclovir itself (1), these include 9-(2-hydroxyethylthiomethyl)guanine (2) [12], 8-substituted derivatives of acyclovir (3) [13], BW-759 (also known as DHPG) [14], 2′NDG [15,16], BIOLF-62 [17,18] which is chemically 9-{[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl}guanine (4), (*RS*)-iNDG (as well as the separate *R*- and *S*-enantiomers of iNDG), which corresponds to 9-(2,3-dihydroxy-1-propoxymethyl)guanine (5) [19–21], 9-{[1,3-dihydroxy-2-(hydroxymethyl)-2-propoxy]methyl}guanine (6) [22], 9-(4-hydroxybutyl)guanine (HBG) (7) [23,24], (*RS*)-9-(3,4-dihydroxybutyl)guanine (DHBG) (8) (as well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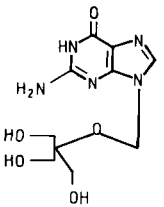
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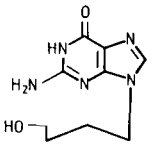
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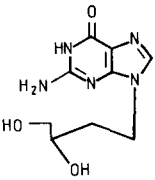
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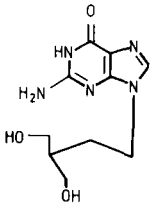
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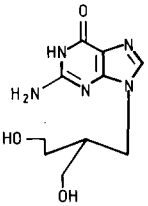
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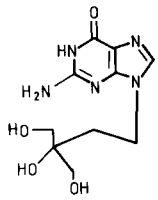
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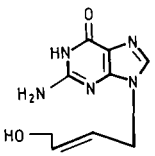
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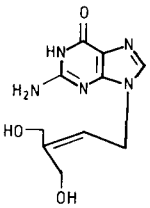
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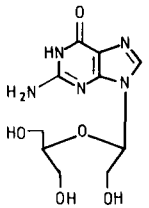
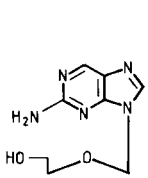

(14)

as the separate *R*- and *S*-enantiomers of DHBG, of which (*R*)-DHBG corresponds to buciclovir (BCV) [24,25]), and several new derivatives of HBG and DHBG (9–13) [26]. Of the butenol analogues (12), the *cis*-isomer proved active but the *trans* was inactive [26]. 2′,3′-*seco*-Guanosine (14), made by the action of periodate on guanosine followed by sodium borohydride reduction,

is another example of the many acyclic guanosine analogues which proved entirely inactive [12].

Metabolic precursors of acyclovir include 6-deoxyacyclovir [A515U, 2-amino-9-(2-hydroxyethoxymethyl)purine (15)], which is activated by xanthine oxidase [27], and A134U [2,6-diamino-9-(2-hydroxyethoxymethyl)purine (16)] [28] or 2-amino-6-chloro-9-(2-hydroxyethoxymethyl)purine (17), both of which are converted to ACV by adenosine deaminase [29]. Similarly, 2,6-diamino-9-{[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl}purine (18) is assumed to be active as an antiherpetic agent due to prior enzymatic deamination to DHPG [30].



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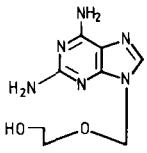

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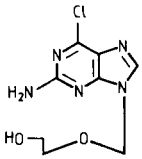

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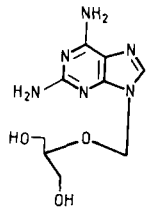
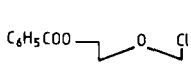


(18)

The chemical synthesis of acyclovir and its analogues can be considered together, as all are made by reacting a suitable guanine precursor with an activated compound which is to form the side-chain at N-9 of the purine ring. The only problem is either trying to direct the reaction regioselectively to N-9 rather than to N-7 or to separate the resulting isomeric forms.

The original preparation of acyclovir (1) [5,31] involved the condensation of 2,6-diaminopurine with 1-benzoyloxy-2-chloromethoxyethane (19), which gives the N-9 substituent which can then be converted into acyclovir by a standard series of reactions used for converting 2,6-diaminopurine into 2-amino-6-hydroxypurine (guanine). The trimethylsilyl derivative of guanine has also been reacted with compound (19) in the presence of triethylamine followed by deblocking to give the required compound [31]. Alternatively, compound (19) has been reacted with guanine in dimethyl sulphoxide in the presence of sodium hydride to generate the anion [32].



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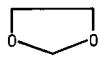


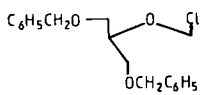
(20)

1,3-Dioxolane (20) has been reacted with trimethylsilyl iodide to give iodomethyl trimethylsilyloxyethylether (21) which is then reacted with a purine anion at −63° C [33–35]. Alternatively, 1,3-dioxolane has been reacted with acetyl bromide to give (2-acetoxyethoxy)methyl bromide (22) which can be reacted with trimethylsilylated purines to give the desired product [36].

Several methods of preparation of DHPG (4) have been reported [14–18,22], but all involve the condensation of N^2,9-diacetylguanine with

(21)

(22)



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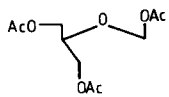
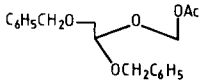


(24)

either 1,3-dibenzyloxy-2-chloromethoxypropane (23) [17,18,22], 2-acetoxymethoxy-1,3-diacetoxypropane (24) [14,15] or 1,3-di-*O*-benzyl-2-acetoxymethoxyglycerol (25) [14]. The cyclic GMP analogue, 2′-nor-cGMP (26), has recently been synthesized by the reaction of 2′NDG with phosphorus oxychloride in triethyl phosphate [37]; unlike 2′NDG, 2′-nor-cGMP does not owe its activity against HSV to the action of virus-specified thymidine kinase.



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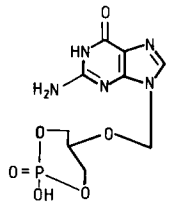


(26)

In addition to 2′-nor-cGMP, the cyclic pyrophosphate of 2′NDG and the cyclic phosphates of (*R*)- and (*S*)-iNDG have also been synthesized [38].

The synthesis of DHBG (8) is performed by condensation under basic conditions of 2-amino-6-chloropurine and an ω-haloalkyl derivative, but no details have been published [24].

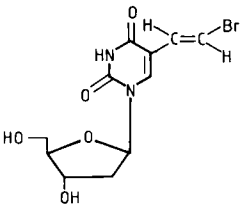
Radiolabelled compounds have been made for metabolic studies. These present little problem; they usually involve starting with a suitably labelled purine derivative.

In order to increase the solubility or alter the pharmacokinetics, aminoacyl esters [39], phospholipid conjugates [38,40] and polymer-linked derivatives [41] of acyclovir have been synthesized, although undoubtedly several other

derivatives must have been prepared, but their syntheses have never been published.

BROMOVINYLDEOXYURIDINE ANALOGUES

The synthesis of antiviral properties of 5-vinylpyrimidine nucleoside analogues have recently been the subject of a comprehensive review [42] and, here, emphasis will be placed upon the parent compound, BVDU (27), and the vinyl



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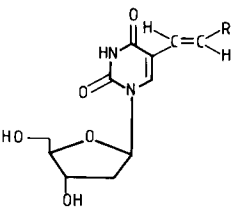
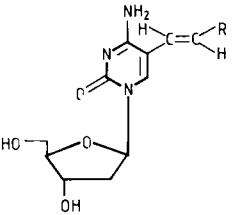


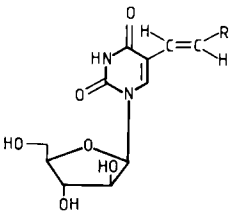
R = H, F, Cl, I, CH_3, CF_3, ...

(28)



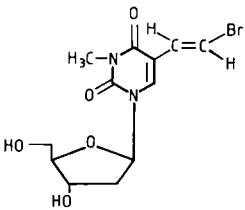
R = H, Br, I, ...

(29)



R = H, Br, Cl, ...

(30)



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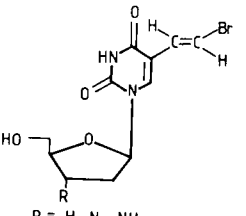
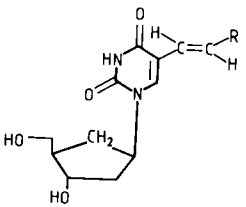


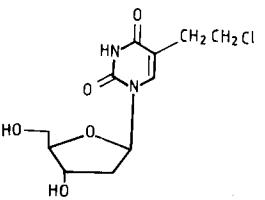
R = H, N_3, NH_2, ...

(32)



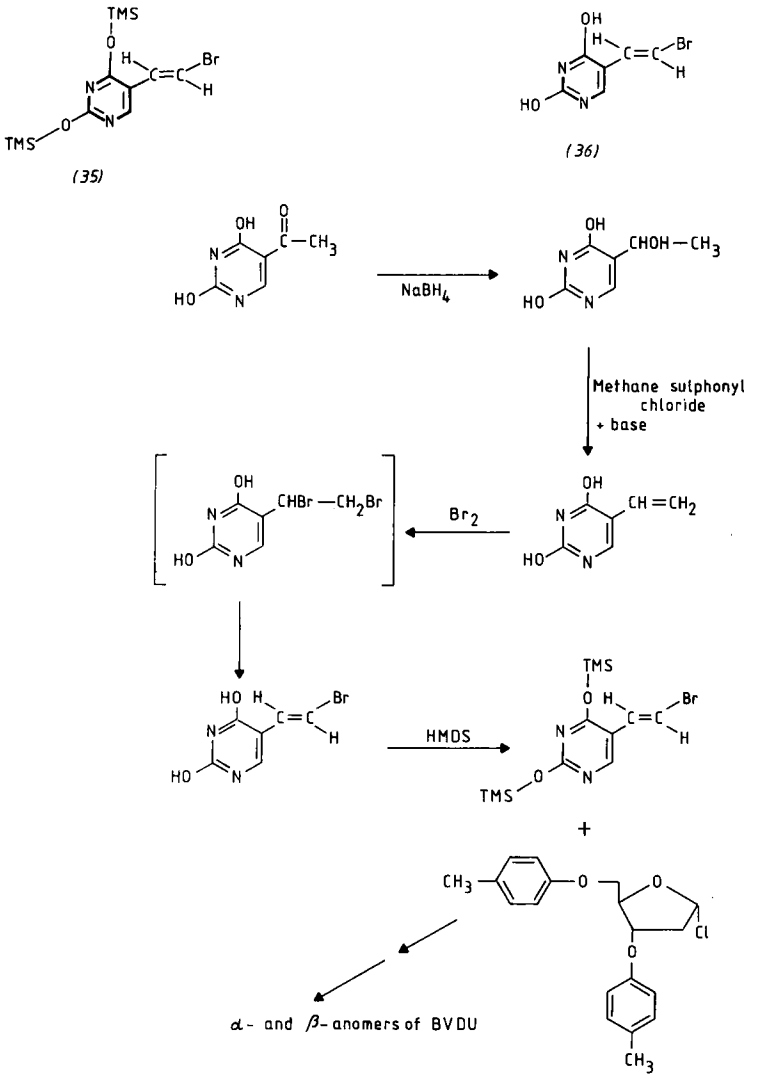
R = Br, I

(33)



(34)

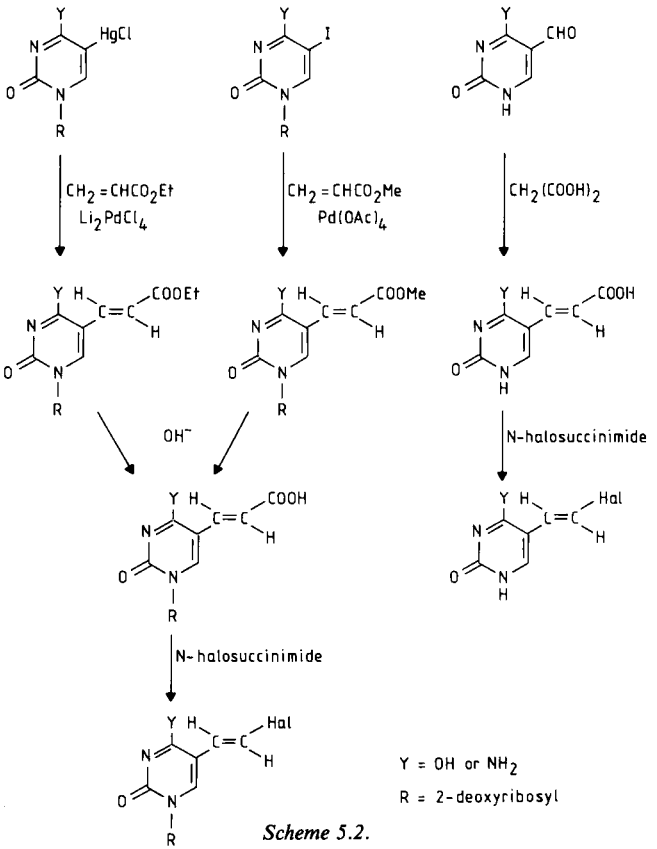
C^2-substituted (28)-, cytidine (29)-, arabinosyl (30)-, N^3-substituted (31)-, 3′-substituted (32)- and carbocyclic (33) derivatives thereof. The 5-(2-halogenoalkyl)pyrimidine nucleoside analogues [43], of which the 2-chloroethyl derivative CEDU (34) emerged as the most effective HSV inhibitor [44], can be regarded as BVDU analogues with a saturated C^5 side-chain.



Scheme 5.1.

There is a great variety of methods available for the synthesis of 5-substituted pyrimidine nucleoside analogues. They can be made either by condensing a suitably protected heterocyclic base with a sugar derivative or by transformation of a preformed nucleoside. The advantage of the latter method is that the problem of separating the α- and β-anomers usually produced in a 2′-deoxynucleoside condensation reaction is avoided, although recent research has shown that predominantly, if not exclusively, β-2′-deoxynucleoside can be produced when care is taken in choosing the proper experimental conditions [45].

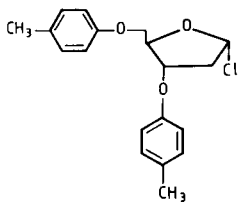
(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU) (27) was first synthesized by condensing the bistrimethylsilyl derivative (35) of (*E*)-5-(2-bromovinyl)uracil (BVU) (36) with 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (37) (*Scheme 5.1*) [46], followed by anomer separation; but condi-



Scheme 5.2.

tions have recently been reported under which only the β-nucleoside is formed [45]. The base (36) had been prepared by the addition of bromine (followed by spontaneous elimination of HBr) to 5-vinyluracil [47]. However, the yield from readily available starting materials was very low (about 5%), and other methods, including that used on a kilogram scale, have usually started with a preformed nucleoside. The 5-(2-halogenovinyl)uracils and -cytosines are conveniently prepared from the corresponding 5-formylpyrimidines and malonic acid. The intermediate 5-(2-carboxyvinyl)pyrimidines give the desired products on reaction with *N*-halosuccinimide (*Scheme 5.2*) [48].

The first commercially viable synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine started with 5-iodo-2′-deoxyuridine or 2′-deoxyuridine and involved the formation of intermediate palladium compounds following the pioneering work of Heck [49] and applied to the nucleoside field by Bergstrom and Ruth [50]. Either lithium palladium chloride is allowed to react with a 5-chloromercurinucleoside [51], or the 5-palladium intermediate can be made *in situ* by the action of a catalytic amount of palladium acetate in the presence of triethylamine and triphenylphosphine on a 5-iodonucleoside (*Scheme 5.2*). Thus, starting from 5-iodo-2′-deoxyuridine and methyl acrylate and using



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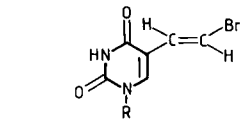
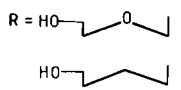




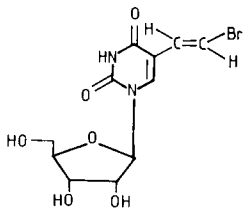
(38)

palladium acetate, kilogram quantities of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine can be made in three steps with an overall yield of around 40% (*Scheme 5.2*). The same procedure can be used for the preparation of the other (*E*)-5-(2-halovinyl)-2′-deoxyuridines (28) and 2′-deoxycytidines (29) [48].

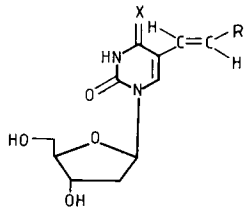
A selective bromination procedure for the preparation of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine starting from 5-ethyl-2′-deoxyuridine has been described by Bärwolff and Langen [52]. For this method a yield of 52% (from 3′,5′-di-*O*-acetyl-5-ethyl-2′-deoxyuridine) is claimed [53].

At the nucleoside level, a Wittig reagent has been used so that by treatment of the triphenylphosphonium salt of 1-β-D-*arabino*-furanosyl-5-chloromethyluracil with butyllithium and paraformaldehyde, the corresponding 5-vinylnucleoside was isolated, and then converted to the *ara*-nucleoside (30) of (*E*)-5-(2-bromovinyl)uracil in the usual way [54,55].

As in the case of acyclovir, various analogues of BVDU have been made and the syntheses of some of these have been discussed elsewhere [42]. In all cases, the corresponding cytidine derivatives can be made from the uridine compounds using the method described by Sung, which proceeds *via* a triazalo intermediate [56]. Thus, the *Z*-isomer of BVDU [57], BVDU acyl esters [58,59], N^3-methyl-BVDU (31) [60], O^2,5′-anhydro–BVDU [61], 3′-substituted BVDU derivatives (32) [62], carbocyclic BVDU (33) [63], acyclic (2-hydroxyethoxymethyl, 4-hydroxybutyl) derivatives of BVDU (38) [13,64], the ribonucleoside counterpart of BVDU, BVRU (39) [65], and the (2-deoxy-2-fluoro-β-D-*arabino*-furanosyl)uracil (FBVAU) and -cytosine (FBVAC) (40) derivatives [66,67] have all been reported.



(39)

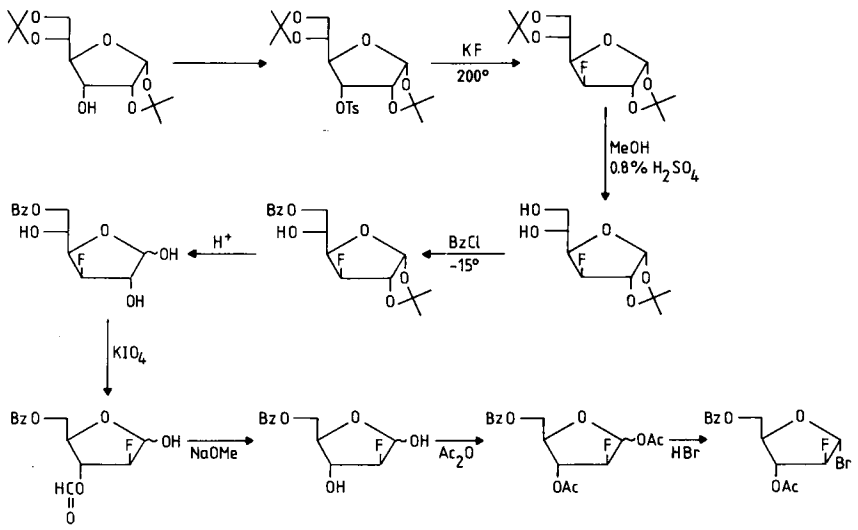


(40)

Radiolabelled BVDU has also been prepared for metabolic studies. [2-^{14}C]BVDU was prepared from 5-ethyl-2′-[2-^{14}C]deoxyuridine [53], and the carbon of the vinyl side-chain has been labelled using [^{14}C]methyl acrylate (R.L. Dyer, personal communication). The synthesis of [^{82}Br]BVDU has been reported [68]. (*E*)-5-(2-[^{125}I]iodovinyl)- and (*E*)-5-(2-[^{131}I]iodovinyl)-2′-deoxyuridine have also been synthesized [68–70].

2′-FLUORINATED PYRIMIDINE NUCLEOSIDE ANALOGUES

Several years ago, the synthesis (in nine steps) of a suitably protected 2-deoxy-fluoro-*arabino*-furanosyl bromide was reported (*Scheme 5.3*) [71], a key intermediate in the subsequent syntheses of a number of 1-(2-deoxy-2-fluoro-β-D-*arabino*-furanosyl)pyrimidines, some of which possess potent antiviral activity. From the many nucleosides made containing this sugar moiety, most attention has been focussed on three compounds: 1-(2-deoxy-2-fluoro-β-D-*arabino*-furanosyl)-5-iodocytosine (41) (FIAC), 1-(2-deoxy-2-fluoro-β-D-*arabino*-furanosyl)-5-methyluracil (42) (FMAU) and 1-(2-deoxy-2-fluoro-β-D-*arabino*-furanosyl)-5-iodouracil (FIAU) (43) [72]. FMAC (44),



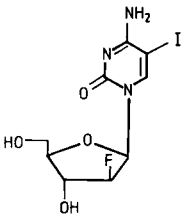
Scheme 5.3.

FEAU (45) and FEAC (46) also exhibit interesting antiviral properties [67], as do the α-monofluoro- and α,α-difluoro derivatives of FMAU, termed F-FMAU (47) and F_2-FMAU (48), respectively [73]. In addition to the 2′-fluorinated compounds, some 1-(2-deoxy-2-chloro-β-D-*arabino*-furanosyl)- and 1-(2-deoxy-2-bromo-β-D-*arabino*-furanosyl)pyrimidine nucleosides have also been synthesized [74].

The difficulty in synthesizing the carbohydrate moiety of FIAC, FMAU and its congeners was recognized many years ago [75,76], and, although the present synthesis is no doubt routine for a leading carbohydrate research laboratory, it has meant that, to the best of our knowledge, nobody else has ever repeated this synthesis, nor has anyone been able to improve upon it. Thus, all the chemical work published comes from one laboratory.

As mentioned above, the key intermediate is the suitably protected sugar 3-*O*-acetyl-5-*O*-benzoyl-2-deoxy-2-fluoro-D-*arabino*-furanosyl bromide which, when condensed with an heterocyclic base, produces almost exclusively the β-nucleoside so that, when the sugar is available, the synthesis of a range of nucleosides presents few problems [66,67].

The synthesis of the sugar moiety starting from di-*O*-isopropylidine-α-D-*allo*-furanose is given in *Scheme 5.3*. The corresponding 2-bromo-2-deoxy and 2-chloro-2-deoxy sugars have also been prepared, as mentioned above [74].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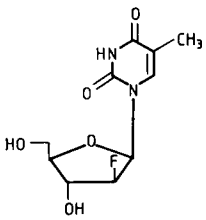
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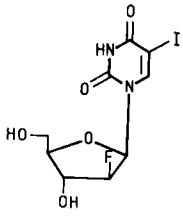
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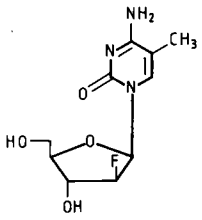
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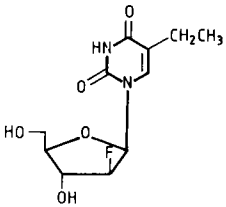
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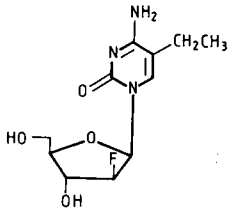
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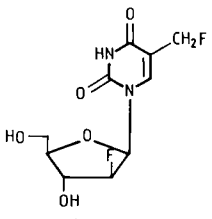
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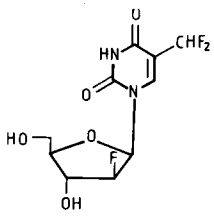
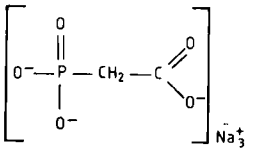

(48)

Radiolabelled material can be readily made available providing that the label is in the heterocyclic ring. Thus, [^{125}I]FIAC, [2-^{14}C]FIAC, [^{11}C]FMAU and [2-^{14}C]FMAU have all been synthesized using the correspondingly labelled heterocyclic base in the condensation reaction [77–80].

PHOSPHONOFORMATE (FOSCARNET)

The chemical synthesis of phosphonoformate (PFA) and phosphono compounds in general goes back to the early history of organic chemistry. Much of the methodology was established by the Russian school of phosphorus chemistry in the early years of this century [81]. The first reported synthesis of phosphonoacetate (49) and phosphonoformate (50) was by Nylér in 1924 [82].



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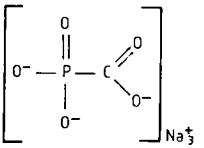
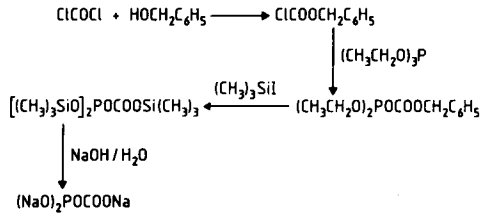


(50)

Phosphonoacetate is commercially available and a recent publication [83] outlines a one-pot synthesis of phosphonoformate which is suitable for making radiolabelled material. Following the normal Arbuzow reaction (*Scheme 5.4*),



Scheme 5.4.

iodotrimethylsilane is used to dealkylate both phosphonic esters [84] and carboxylic benzyl esters [85] under mild conditions. Previously, the use of basic [86] or acidic hydrolysis [82] conditions had given very low yields.

ANTIVIRAL ACTIVITY *IN VITRO*. STRUCTURE-ACTIVITY RELATIONSHIP

Routinely, potential antiherpes compounds are examined for their inhibitory effects on HSV-1 and HSV-2, and only the most promising ones are further investigated for their inhibitory effects on VZV, CMV (cytomegalovirus) and EBV (Epstein-Barr virus). *Table 5.1* summarizes the 50% inhibitory doses (ID_{50} value) obtained with compounds (1)–(50) in the various herpesvirus assay systems.

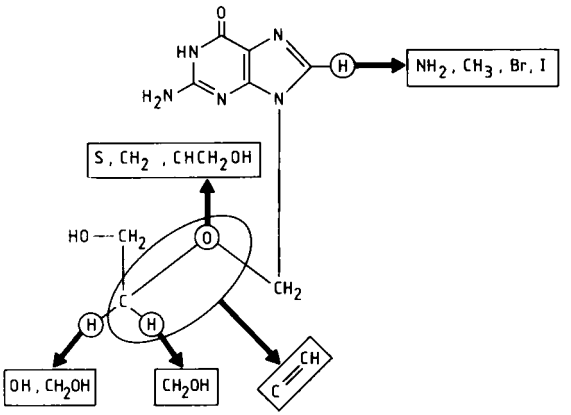
As the most potent inhibitors of HSV-1, BVDU (27) and the 2′-fluorinated pyridimine nucleoside analogues (41)–(46) with ID_{50} values in the range of 0.003–0.03 μM emerged. The latter were equally inhibitory to HSV-2, whereas BVDU and its analogues (28, 29, 30, 32 and 33) were much less inhibitory to HSV-2 than to HSV-1. The reason for the differential susceptibility of HSV-1 and HSV-2 towards BVDU has been discussed previously [42].

Table 5.1. COMPARATIVE POTENCY OF SELECTED ANTIHERPES COMPOUNDS AGAINST HSV-1 AND HSV-2 *IN VITRO*

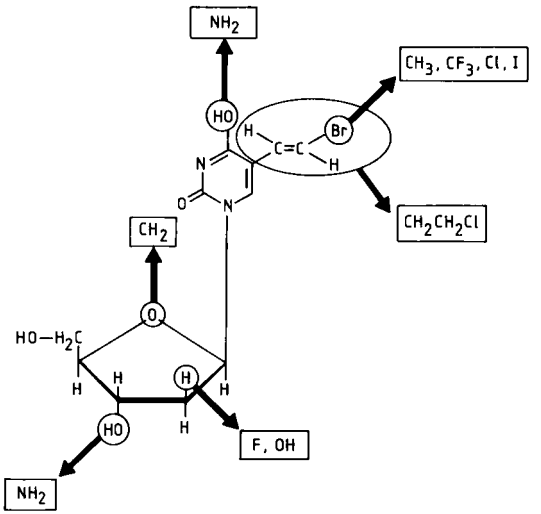
Compound	*Minimum inhibitory dose: ID_{50} value (μM)*					*References*[a]
	HSV-1	*HSV-2*	*VZV*	*CMV*	*EBV*	
1 ACV	0.1	0.1	4	10–100	0.3	[87,92]
2	0.5	–	–	–	–	[12]
3	1	1	–	–	–	[13]
4 DHPG	0.2	0.3	5	0.1–1	0.05	[87,92]
5 (*S*)-iNDG	1.7	3.5	>90	>90	–	[19,38]
5 (*R*)-iNDG	90	90	–	>90	–	[19,38]
6	19	–	–	–	–	[22]
7 HBG	2	8	–	–	–	[23]
8 (*S*)-DHBG	8	20	–	–	–	[88]
8 (*R*)-DHBG, BCV	1	4	–	–	–	[88]
9	0.2	5	–	–	–	[26]
10	0.1	3	0.4	–	–	[26]
11	20	–	–	–	–	[26]
12 *cis*	0.6	–	–	–	–	[26]
12 *trans*	>100	–	–	–	–	[26]
13	3	–	–	–	–	[26]
14	>250	–	–	–	–	[12]
15 6-deoxyacyclovir	>225	–	–	–	–	[27]
16	10	–	–	–	–	[6]
17	10	100	–	–	–	[13]
18	97	390	–	–	–	[38]
26 2′-nor-cGMP	10–20	1–2	1–2	2	–	[37,38]
27 BVDU	0.003	3	0.007	10	0.06	[87,92]
28 VDU	0.3	0.3	–	–	–	[42]
29 BVDC	0.2	30	0.07	–	–	[87]
30 BV*ara*U	0.1	100	0.004	–	–	[87]
31	20	–	–	–	–	[89]
32 3′-NH_2-BVDDU	0.3	100	0.1	–	–	[90]
33 C-BVDU	0.1	30	0.1	–	–	[63]
34 CEDU	0.5	2	0.1	100	–	[44]
36 BVU	20	>2000	–	–	–	[65]
38	>1000	>1000	–	–	–	[13,64]
39	20	>2000	–	–	–	[65]
40 FBVAU	0.067	0.13	–	–	–	[67]
40 FBVAC	0.068	0.037	–	–	–	[67]
41 FIAC	0.024	0.014	0.01	0.3	0.005	[66,92–94]
42 FMAU	0.01	0.023	0.01	0.1	0.006	[67,92–94]
43 FIAU	<0.3	–	–	0.2	0.005	[72,92–94]
44 FMAC	0.006	0.026	–	0.2	–	[67,94]
45 FEAU	0.024	0.24	–	45	–	[67,93]
46 FEAC	0.035	0.32	–	–	–	[67]
47 F-FMAU	0.21	0.18	–	–	–	[73]
48 F_2-FMAU	0.48	1.04	–	–	–	[73]
49 PAA	20	<100	<100	<100	<100	[26,91]
50 PFA	10	<100	<100	<100	<100	[26,91]

[a] Only the most representative references are indicated.

The most potent inhibitors of VZV were BV*ara*U (30), BVDU (27) and IVDU (28) [42], followed by FIAC (41) and FMAU (42). It is noteworthy that these compounds inhibited the replication of VZV at a concentration that was 500- to 1000-fold lower than the ID_{50} of ACV (1) or DHPG (4) for VZV.

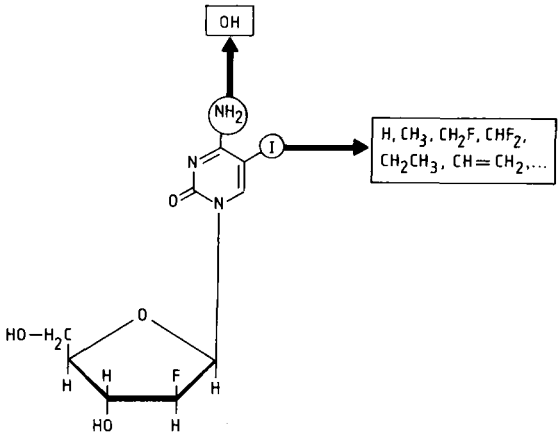


Scheme 5.5.

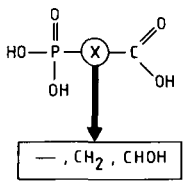


Scheme 5.6.

The inhibitory effects of the ACV, BVDU and FIAC analogues on HSV-1, HSV-2 and VZV replication were achieved at concentrations that were significantly lower than the concentrations required to affect normal cell growth or metabolism. Quite often, selectivity indexes of 1,000–10,000 were noted, and in their activity against VZV, BVDU, IVDU and BV*ara*U reached even a selectivity index of about 50,000 [42].



Scheme 5.7.



Scheme 5.8.

The structural modifications which appeared compatible with significant activity against HSV and VZV are presented in *Scheme 5.5* (ACV analogues), *Scheme 5.6* (BVDU analogues), *Scheme 5.7* (FIAC analogues) and *Scheme 5.8* (PFA analogues). These schemes illustrate the portions of the molecules which are tolerant to chemical substitutions without (significant) loss of antiviral activity. Further details on the structural parameters that govern the antiviral activity of ACV analogues, BVDU analogues and PFA analogues can be found

in the reviews by De Clercq and Walker [42], Öberg [95] and a team of eleven authors [26]. However, too few congeners have been synthesized, especially within the FIAC series, to permit a full structure-function analysis.

That steric factors play an important rôle in the antiviral activity of the ACV, BVDU and their analogues is clearly demonstrated by (i) the inactivity of *trans*, as compared with the *cis*, butenol derivative of ACV (12), (ii) the much greater activity of the *E* (*Entgegen*) than of the *Z* (*Zusammen*) isomers of the BVDU analogues [42], and (iii) the marked differences in activity between the *S*- and *R*-enantiomers of iNDG (5) and DHBG (8).

The activity of ACV, BVDU and FIAC and their analogues against HSV-1, HSV-2 and VZV is dependent upon the action of a virus-induced dThd(dCyd) kinase. As a consequence, these compounds are not active against dThd(dCyd) kinase-deficient (TK^-) mutants of HSV or VZV. A notable exception to this rule is 2′-nor-cGMP (26), which has activity against a broad range of DNA viruses, including those that do not encode for a specific dThd(dCyd) kinase [37].

CMV is not known to encode for its own dThd(dCyd) kinase. Yet, it is quite susceptible to the inhibitory effects of the 2′-fluorinated pyrimidine nucleoside analogues [93,94] and DHPG [14,16,96–99]. The ID_{50} of DHPG for CMV is about 100-fold lower than that of ACV [99]. The anti-CMV action of DHPG is highly specific, but reversible; thus CMV replication resumes upon removal of the drug [97].

EBV is also sensitive to inhibition by DHPG [100], and in this case the inhibitory effect seems to persist following drug removal. When a series of nucleoside analogues were compared for their inhibitory effects on the replication of EBV, the relative potencies were FIAC = FIAU > FMAU > DHPG > BVDU > ACV and their selectivity indexes were BVDU > DHPG > FIAC > ACV > FIAU > FMAU [92].

The antiviral activity spectrum of BVDU and DHPG is not limited to the human herpesviruses; it also extends to herpesviruses of veterinary importance such as pseudorabies virus (suid herpesvirus type I, Aujeszky's disease virus), infectious bovine rhinotracheitis virus (bovid herpesvirus type I), simian varicella virus and equine rhinopneumonitis virus (equid herpesvirus type I) [42,101,102].

Of great importance also is the recent finding that PFA inhibits the replication of human T-cell lymphotropic virus type III (HTLV-III) *in vitro* at a concentration (100–400 μM) which is readily attainable in the serum [103]. The inhibitory effect of PFA on HTLV-III replication is likely to be due to an inhibition of the HTLV-III associated reverse transcriptase, since PFA has long been recognized as a potent reverse transcriptase inhibitor [95,104].

MECHANISM OF ANTIVIRAL ACTIVITY

Although the ACV, BVDU and FIAC analogues belong to structurally different classes, they exhibit remarkable similarities in their mechanism of action [87]. With the exception of 2′-nor-cGMP (26), they all require the interaction with a virus-specified dThd(dCyd) kinase which converts the compound to its monophosphate form (first phosphorylation step). The acyclic guanosine analogues (i.e., ACV, DHPG) are then further phosphorylated by a cellular GMP kinase (second phosphorylation step), whereas for BVDU, FIAC and their analogues the second phosphorylation step is carried out by the same enzyme that was responsible for the first phosphorylation step, and which can therefore be regarded as a dThd(dCyd)-dTMP kinase. The primary target for the antiviral activity of the antiherpes compounds would be the DNA polymerization reaction, and at this level the nucleoside triphosphate analogues may act as either inhibitor or substrate [87]. PFA does not appear to need any previous phosphorylation; it can interact directly with the DNA polymerase.

The kinetic constants for the enzymatic steps involved in the mechanism of action of the antiherpes compounds are listed in *Table 5.2*. From *Table 5.2* it is clear that all compounds listed have a much greater affinity for the HSV-1 dThd(dCyd) kinase than for the cellular enzyme. This explains their preferential phosphorylation by the virus-infected cell and contributes to their selectivity as antiviral agents. BVDU may gain a further increase in selectivity from the second phosphorylation step, since BVDU 5′-monophosphate has a higher affinity for the viral dTMP kinase than for the cellular dTMP kinase [108].

This contrasts with FIAC 5′-monophosphate, which has a poor affinity for both cellular and viral dTMP kinase, and FMAU-MP and FIAU-MP, which have a much greater affinity for the cellular than for the viral enzyme [108]. *In vivo* metabolic studies have indicated that FIAC is rapidly metabolized, by deiodination and deamination, to FAC, FIAU and FAU [113,114]. Even FMAU can be formed metabolically from FIAC [115]. In HSV-1-infected cells, FIAC would be phosphorylated by the viral dThd(dCyd) kinase to FIAC-MP [116], and, following deamination, be processed as a dThd analogue (117). The active form of FIAC would thus correspond with the 5′-triphosphate of its metabolites, FIAU or FAU.

Since the initial phosphorylation by the viral dThd(dCyd) kinase is restricted to virus-infected cells, triphosphate analogues of ACV, DHPG, BCV, BVDU and others will be formed only in virus-infected cells and not (to a detectable extent) in uninfected cells. With the exception of 3′-NH_2-BVDDU (32) triphosphate, all other triphosphate analogues (1,4,7,8,27,30,41–43) as well as PFA have a much greater affinity for HSV-1 DNA polymerase than for cellular DNA

Table 5.2. PRINCIPAL STEPS IN THE MECHANISM OF ANTIVIRAL ACTION OF SELECTED ANTIHERPES COMPOUNDS

Compounds	*Kinetic constants: K_i (μM)*[a]							*References*
	First phosphorylation step		*Second phosphorylation step*			*DNA polymerase*		
	dThd (dCyd) kinase		*GMP kinase*	*dTMP kinase*				
	HSV-1	*Cytosol*	*Cellular*	*HSV-1*	*Cellular*	*HSV-1*	*Cellular α*	
ACV (1)	173	>250	316	–	–	0.0014	1.1	[16,26]
						0.07	0.38	[105]
						0.006	0.37	[106]
DHPG (4)	13	>250	22	–	–	0.05	2.5	[16,26,106]
						0.29	23.8	[105]
HBG (7)	2	>250	–	–	–	0.12	34	[26]
BCV (8)	1.5	>250	–	–	–	0.76	204	[26]
BVDU (27)	0.24	>150	–	26	400	0.25	3.6	[42,107–109]
						0.068	3.6	[110]
BV*ara*U (30)	0.94	>100	–	–	–	0.14	0.32	[42,111]
						0.013	0.29	[110]
3′-NH_2-BVDDU (32)	1.9	> 50	–	–	–	0.13	0.1	[90]
FIAC (41)	1.09	>100	–	320	>900	0.26	2.7	[108,111,112]
						0.028	1.25	[110]
FMAU (42)	0.59	>100	–	495	0.3	0.048	1.2	[108,110,111]
FIAU (43)	0.68	26	–	132	1.4	–	–	[108,111]
PFA (50)	–	–	–	–	–	0.3	40	[95]

[a] For dThd kinase, dTMP kinase and DNA polymerase; for GMP kinase the K_m (μM) is indicated.

polymerase (*Table 5.2*), the difference reaching 2 to 3 orders of magnitude in some cases. These differential effects of the triphosphate analogues at the DNA polymerase level may further contribute to their selectivity as antiviral agents.

That ACV, DHPG, BVDU and their congeners are effectively phosphorylated to their triphosphates in HSV-1-infected cells has been amply demonstrated [118,119]. In fact, DHPG-TP persists for a much longer time in these cells than ACV-TP, and this finding may be a key factor to the superior antiviral potency of DHPG *in vivo*.

Of the compounds listed in *Table 5.2*, some are incorporated into DNA of HSV-1 infected cells (i.e., (1,4,27,41–43)) [87], whereas others (i.e., (8)) are seemingly not [26]. ACV serves as a chain terminator when incorporated into DNA, whereas DHPG would enter both internal and terminal linkages [120,121]. Like BVDU itself, carbocyclic BVDU (33) is readily incorporated into both viral and cellular DNA of HSV-1-infected cells [69]. Not serving as an alternate substrate for DNA polymerase [110], BV*ara*U (30) is probably not incorporated into DNA, or, at the most, incorporated at the 3′-terminal, thus leading to chain termination [122]. Considering the widely different patterns of incorporation of the antiherpes compounds into DNA, it is at present difficult to assess to what extent this incorporation contributes to their antiviral activity.

That the mechanism of antiviral activity of ACV, BVDU and their analogues is essentially based upon a specific interaction with three virus-encoded enzymatic functions [dThd(dCyd)) kinase-dTMP kinase-DNA polymerase] is further supported by the existence of drug-resistant HSV mutants with mutations in either of the following loci (i) dThd(dCyd) kinase, (ii) dTMP kinase and (iii) DNA polymerase [123–125]. Of particular clinical importance may be dThd(dCyd) kinase mutants with altered substrate specificity: these are mutants expressing a dThd(dCyd) kinase which is able to phosphorylate some compounds but not others. Mutants based on an altered or deficient DNA polymerase have so far not been isolated in the clinic.

As mentioned above, the synthesis of viral DNA is undoubtedly the prime target for the antiviral action of ACV, DHPG, BVDU and their analogues. This does, however, not exclude the possibility that these compounds may interfere with yet other processes: i.e., BVDU has recently been shown to inhibit the glycosylation of herpesvirus glycoproteins [126,127], and BVDU may do so by interfering with the synthesis of the oligosaccharide-dolichol phosphate precursor or the processing of N-linked or O-linked oligosaccharides [128]. Such mode of action would require a previous conversion of BVDU to a nucleotide metabolite that competes with the formation or action of a usual UDP-sugar complex involved in carbohydrate synthesis. The nature of such putative BVDU metabolite is being searched for.

ANTIVIRAL ACTIVITY *IN VIVO*

The potency of an antiviral agent *in vitro* is not necessarily predictive of its potency *in vivo*, since the latter is determined by several factors; thus, besides the intrinsic activity of the compound, its pharmacokinetic profile, route of administration (topical or systemic), absorption from the gut (if administered perorally), penetration through the skin (if administered topically), tissue distribution (i.e., penetration into the brain), presence of competing metabolites (i.e., dThd) in the peripheral tissues, metabolism (i.e., phosphorylation rate) in the peripheral tissues, and clearance from the bloodstream. These factors may explain why the *in vitro* minimum effective doses may deviate from the *in vivo* minimum effective doses and why the latter may vary considerably depending on the experimental conditions for evaluating *in vivo* activity.

Table 5.3 summarizes the (minimum) effective doses for several selected antiherpes compounds in a number of animal model infections. Minimum effective doses were not determined in all systems, so that direct comparison of the relative efficacy of the compounds is not always possible; but, where the compounds were compared side by side [16,38,137], DHPG (4) appeared clearly more effective at lower dosage regimens than ACV (1). The increased potency of DHPG over ACV may be related to an increased phosphorylation rate, hence bioavailability of the active triphosphate form. For 2′-nor-cGMP (26), the minimum effective dose was similar to that of DHPG [37,38]; (*S*)-iNDG (5), however, was less efficacious than DHPG, but comparable with or more active than ACV [19].

Although HBG (7) and DHBG (8) have similar antiherpes activities *in vitro*, only (*R*)-DHBG (BCV) proved therapeutically effective *in vivo* [88]. The lack of activity of HBG could be attributed to an extremely fast clearance (upon systemic administration) and antagonizing effect of dThd (following topical administration).

For BVDU (27), significant protection has been noted in a variety of animal models [42], the most striking being the effect of BVDU 0.1% eye-drops against HSV-1 keratitis and of systemic BVDU at 1–15 mg/kg per day against simian varicella. How BV*ara*U compares with BVDU in the latter model cannot be assessed from the published data [138,141], but in the topical treatment of HSV-1 keratitis and cutaneous HSV-1 lesions, BVDU was clearly more efficacious than BV*ara*U [139,142].

CEDU (34) was at least as effective as BVDU in the topical treatment of herpetic skin and eye lesions (minimum effective dose as low as 0.1%) [44,142]. In the systemic treatment of HSV-1 infections, CEDU appeared to be effective at a 10-fold lower dose than either ACV or BVDU [44].

Table 5.3. ANTIVIRAL ACTIVITY OF SELECTED ANTIHERPES COMPOUNDS *IN VIVO*

Compound	*(Minimum) effective dose (mg/kg per day)*[a]						*References*
	Systemic HSV-1 (or HSV-2) infection	*Genital herpes (HSV-2) infection*	*Herpetic (HSV-1) keratitis*	*Herpetic (HSV-1 or HSV-2) encephalitis*	*Mucocutaneous HSV-1 (or HSV-2) infection*	*Systemic VZV infection*	
ACV (1)	12.5–50	12.5	1%	100	12.5 0.35%	45	[6,16,38,44,129,130]
DHPG (4)	0.8	0.8	1%	10	0.2 0.06%	–	[15,16,38,131–137]
(*S*)-iNDG (5)	12.5	–	–	–	12.5 0.25%	–	[19]
BCV (8)	30	–	3%	–	5%	–	[25,88]
2′-nor-cGMP (26)	0.8	0.8	–	–	–	–	[37,38]
BVDU[b] (27)	10	–	0.1%	50–100	50 0.1%	1	[42,44,138,139]
BV*ara*U[b] (30)	–	–	0.1%	50–100	10%	20	[42,139–143]
CEDU (34)	8.5	–	0.1%	35	0.1%	–	[44,142]
FIAC (41)	10	50	0.1–1%	9.3	–	–	[144–147]
FMAU (42)	1	50	0.02–0.2%	0.12	–	–	[144,146–148]
PFA (50)	500	3%	3%	800	0.15%	200	[95]

[a] For systemic treatment; for topical treatment the (minimum) effective doses are indicated in %.
[b] Data for BVDU and BV*ara*U refer to HSV-1 infections only.

The lowest minimum effective dose recorded for any antiherpes agent was 0.12 mg/kg per day with FMAU in the treatment of HSV-2 encephalitis in mice [146]. The maximum tolerated dose was 400 mg/kg per day, so that in this model infection, FMAU achieved a therapeutic index of 3333. When evaluated under the same conditions, the minimum effective dose of ACV was 33 mg/kg per day and its therapeutic index only 12 [146].

PFA (50) is particularly effective upon topical administration, where a 0.15% cream may suffice to promote the healing process. To achieve a protective activity upon systemic administration, relatively high doses (at least 200 mg/kg per day) are required [95].

Important considerations in the realization of the full potential of the antiviral agents is the frequency of administration (1–5 times per day) for both topically and systemically administered drugs, and the choice of the vehicle for topically applied drugs. In this sense, DMSO (dimethyl sulphoxide) is a more effective vehicle for ACV [149] and BVDU [150] than either poly(ethylene glycol) or Beeler base, and the efficacy of the BVDU-DMSO formulation can be further enhanced if 5% Azone (1-dodecylazaacycloheptan-2-one) is added [139].

A general principle valid for all antiherpetic agents known to date is that they are not effective against latent HSV or VZV infections, or for that matter, against any latent virus infection, and thus not amenable to eradicating the infection during its dormant state. Establishment of a latent infection can be prevented, but only if treatment is initiated shortly after the infection, that is, before the virus has colonized the sensory ganglia.

CLINICAL STUDIES

Clinical studies have been conducted with acyclovir analogues (6-deoxyacyclovir, DHPG), BVDU, FIAC and PFA. The acyclovir prodrug, 6-deoxyacyclovir (15), when administered orally as a single dose of 50 mg, achieved plasma concentrations of acyclovir which were comparable with those produced by 400 mg acyclovir [151,152]. The higher plasma ACV levels thus achieved would bring more members of the herpesvirus group, and in particular VZV, within the range of effective oral chemotherapy. Oral acyclovir itself has not been shown effective against VZV infections. Whether its prodrug would be an effective and safe drug for the oral treatment of VZV infections is now being examined. Also in progress are several clinical studies aimed at establishing the value of DHPG (4) in the systemic (intravenous) treatment of CMV infections in immunosuppressed patients.

BVDU (27) has been the subject of various clinical trials which point to its efficacy and safety in the topical treatment (as 0.1% eyedrops) of herpetic keratitis [153,154], the systemic treatment (at 3 × 2.5 mg/kg per day orally for 5 days) of mucocutaneous herpes simplex and varicella-zoster infections in immunosuppressed patients (including cancer patients and bone-marrow transplant recipients) [155,156], the systemic treatment (at 3 × 5 mg/kg per day orally for 5 days) of varicella or zoster in children with cancer (i.e., acute lymphoblastic leukemia) [157,158], and the combined topical (0.1% eyedrops) and systemic (3 × 2.5 mg/kg per day orally) treatment of ophthalmic zoster [153,159]. As a rule, all patients recovered promptly from their HSV-1 or VZV infections upon BVDU treatment. In most patients, progression of the disease was arrested within 1–3 days of starting BVDU treatment, and in no case did it appear necessary to prolong systemic treatment with BVDU for more than 5 days. Neither adults nor children showed any evidence of drug toxicity for bone marrow, liver, kidney or any other organ.

In a phase I clinical trial, FIAC (41), when administered intravenously at a dose of at least 120 mg/m^2 per day appeared to stabilize cutaneous zoster lesions within 2–3 days after starting therapy; however, at a 5-fold higher dose (600 mg/m^2 per day), toxic side-effects (nausea, vomiting, myelosuppression) became evident [160]. When FIAC was compared with Ara-A in a phase II double-blind trial in immunosuppressed patients with VZV infections, where both drugs were administered intravenously at 400 mg/m^2 per day for 5 days, FIAC proved therapeutically superior to Ara-A, as judged from the time needed for crusting (3 days *versus* 7 days) or last new lesion formation (2 days *versus* 5 days) [161]. Pharmacokinetic studies in immunosuppressed patients with herpesvirus infections [162] have shown that FIAC is rapidly deaminated to FIAU (43) and that the antiviral activity of FIAC *in vivo* is due to this metabolite.

Double-blind studies with PFA (foscarnet, 50) have indicated that the drug has therapeutic activity compared with placebo in the topical treatment of recurrent herpes labialis [95] and recurrent herpes genitalis [163], when applied topically as a 3% or 0.3% cream, respectively. The potentials of PFA in the systemic treatment of severe CMV infections, i.e., cytomegalovirus pneumonitis, in allograft recipients have only recently been appreciated [164,165]. The favourable clinical responses obtained with intravenous PFA (70–170 mg/kg per day for 4–21 days) are such that PFA should be further evaluated in the treatment of CMV infections, preferably starting at the earliest possible stage of the disease.

CONCLUSION

The rather empirical discovery of ACV (1) as a potent and selective antiherpes agent has prompted the search for other, and possibly more effective, antiviral agents. This search yielded several compounds which appear to have an advantage over acyclovir in that they (i) exhibit more potent antiviral activity, particularly against VZV (i.e., BVDU (27), BV*ara*U (30) and FIAC (41)), (ii) possess a broader activity spectrum encompassing CMV (i.e., DHPG (4), 2′-nor-cGMP (26) and PFA (50)), (iii) are better absorbed when administered orally (i.e., BVDU (27), 6-deoxyacyclovir (15)), (iv) achieve *in vivo* activity at much lower concentrations, probably because of a more efficient phosphorylation rate (i.e., DHPG (4) and its 2.6-diamino precursor (18), and 2′-nor-cGMP (26)), or (v) may be effective against herpesvirus mutants which have become resistant to ACV as the consequence of an altered substrate specificity (i.e., DHPG (4), BVDU (27)) or total deficiency of their dThd kinase (i.e., 2′-nor-cGMP (26), PFA (50)).

ACV has been world-wide licensed and marketed for the topical and systemic treatment of various HSV-1, HSV-2 and VZV infections. The newer compounds have not been licensed yet. Initial clinical results point to their efficacy and usefulness in the topical treatment of herpetic eye infections (BVDU), the systemic treatment of VZV infections (BVDU perorally; FIAC intravenously) and the systemic treatment of CMV infections (DHPG, PFA; both intravenously). From their *in vitro* activity against EBV replication, BVDU, DHPG and some 2′-fluorinated pyrimidine nucleosides may also be considered as potential candidates for the treatment of EBV-associated diseases, i.e., infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma. Thus, current progress in the development of antiherpes agents extends to the whole range of medically important herpesvirus infections, including HSV-1, HSV-2, VZV, CMV and EBV.

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6 Chemical and Biological Aspects of Sparsomycin, an Antibiotic from *Streptomyces*

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INTRODUCTION

Sparsomycin (1), a bacterial metabolite, has attracted considerable interest because of its structure and its biological activity. The monoxodithioacetal moiety $RS(O)CH_2SMe$ is one of its diverse functions. This moiety is rarely encountered in natural products but has recently attracted the attention of organic chemists due to its synthetic utility.

Sparsomycin is a strong inhibitor of protein biosynthesis, its site of interaction being the large ribosomal subunit, which prevents peptide transfer by interfering with the peptidyl transferase centre. Valuable information on the structure of this peptidyl transferase centre and of the ribosomes as a whole has been obtained from studies using sparsomycin.

A Phase I clinical study on sparsomycin was initiated in 1964 – two years after its isolation – in view of its observed antitumour activity. This study was discontinued, since a sparsomycin-related retinopathy was observed. Recent evidence suggests, however, that this retinopathy is found only in very sick animals and that with healthy animals sparsomycin does not pass through the blood-retina barrier. It was observed recently that sparsomycin potentiates the activity of cisplatin, which has achieved prominence as an anticancer drug.

An intense investigation of a series of analogues of sparsomycin was initiated because of these observations. Synthetic routes were developed as a preliminary to such a study. Only two of all the analogues prepared so far were found to have a therapeutic index broader than that of sparsomycin itself.

A decision on whether sparsomycin – or its most promising analogue – shows sufficiently auspicious activity to warrant further clinical development as an anticancer drug will be required shortly. This antibiotic has achieved prominence in the past 20 years as a tool to study the protein biosynthesis machinery.

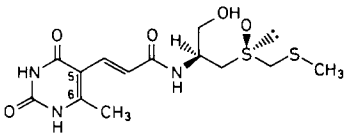
A comprehensive review of sparsomycin, its chemistry, biological activity and potential as an anticancer drug, is timely. This chapter presents the first extensive review of this topic.

CHEMISTRY

ISOLATION AND STRUCTURE ELUCIDATION

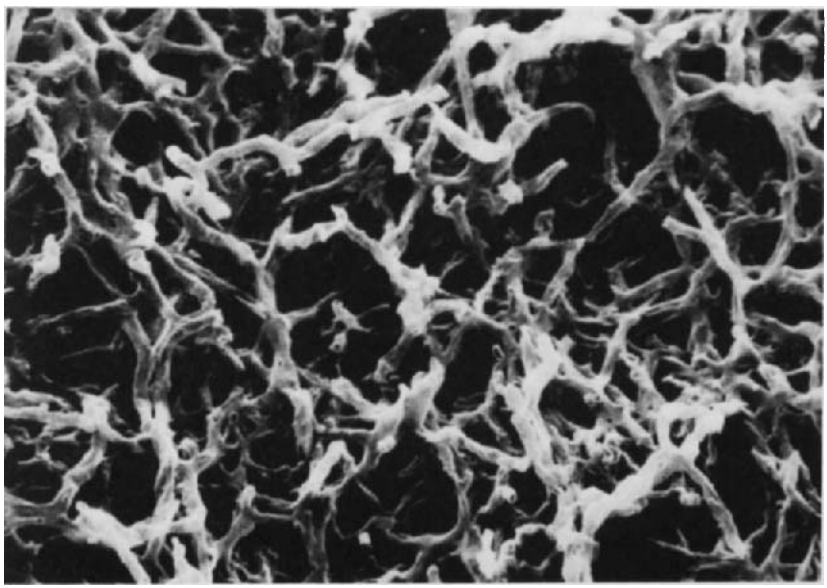
Sparsomycin was detected initially in beers, by antibacterial bioautographic techniques, and was isolated subsequently in 1962 from a *Streptomyces sparsogenes* fermentation broth [1,2]. The antibiotic was found to be active

against KB human epidermoid carcinoma cells, in tissue culture [1]. The microorganism *S. sparsogenes* is characterized microscopically by having many coremia with branches (see photograph). The sporophores are clustered along the hyphae and terminate in tight spirals of a variable number of turns [1]. The isolation of the metabolite was reported also from the culture filtrate of



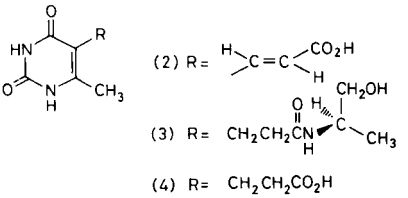
Sparsomycin (1)

Streptomyces cuspidosporus [3,4] a few years later. The fermentation broths from which sparsomycin was isolated contained also several other substances displaying antibiotic activity. The most important of these was the antibiotic tubercidin, a ribofuranosylpyrimidine [5]. The separation of sparsomycin from tubercidin was achieved by use of partition chromatography. Final purification of the drug was effected by countercurrent distribution.



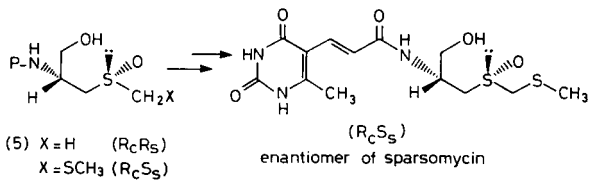
Streptomyces sparsogenes (× 3,250 as shown)

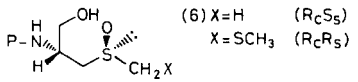
The structure of sparsomycin remained unresolved until 1970, when Wiley and MacKellar [6,7] reported the results of synthetic, spectroscopic and degradation studies. Mild acidic hydrolysis of sparsomycin resulted in the formation of formaldehyde and in the isolation of a compound (2) having the molecular formula $C_8H_8N_2O_4$ (*Scheme 6.1*). The structure of (2) was confirmed by comparison of its spectral and analytical properties with those of the



Scheme 6.1. Structure elucidation of sparsomycin (1) [6,7].

synthesized compound. Treatment of sparsomycin with Raney nickel yielded 2 mol of methane per mol of sparsomycin and a compound (3) having the molecular formula $C_{11}H_{17}N_3O_4$. Alkaline hydrolysis of (3) followed by acidification gave another eight-carbon acid (4) which appeared to be the analogue of (2), in which the *trans*-olefin system was reduced. A second product from this hydrolysis proved to be D-2-aminopropanol. The absence of a basic group in sparsomycin indicated that D-2-aminopropanol and (2) had to be combined through an amide linkage to give structure (3). It was concluded from ^{1}H-NMR spectroscopy that a $C_2H_5OS_2$ moiety is attached through a sulphur atom, which is cleaved by Raney nickel reduction, at the methyl carbon atom. This moiety is present as $MeSCH_2S(O)$ in sparsomycin, confirming the evolution





Scheme 6.2. Chirality of sparsomycin [8].

of 2 mol of methane following Raney nickel reduction and the formation of formaldehyde after acid hydrolysis.

In summary, the molecule features a modified uracil ring, a *trans*-olefin bond, a chiral carbon atom which was shown to have the *S* configuration and a chiral sulphur atom, the chirality of which remained unspecified.

Elucidation of the chirality of this sulphur atom was accomplished as follows. In studies relating to a total synthesis of sparsomycin, two sulphoxides (5) and (6) were prepared that differed only in the chirality of the sulphoxide function. From chiroptical studies and single-crystal X-ray analysis it was concluded [8] that the stereoisomer (5) – a precursor for the enantiomer of sparsomycin – has the chirality depicted in *Scheme 6.2*. It was concluded from this that sparsomycin has the S_cR_s chirality.

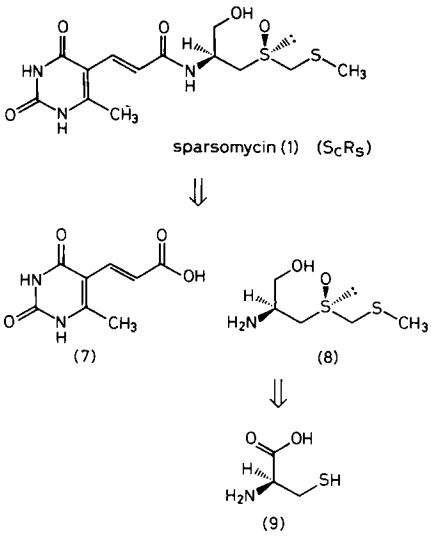
SYNTHESIS

The compound has attracted much attention since its discovery, not only because of its biological activity, but also for its unique structural characteristics and synthetic challenge.

Although sparsomycin is accessible from natural sources in limited amounts [2–4], a total synthesis is desirable for several reasons. One important reason is the fact that a synthesis has been needed to confirm the assigned structure and to determine the chirality of the sulphoxide. Secondly, an efficient synthesis would provide sparsomycin in sufficient quantities for further clinical testing and for other studies regarding its biological activity. Thirdly, small alterations in a flexible synthesis would permit the preparation of a number of analogues for structure-activity relationship studies. Finally, a total synthesis of (1) constitutes a challenge, since the formaldehyde monoxodithioacetal group $RS(O)CH_2Me$ is one of its diverse functionalities. This synthetically complex moiety is rarely encountered in nature: the only other natural examples are γ-glutamylmarasmine and SE-3 [9].

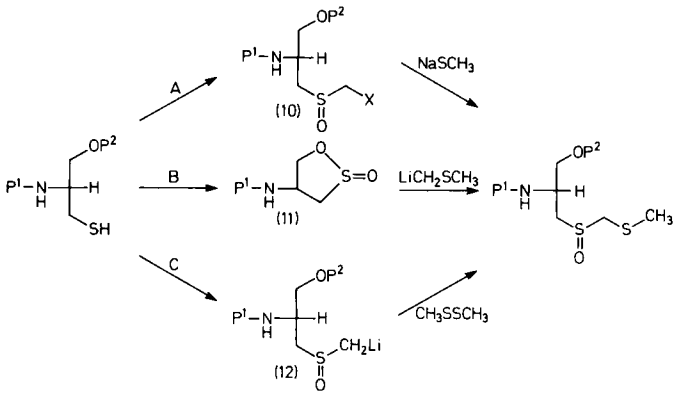
Total syntheses of sparsomycin were investigated concurrently by the research groups of Ottenheijm and Helquist. (For another recent study directed towards the synthesis of sparsomycin, see [10]). The strategy used in all of the routes leading to sparsomycin was based on the retrosynthetic *Scheme 6.3*. Sparsomycin can be considered to be an amide derived from the coupling of β-(6-methyluracilyl)acrylic acid (7) and the amine (8). The latter can be viewed as a derivative of D-cysteine (9), having a reduced CO_2H function and its sulphydryl function oxidized and alkylated.

Component (7) was prepared from C(5)-substituted 6-methyluracil according to described procedures [6,7,11,12]. The synthesis of the amine



Scheme 6.3. Retrosynthetic analysis of sparsomycin.

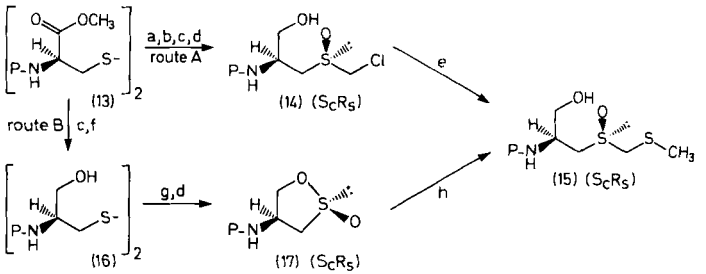
component (8) was more challenging, primarily because of the unusual monoxodithioacetal moiety, for which new methodologies had to be developed. The three fundamentally different approaches that were used successfully in the synthesis of protected derivatives of (8) are depicted schematically in *Scheme 6.4*. In the first approach (route A) the reaction of an α-chlorosulphoxide derivative of cysteine (10) with sodium methyl mercaptide was



Scheme 6.4. Approaches to sparsomycin (1).

employed [11]. The second approach (route B) features the nucleophilic ring opening of the cyclic sulphinate ester or γ-sultine (11) [13]. In the third approach (route C), which has been explored successfully by Helquist and Shekhani [12], the sulphenylation of an α-sulphinyl carbanion (12) was used.

The first two approaches (routes A and B), both starting from D-cystine, are shown in greater detail in *Scheme 6.5*. The reaction of the *N*-protected D-cystine methyl ester (13) with chlorine and acetic anhydride gave a sulphinyl chloride, which was treated subsequently with diazomethane to give an α-chlorosulphoxide (route A). Following reduction of the methyl ester, the two diastereomeric α-chlorosulphoxides were separated by column chromatography. The required stereoisomer (14) was reacted with sodium methyl mercaptide to give the *N*-protected amine (15), the *N*-protecting group of which was removed subsequently. Coupling of the unprotected amine (8) with the acid (7), (see *Scheme 6.3*), was accomplished by the use of standard methods [11] to yield a compound which was identical in all physicochemical and biological respects with an authentic sample of sparsomycin.

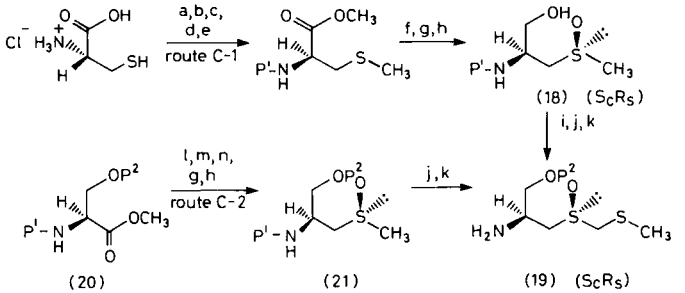


Scheme 6.5. Synthesis of sparsomycin by Ottenheijm and co-workers [11,13,14]. P = Boc or Cbo. Reagents: (a) Cl_2, Ac_2O; (b) CH_2N_2; (c) $LiBH_4$; (d) separation of diastereomers; (e) NaSMe; (f) I_2, pyridine; (g) NCS, AcOH, pyridine; (h) $LiCH_2SMe$.

The attractiveness of the second route, i.e., route B, relies on the use of the sultine (17). This compound has a sulphur atom activated toward nucleophilic attack and has simultaneously a protected alcohol function. Reduction of the cystine methyl ester (13) gave the alcohol (16), which was treated with *N*-chlorosuccinimide and acetic acid to give two diastereomeric sultines. Separation of the diastereomers and subsequent reaction of the required isomer (17) with the anion of dimethyl sulphide gave the *N*-protected amine (15) stereospecifically.

Approach C is shown in more detail in *Scheme 6.6*. Route C-1 involves a racemization and resolution sequence as a consequence of starting with L-cysteine. The monoxodithioacetal function of (19) was prepared by reaction

of the anion of the appropriate methyl sulphoxide (18) with dimethyl disulphide. Compound (18) was obtained from *N*-protected *S*-methyl-D-cysteine methyl ester by reduction of the ester function, subsequent oxidation of the sulphide and separation of the resultant diastereomers by column chromatography. The amine (19) was then coupled with acid (7), as described for route A.

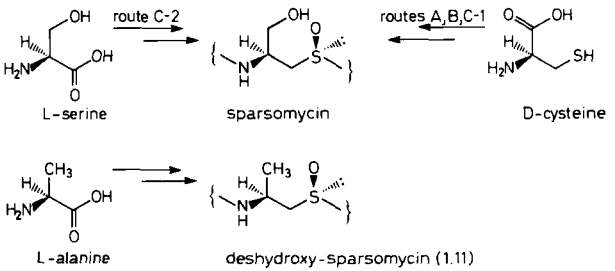


Scheme 6.6. Synthesis of sparsomycin (1) by Helquist and co-workers [12,15]. P^1 = Cbo; P^2 = THP or MOM. Reagents: (a) NaOEt, CH_3I, aq. HI; (b) HOAc/reflux, aq. HCl/reflux; (c) $ClCO_2CH_2Ph$; (d) ephedrine (resolution); (e) CH_2N_2; (f) $LiBH_4$; (g) $NaIO_4$; (h) separation of diastereomers; (i) DHP, TsOH; (j) Na, liq. NH_3; (k)LDA, MeSSMe; (l) DIBAH, $NaBH_4$; (m) TsCl; (n) NaSMe.

A more elegant approach, thus avoiding the inefficient resolution step, involves a formal 'inversion' of the configuration of L-serine by selective manipulation of the functional groups surrounding the chiral carbon atom. The carboxyl group of L-serine can, in effect, be converted into a mercaptomethylene moiety, thus permitting entry into the D-cysteine system without actually operating upon the chiral centre itself; the configuration at the carbon atom of sparsomycin corresponds with the chirality of D-cysteine. The principle of this approach is shown in *Scheme 6.7*, route C-2. The use of L-amino acids is attractive with respect to their availability and cost.

Route C-2 is shown in more detail in *Scheme 6.6*. Reduction of the ester function of the L-serine derivative (20), formation of the tosylate, followed by reaction with sodium methyl mercaptide and subsequent oxidation with sodium metaperiodate generates two diastereomeric methyl sulphoxides. The relevant sulphoxide (21) was converted into (19) by the same sequence of reactions as described for the conversion of *O*-protected (18) into (19).

This principle of a formal 'inversion' has also been used successfully for the synthesis of dehydroxysparsomycin (1.11), as shown in *Scheme 6.7* [16]. In conclusion, this concept furnishes the possibility of replacing the hydroxymethylene group of sparsomycin by alkyl or alkylaryl chains by starting from the L-amino acids, valine, isoleucine or phenylalanine.



Scheme 6.7. Amino acids in the synthesis of sparsomycin.

The investigations in the aforementioned synthetic routes leading to sparsomycin unequivocally provided definite proof of the structure of sparsomycin. These routes are capable of providing practical quantities of sparsomycin for biochemical and preclinical testing. Moreover, these routes are flexible enough to allow the synthesis of structural analogues of sparsomycin.

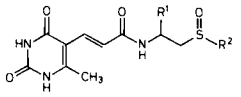
SYNTHESIS OF ANALOGUES

The active interest shown in the synthesis of sparsomycin analogues has undoubtedly been prompted by the usefulness of sparsomycin as a potent protein synthesis inhibitor and as a potential anticancer drug. All of the various syntheses of derivatives related to the drug followed the retrosynthetic *Scheme 6.3*. Thus, the target compound was synthesized by coupling an amino cysteinyl derivative with an acid, thereby establishing the amide linkage present in sparsomycin. Initial attempts often started with either L-cystine or a racemic mixture of cystine, due to the high cost of D-cystine.

In *Tables 6.1–6.7* the analogues prepared so far are listed; their biological activities are discussed in the section 'Analogues as inhibitors of protein synthesis'. In *Table 6.1* analogues are presented having structural variations at the bivalent sulphur atom or at the hydroxymethylene function, whilst leaving the $S(O)CH_2S$ moiety intact. These variations permitted conclusions to be drawn about the rôle these two side-chains play in the biological activity of sparsomycin.

Table 6.2 gives a compilation of derivatives in which the CH_2SMe moiety is replaced by a variety of substituents. The effect of the bivalent sulphur atom and the influence of hydrophobic substituents on the inhibitory activity were investigated using these synthetic analogues. In *Table 6.3* compounds are listed in which the monoxodithioacetal function has been replaced by an SR function and amongst these are derivatives for which R = $SCH_2S(O)Me$. The necessity

Table 6.1. SPARSOMYCIN DERIVATIVES



Compound	R^1	R^2	*Chirality at*		*Reference*
			C	*S*	
(1.1)	CH_2OH	CH_2SMe	*R*	*S*	[11]
(1.2)	CH_2OH	CH_2SMe	*S*	*S*	[11]
(1.3)	CH_2OH	CH_2SMe	*R*	*R*	[11]
(1.4)	CH_2OH	$CH_2S(CH_2)_2Me$	*S*	*R*	[16]
(1.5)	CH_2OH	$CH_2S(CH_2)_3Me$	*S*	*R*	[16]
(1.6)	CH_2OH	$CH_2S(CH_2)_4Me$	*S*	*R*	[16]
(1.7)	CH_2OH	$CH_2S(CH_2)_7Me$	*S*	*R*	[17]
(1.8)	CH_2OH	CH_2SCH_2Ph	*S*	*R*	[29]
(1.9)	CH_2OAc	CH_2SMe	*S*	*R*	[16]
(1.10)	CH_2OMe	CH_2SMe	*S*	*R*	[16]
(1.11)	Me	CH_2SMe	*S*	*R*	[16]
(1.12)	Me	CH_2SMe	*S*	*S*	[16]
(1.13)	Me	$CH_2S(CH_2)_7Me$	*S*	*R*	[16]

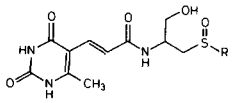
for the presence and position of the sulphoxide function in the molecule was correlated using these structural variations.

Table 6.4 lists derivatives in which the hydroxymethylene group is substituted by hydrogen and/or the entire $CH_2S(O)CH_2SMe$ moiety is replaced by alkyl, aryl or benzylamide groups. The syntheses of these analogues enable theories about the effect of such radical changes in the structure of sparsomycin on the biological activity to be postulated.

In *Table 6.5* are listed compounds that lack the ethylene moiety of the uracilylacryloyl portion of sparsomycin.

Analogues that have the uracil nucleus replaced by aryl groups and some unusual analogues are listed in *Tables 6.6* and *6.7*, respectively. The compound (7.5) having a *cis*-double bond was synthesized to investigate the effect of this change on the biological activity of the drug. It was observed that *trans*- to *cis*-isomerization occurs under the influence of laboratory light or daylight [7,17]. The derivative (7.6) without the methyl group on the uracil ring was prepared in order to study whether this group is essential to assure a certain preferential conformation of the molecule necessary for the exhibition of the reported activity.

Table 6.2. SPARSOMYCIN DERIVATIVES



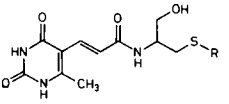
Compound	*R*	*Chirality at*		*Reference*
		C	*S*	
(2.1)	Me	*R*	*R/S*	[73]
(2.2)	Me	*R/S*	*R/S*	[73]
(2.3)	$(CH_2)_2Me$	*S*	*R*	[29]
(2.4)	iPr	*R*	*R/S*	[73]
(2.5)	Bu	*R/S*	*R/S*	[73]
(2.6)	$(CH_2)_9Me$	*S*	*R*	[29]
(2.7)	$(CH_2)_9Me$	*R/S*	*R/S*	[73]
(2.8)	$CH_2CH=CH_2$	*R/S*	*R/S*	[34]
(2.9)	$CH_2CH=CHMe$	*R/S*	*R/S*	[34]
(2.10)	CH_2Cl	*S*	*R*	[29]
(2.11)	CH_2Cl	*S*	*S*	[29]
(2.12)	$CH_2S(O)Me$	*R/S*	*R/S*	[73]
(2.13)	Ph	*R/S*	*R/S*	[34]
(2.14)	CH_2-2-furyl	*R/S*	*R/S*	[34]
(2.15)	CH_2Ph	*R/S*	*R/S*	[34]
(2.16)	CH_2Ph	*R*	*R/S*	[73]
(2.17)	C_6H_4-4-OH	*R/S*	*R/S*	[34]
(2.18)	$CH_2C_6H_4$-3-CF_3	*R/S*	*R/S*	[34]
(2.19)	$CH_2C_6H_4$-3-OMe	*R/S*	*R/S*	[34]
(2.20)	$CH_2C_6H_4$-4-OMe	*R/S*	*R/S*	[34]
(2.21)	$CH_2C_7H_4O_2$-2-Cl	*R/S*	*R/S*	[34]

BIOLOGICAL ACTIVITY *IN VITRO*

INHIBITION OF PROTEIN SYNTHESIS

Sparsomycin, isolated originally from cultures of *Streptomyces sparsogenes* [5], shows antiviral activity [104], inhibitory activity to growing Gram-positive and Gram-negative bacteria, a number of fungi and to KB human epidermoid carcinoma cells [1]. It also causes significant growth inhibition in about 50% of the tumours tested *in vivo* [1]. The broad spectrum of activity exhibited by sparsomycin, especially that towards tumours, prompted additional studies to elucidate its mode of action.

Table 6.3. SPARSOMYCIN DERIVATIVES

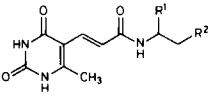


Compound	*R*	*Chirality at*		*Reference*
		C	*S*	
(3.1)	Me	*R*		[73,74]
(3.2)	Me	*S*		[74]
(3.3)	Me	*R/S*		[73]
(3.4)	Pr	*R*		[39]
(3.5)	Pr	*S*		[39]
(3.6)	Pr	*R/S*		[39]
(3.7)	iPr	*R*		[73]
(3.8)	Bu	*R/S*		[39,73]
(3.9)	$(CH_2)_9Me$	*R/S*		[73]
(3.10)	CH_2Ph	*R*		[73]
(3.11)	CH_2Ph	*R/S*		[39]
(3.12)	CH_2SMe	*S*		[17]
(3.13)	CH_2SMe	*R*		[17]
(3.14)	CH_2SMe	*R/S*		[39]
(3.15)	$CH_2S(O)Me$	*S*	*R/S*	[17]
(3.16)	$CH_2S(O)Me$	*R*	*R/S*	[17]

The different metabolic parameters altered by this antibiotic in *Escherichia coli* cells have been studied [18,19]. Protein synthesis was affected at a lower drug concentration than was the synthesis of DNA and RNA. Cell growth and DNA synthesis were inhibited in parallel, in the presence of the drug, whilst RNA accumulated as tRNA. Protein synthesis was inhibited at a rate faster than cell growth. The release of amino acids into the growth medium due to growth inhibition was also reported. This interesting result has, unfortunately, not been explored more extensively in subsequent investigations. These results were confirmed, and the isolation was reported of a sparsomycin-resistant strain of *E. coli* apparently affected in the cell envelopes [20].

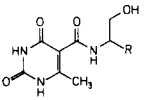
Preferential inhibition of protein synthesis by sparsomycin has also been reported in growing L cells [21] and in mouse livers [22]. Sparsomycin does not, however, inhibit protein synthesis in intact reticulocytes *in vitro*, probably due to permeability problems [23].

Table 6.4. SPARSOMYCIN DERIVATIVES



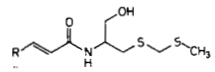
Compound	*R^1*	*R^2*	*Chirality at C*	*Reference*
(4.1)	H	Ph	*R/S*	[76]
(4.2)	H	C_6H_4-4-Me	*R/S*	[76]
(4.3)	H	C_6H_4-4-OMe	*R/S*	[76]
(4.4)	H	C_6H_4-4-Br	*R/S*	[76]
(4.5)	H	$CONHCH_2Ph$	*R/S*	[76]
(4.6)	H	$CONHCH_2C_6H_4$-4-Me	*R/S*	[76]
(4.7)	H	$CONHCH_2C_6H_4$-4-OMe	*R/S*	[76]
(4.8)	H	$CONHCH_2C_6H_4$-4-Br	*R/S*	[76]
(4.9)	H	$CONHCH_2C_6H_3$-3,4-Cl_2	*R/S*	[76]
(4.10)	CH_2OH	H	*R*	[74]
(4.11)	CH_2OH	H	*S*	[74]
(4.12)	CH_2OH	OH	*R*	[74]
(4.13)	CH_2OH	OH	*S*	[74]
(4.14)	CH_2OH	CH_2SMe	*S*	[74]
(4.15)	CH_2OH	CH_2SMe	*R*	[74]
(4.16)	CH_2OH	Ph	*R/S*	[76]
(4.17)	CH_2OH	C_6H_4-4-Me	*R/S*	[76]
(4.18)	CH_2OH	C_6H_4-4-OMe	*R/S*	[76]
(4.19)	CH_2OH	C_6H_4-4-Br	*R/S*	[76]

Table 6.5. SPARSOMYCIN DERIVATIVES



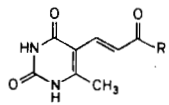
Compound	*R*	*Chirality at C*	*Reference*
(5.1)	H		[82]
(5.2)	Me	*S*	[82]
(5.3)	Me	*R*	[82]
(5.4)	CH_2OH	*S*	[82]
(5.5)	CH_2OH	*R*	[82]
(5.6)	CH_2SMe	*S*	[82]
(5.7)	CH_2SMe	*R*	[82]
(5.8)	CH_2CH_2SMe	*S*	[82]
(5.9)	CH_2CH_2SMe	*R*	[82]

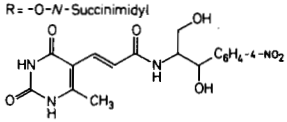
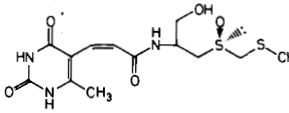
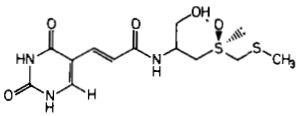
Table 6.6. SPARSOMYCIN DERIVATIVES



Compound	*R*	*Chirality at C*	*Reference*
(6.1)	Ph	*R*	[97]
(6.2)	2-furyl	*R*	[97]
(6.3)	2-MeC_6H_4	*R*	[97]
(6.4)	4-MeC_6H_4	*R*	[97]
(6.5)	2-ClC_6H_4	*R*	[97]
(6.6)	2,5-$(MeO)_2C_6H_3$	*R*	[97]

Table 6.7. SPARSOMYCIN DERIVATIVES



Compound		chirality at C	chirality at S	reference
(7.1)	R = $NHCH_2CH_2OH$			[74]
(7.2)	R = $N(CH_3)_2$			[77]
(7.3)	R = -O-*N*-Succinimidyl			[77]
(7.4)		S,S		[77]
(7.5)		S	R	[17]
(7.6)		S	R	[29]

These initial observations indicated that protein synthesis was probably the target of sparsomycin. Various *in vitro* protein-synthesizing systems were employed to determine which of the various stages in the metabolic process was affected by the drug. The assays used and results reported are discussed briefly here.

Inhibition of natural mRNA-depending assays

It was quickly shown, using a reticulocyte lysate [23], that sparsomycin inhibited the incorporation of amino acids into proteins directed by the endogenous mRNA preventing, simultaneously, the run-off of the polysomes present in the sample.

Similar results were obtained using cell lysates from mouse livers [22], yeast [24], plasma cell tumours [25] and *E. coli* systems directed by MS 2 mRNA [26]. In all these examples, ED_{50} values of approximately 1 μM were obtained.

The translation of mRNA from encephalomyocarditis virus is also blocked by the drug [27]. It is interesting that in this case, the formation of the first dipeptide coded by the mRNA, i.e., Met-Ala, is resistant to sparsomycin concentrations as high as 200 μM.

Inhibition of synthetic mRNA coded assays

The translation of natural mRNA requires complex reaction mixtures, and strict ionic conditions, in order to initiate the process at the correct starting codons in the mRNA. The requirements are simpler when synthetic mRNAs, not requiring a physiological initiation, are translated.

It has been reported [21,26] that, in bacterial systems, sparsomycin inhibits the polymerization of the cognate amino acids directed by polyuridylic, polyadenylic and polycytidylic acids and by heteropolymers containing different proportions of nucleotides. The extent of inhibition, however, was found to be dependent upon the polynucleotide used as messenger, being inversely proportional to the uridylic acid content of the RNA.

It was also found that the extent of the effect of sparsomycin decreases with sample incubation time in these systems, suggesting that the drug probably affects mainly the initial reaction rate [26]. In general ED_{50} values in the range of 1 μM are found for polyphenylalanine synthesis inhibition. The inhibition of polyphenylalanine synthesis by sparsomycin has also been reported in systems derived from *Saccharomyces cerevisiae* [28] and in the archaebacteria *Sulfolobus solfataricus* [29].

The inhibition of the synthetic polynucleotide translation confirmed that protein synthesis is highly sensitive to sparsomycin, but strongly suggested that the drug is not an inhibitor of the initiation step of this process. It was concluded from this as well as from the 'freezing' of polysomes in cell lysates, that one of the reactions involved in the elongation or termination of the peptide chain synthesis is the target of sparsomycin. This conclusion was supported by the finding that the antibiotic does not affect other steps of the synthetic process

such as amino acid acylation of tRNA [21], formation of the ternary complex aminoacyl-tRNA-elongation factor Tu-GTP [26] and binding of the mRNA to the ribosomes [26].

Effect of sparsomycin on peptide bond formation

The formation of the peptide bond, the central step of protein biosynthesis, is carried out at the peptidyl transferase centre of the ribosome. In this active centre, part of the large ribosomal subunit [30], the transfer of the peptidyl residue from the peptidyl-tRNA bound to the P-site, to the aminoacyl-tRNA bound to the A-site, takes place. This reaction has been studied extensively using different model systems which have also been useful for investigating the mechanism of sparsomycin action. The data available from these assays are now summarized.

Inhibition of dipeptide synthesis

The peptide bond formation can be tested with assays that use an analogue of the peptidyl-tRNA as a donor, either polylysyl-tRNA of *N*-acetylphenylalanyl-tRNA. The corresponding aminoacyl-tRNAs, Lys-tRNA and Phe-tRNA, are used as acceptors on ribosomes coded by poly(A) and poly(U), respectively. Both assays are sensitive to sparsomycin in bacterial [31] as well as in mammalian systems [32]. When the conditions employed allow the Phe-tRNA to act as a donor as well as an acceptor in the poly(U)-directed reaction, however, the formation of the dipeptide Phe-Phe is not blocked by sparsomycin [32].

Reaction with puromycin

The antibiotic puromycin, functioning as an analogue of the 3′ end of the aminoacyl-tRNA (see *Figure 6.1*), is used extensively as an acceptor in model assays for peptide bond formation. In this assay the reaction is simplified considerably, since the requirements for the binding of a large molecule, e.g., the aminoacyl-tRNA to the ribosome, are eliminated. The effect of sparsomycin on several of these puromycin reactions has been tested.

Inhibition of the puromycin reaction on polysomes

Polysomes carrying peptidyl-tRNA at the ribosomal P-site react with puromycin to form peptidyl-puromycin, which is released from the particles.

When radioactively labelled antibiotic is used, the peptide bond formation is easily studied by following the peptide-bound radioactivity.

Sparsomycin inhibits this reaction of puromycin in native polysomes from *E. coli* [26,33,34], rabbit reticulocytes [35], rat liver [33], HeLa cells [34] and *S. cerevisiae* [24] as well as in synthetic mRNA polysomes [26]. It was demonstrated clearly that sparsomycin is a competitive inhibitor of puromycin in the peptide release reaction when the results of the assays were analysed kinetically [26,33,36]. They indicate a K_i of circa 0.1 μM [36] and that the process is inhibited by one molecule of drug per peptidyl-tRNA-ribosome complex [36]. The extent of inhibition of the puromycin reaction on polysomes is also affected by the incubation time as in the case of the polymerization assay.

Inhibition of the reaction of puromycin with N-*blocked aminoacyl-tRNA*

N-Blocked aminoacyl-tRNAs bound to the P-site on the ribosome react with puromycin, forming *N*-acetylaminoacylpuromycin. This reaction is also sensitive to sparsomycin and the kinetic characteristics of the inhibition are similar to those found for the puromycin reaction on polysomes in *E. coli* [33,37–39] and in *S. cerevisiae* [28].

Inhibition of the fragment reaction

Monro and Marcker [40] tested peptide bond formation using *N*-acetyl-aminoacylpentanucleotide and puromycin, as donor and acceptor substrates, respectively. The reaction required only the presence of the large ribosomal subunit and an alcohol to increase the affinity of the ribosome for the 3′-terminal oligonucleotide. This assay, termed 'fragment reaction', eliminates the requirements for natural substrates. All the available data indicate that it takes place at the peptidyl transferase centre, which is in agreement with the normal mechanism for peptide bond formation [40].

This reaction is sensitive to sparsomycin [41] and this sensitivity was the definitive proof of the action of sparsomycin at the peptidyl transferase centre of the bacterial ribosome. Similar inhibitory results were obtained later using ribosomes from human tonsils [42], yeast [28,42], wheat germ [43] and mammalian cells [44].

Model reactions using unnatural substrates, although convenient for analyzing the mechanism of antibiotic action, do not portray the real situation inside the cell. This must be considered when comparing the results from the various tests. All the kinetic parameters showing the competitive character of sparsomycin inhibition, for example, have been obtained using puromycin as

the acceptor substrate. The differences between this nucleoside antibiotic and the real substrate, i.e., aminoacyl-tRNA, are obvious (see *Figure 6.1*). To what extent the facts are applicable to the situation *in vivo* is questionable.

It is not surprising therefore that, although detailed comparative studies are lacking, the data available on ED_{50} values seem to indicate that the puromycin reaction is more sensitive to sparsomycin than the polymerization tests. These differences might reflect, as will be discussed later, the different effects of sparsomycin on the interaction of each of the two types of substrate with the ribosome.

The differences among the various types of test would be of special importance when comparing the activity of different sparsomycin derivatives. It is conceivable that modification of the drug alters its activity differently, depending on the substrate tested. Evidence available from the literature confirms this possibility and will be commented upon in the section 'Analogues as inhibitors of protein synthesis'.

In summary, the collective results from the various assays employed agree reasonably well and show clearly that sparsomycin is an inhibitor of the peptide bond formation step in protein synthesis.

Effect of sparsomycin on binding of peptidyl transferase substrates

The reaction mechanism of the simplest model assay for peptide bond formation can be differentiated into three stages – the binding of the donor substrate, the binding of the acceptor substrate and the transfer reaction – and, in principle, any of these can be affected by sparsomycin.

The effect of sparsomycin on the binding of the peptidyl transferase substrates was studied initially using Phe-tRNA and poly(U)-coded ribosomes [45], as well as polylysyl-tRNA and poly(A)-coded ribosomes [26]. The lack of any effect obtained in these two systems suggested that sparsomycin does not affect the interaction of substrates either at the A-site (Phe-tRNA binding) or at the P-site (polylysyl-tRNA binding). Subsequent studies indicated, however, that the drug is able to disturb the enzymatic binding of Phe-tRNA to the A-site, uncoupling the GTP hydrolysis [46]. These results have been confirmed recently [47] by studying the AUGCCC-directed binding of Pro-tRNA to *E. coli* ribosomes. Conversely, stimulation by sparsomycin of *N*-acetyl-Phe-tRNA binding to the P-site directed by poly(U) has been reported [48,49].

The binding of a tRNA molecule to the ribosome is the result of the interaction at several places along the ribosomal A- and P-sites, including the 3′ end of the peptidyl transferase centre, the anticodon loop, the mRNA

binding site on the 30 S subunit and several other points on the 30 S and 50 S subunits. The interpretation of the effects caused by sparsomycin on the binding of the whole tRNA molecule is, therefore, not straightforward.

Binding studies of substrates can be limited to the peptidyl transferase centre by using the 3′ terminal fragments from *N*-acetylaminoacyl-tRNA and aminoacyl-tRNA. Under the appropriate conditions, the *N*-blocked fragment binds to the P-site and the unblocked fragment to the A-site [50–52]. It has been shown [53] that, in the presence of ethanol, sparsomycin strongly stimulates the binding of *N*-acetyl-Leu-ACCAC(U) to *E. coli* ribosomes. Similar results were reported using ribosomes from yeast [28] and wheat germ [43]. Stimulation of *N*-acetyl-Phe- and formyl-Met- fragments, binding to *E. coli* ribosomes in the absence of alcohol, has also been reported [54]. In contrast to these findings, the interaction of the unblocked fragment with the ribosome is strongly inhibited by sparsomycin [51,54,55].

These data indicate that sparsomycin inhibits the formation of peptide bonds by interfering with the binding of substrates at the A- and P-sites of the peptidyl transferase centre. At the A-site the drug inhibits the binding of the small-size substrates, i.e., the unacetylated fragment, and distorts the interaction of the large aminoacyl-tRNA with the ribosome, uncoupling the GTP hydrolysis from the binding process. At the P-site, the binding of both small and large substrates is stimulated by sparsomycin.

Effects of sparsomycin on the interaction of other antibiotics with the ribosome

The peptidyl transferase centre of the ribosome is the target of a considerable number of antibiotics that, like sparsomycin, inhibit the peptide bond formation (for review articles, see Ref. 56). Valuable information on their mode of action, as well as on the structure of the peptidyl transferase active centre, has been derived from studies on their mutual competition for binding to the ribosomes [57]. The results from competition studies using puromycin, chloramphenicol, erythromycin, lincomycin and sparsomycin are summarized (*Table 6.8*) together with the effect of these drugs on the binding of the blocked and unblocked 3′-terminal fragments to bacterial ribosomes.

It is apparent from these data that sparsomycin does not compete for binding with chloramphenicol, lincomycin and erythromycin [58]. These three antibiotics compete, however, among themselves and therefore they seem to share a common binding site on the ribosome. This site, however, cannot be identical for the three drugs, since there are substantial differences in their effect on the binding of peptidyl transferase substrates. Whilst chloramphenicol and lincomycin inhibit the binding of the unblocked fragment, erythromycin has a

Table 6.8. EFFECT OF ANTIBIOTICS ON THE BINDING OF SUBSTRATES TO BACTERIAL RIBOSOMES

Antibiotic	*Substrate*[a]					
	N-Acetylated fragment	*Non-acetylated fragment*	*Puromycin*	*Chloramphenicol*	*Erythromycin*	*Lincomycin*
Sparsomycin	s [50]	+ [50,55]	+ [62]	−[c] [58]	− [58]	− [58]
Chloramphenicol	−[b] [50]	+ [50,55]	± [62]	+ [58]	+ [58]	+ [58]
Erythromycin	−[b] [50]	s [50,55]	s [62]	+ [58]	+ [58]	+ [58]
Lincomycin	± [50]	+ [50]	± [62]	+ [58]	+ [58]	+ [58]
Puromycin		+	+	± [58]	− [58]	± [58]

[a] +, inhibition of binding; ±, partial inhibition of binding; −, no inhibition of binding; s, stimulation of binding.
[b] The antibiotic inhibits the sparsomycin-dependent binding of the fragment.
[c] Sparsomycin inhibits the binding of chloramphenicol to polysomes.

slightly stimulatory effect on this binding [51,54,55,59]. Conversely, the binding of the acetylated fragment is affected by lincomycin, but not by chloramphenicol or erythromycin [58]. These last two drugs, nevertheless, inhibit the binding of the acetylated substrate that takes place in the presence of sparsomycin [53]. These results show that these drugs are apparently unable to compete for binding to the ribosome with the acetylated fragment and with sparsomycin when tested separately, but block the sparsomycin-dependent binding of the same substrate. These observations are rather surprising and difficult to understand at present.

An additional indication of the differences in the binding sites of chloramphenicol, lincomycin and erythromycin is the fact that chloramphenicol alone is capable of interacting with polysomes. The interaction is also sensitive to sparsomycin [60,61].

The binding of puromycin, which – due to its acceptor activity in the peptide bond forming reaction – is the simplest substrate interacting at the ribosomal A-site, is blocked totally by sparsomycin, partially affected by chloramphenicol and lincomycin, and notably stimulated by erythromycin [62].

The combined facts suggest that sparsomycin binds to the ribosomes at a site totally different from that of erythromycin and lincomycin. These share a partially overlapping site, probably affecting both the A- and P-sites on the peptidyl transferase. Chloramphenicol and sparsomycin apparently bind to the A-site, although their binding sites are not identical [57].

The most simple interpretation of the competition experiment results implies a direct hindering effect on the blocked ribosomal sites by these drugs. Alternative modes of action, implying steric effects, are also possible considering the strong co-operation that prevails among the different ribosomal components. At present there is no evidence permitting a choice between these alternatives. A satisfactory choice requires extra data on ribosomal binding site structures. Affinity-labelling studies have proven most valuable in this context [63].

Long-range effects of sparsomycin binding

Although sparsomycin binds to the A-site, the drug also affects the peptidyl transferase, stimulating the binding of P-site substrates such as *N*-acetylated fragments. This sparsomycin-stimulated binding of substrates takes place, however, at a ribosomal region that has different structural characteristics from the normal functional P-site, as indicated by affinity-labelling studies [64]. Antibiotics that do not affect the binding of substrates at the P-site inhibit the sparsomycin-dependent interaction, confirming also that the binding site must be at least partially different in this case (*vide supra*).

There is no current evidence to suggest that sparsomycin interacts directly with the P-site. It can be assumed that the aforementioned stimulation of the binding of P-site substrates is due to allosteric effects which induce a conformational change, thereby increasing the affinity of the ribosome for the substrates at this site. It is therefore probable that sparsomycin action on the P-site will also inhibit the peptide bond formation, independently of the direct effect of sparsomycin on the A-site.

It has been reported [27,32] that the first peptide bond of natural mRNA in some eucaryotes and the unblocked Phe-tRNA in *E. coli* poly(U)-dependent systems show low sensitivity towards sparsomycin. That the A-site is affected by the P-site must be considered to explain this aspect of the overall mechanism of the drug. It is probable that occupancy of the P-site increases the affinity of the A-site for sparsomycin. The α-amino group of the donor substrate at the P-site has to be blocked, however, in order to bring about this effect. Neither Phe-tRNA [32] nor the eucaryotic initiator, Met-tRNA [27], are therefore able to stimulate the binding of substrates to the A-site. Here, sparsomycin cannot compete with the acceptor substrate at the concentrations that usually inhibit peptide bond formation and as a consequence of this, peptide bond formation takes place. When the resulting dipeptidyl-tRNA is translocated to the P-site, the affinity of the A-site for sparsomycin increases and the formation of the second peptide bond is inhibited.

The effect of substrate binding to the P-site on the affinity of the A-site for sparsomycin would explain also why sparsomycin is able to compete for binding with chloramphenicol in polysomes, but not in free ribosomes [60,61].

The evidence that suggests mutual interaction between A- and P-sites during antibiotic binding [61] is presently speculative. Nevertheless, this mutual interaction appears to be the most probable explanation for sparsomycin's action at the P-site.

Structure of the binding site

Little is known about the interaction between ribosomal components and sparsomycin. Affinity-labelling studies indicate that several ribosomal proteins, such as L16, L2, L27, in addition to the 23 S RNA, possibly form part of the peptidyl transferase P-site [65]. It is conceivable that one of these components forms part of the sparsomycin-binding site. It has been reported that protein L23 is involved also at the puromycin-binding site [66]. This drug competes with sparsomycin for ribosomal binding, which suggests that protein L23 could also be involved at the sparsomycin-binding site. It is unreliable to propose theories based on the results obtained using other ligands. Additional evidence

from sparsomycin analogues is still required. It is expected that affinity-labelling studies using these analogues will shortly provide this information.

The analysis of ribosomal alterations on mutant strains can provide structural data on antibiotic-binding sites. For sparsomycin this information is unavailable. Although two sparsomycin-resistant strains have been described, isolated from *E. coli* [20] and mouse mammary carcinoma [67], possible ribosomal alteration studies have not been performed.

Does sparsomycin inactivate ribosomes irreversibly?

The irreversible inactivation of ribosomes by sparsomycin was first reported by Coutsogeorgopoulos, Miller and Hann [38]. The results of studies on the reaction of puromycin with ribosomes bound to nitrocellulose filters indicated that in ribosomes pretreated with sparsomycin the rate of peptide bond formation was only 10% of that observed in untreated ribosomes. It was proposed that the previously reported [26] time-dependent effect of sparsomycin was due to irreversible ribosomal inactivation. The presence of different binding sites for puromycin and sparsomycin was also suggested.

The existence of irreversible ribosomal inactivation by sparsomycin conflicts with the well-established competitive inhibition character of the drug on similar puromycin reactions [26,33,36]. The primarily bacteriostatic rather than bactericidal effect of sparsomycin on *E. coli* cells [18] in addition to its cytostatic activity on fibroblasts [68] do not concur with an irreversible action of the drug.

The observation that ribosomes from sparsomycin-treated mice are less active than those from untreated animals [22] supports the permanent inactivation of the particles. This type of experiment is, however, open to criticism. Inactivation of the ribosomes by long-term effects, as a consequence of a prolonged drug-induced alteration of the cell metabolism, is also possible.

The effect of several sparsomycin analogues on the puromycin reaction confirms the competitive nature of the inhibition [39]. This competitive inhibition proceeds apparently *via* a binding process not involving the sulphoxide group of the molecule. The possible involvement of this functional group in a posterior reaction at the binding site, causing permanent ribosomal inactivation, was proposed. Recent [69] puromycin tests on polysomes have extended these results and showed that only sparsomycin analogues carrying sulphoxide groups with the correct configuration are able to produce the so-called 'preincubation effect', thereby increasing the extent of inhibition upon preincubation with the ribosomes.

Support for the involvement of the sulphoxide group in the preincubation effect of sparsomycin has been obtained using a sparsomycin analogue bearing a phenolic residue which facilitates the *O*-acylation of the sulphoxide. Although this compound is a poor inhibitor of protein synthesis, it shows a preincubation effect that is higher than that observed using sparsomycin [70].

These results, which will be discussed in the section 'Molecular basis of action', are indeed indicative of an irreversible action of sparsomycin. Caution must be exercised in their interpretation, since the treatment of the particles with relatively high concentrations of the drug could favour secondary reactions to take place that are considerably less important than when the drug is added at the initiation of the inhibition process. The results indicating an inactivation of the small subunit of *S. cerevisiae* upon preincubation with sparsomycin [28] are highly significant in this respect. This inactivation is probably due to an unspecific action of the drug. It has been shown clearly to bind specifically to the large ribosomal subunit only [54].

In conclusion, permanent inactivation of ribosomes by sparsomycin is therefore possible. However, permanent inactivation does not necessarily imply irreversible binding of the drug. Thus, virginiamycin M has been shown to inactivate permanently, in a non-enzymatic way, the large ribosomal subunit of bacterial ribosomes without exhibiting an irreversible interaction [71,72].

ANALOGUES AS INHIBITORS OF PROTEIN SYNTHESIS

Most of the structure-activity relationship studies of sparsomycin that appeared prior to its first total synthesis [11] were concerned with analogues in which several structural parameters had been varied simultaneously; mixtures of stereoisomers were frequently studied. The development of total syntheses of sparsomycin [8,11–15] facilitated the preparation of homochiral analogues of the antibiotic carrying single structural variations on specific parts of the molecule. This allowed an unambiguous interpretation of the results with regard to the rôle of the various structural fragments. The analogues are listed in *Tables 6.1–6.7*. Interpretation of part of the available information on structure-activity relationships of sparsomycin is hampered by the fact that many of the analogues have been tested using different assays, *in vitro* as well as *in vivo*.

In *Table 6.9* the results obtained in several peptide bond formation assays in *E. coli* are summarized. (For information regarding peptide bond formation assays in *S. cerevisiae* and *S. solfataricus*, see Ref. 29.) As can be seen from this table, the polyphenylalanine synthesis assay discriminates only partially between the activities of the less active analogues at the concentrations tested.

Table 6.9. INHIBITION OF PEPTIDE BOND FORMATION *IN VITRO* IN *E. COLI* BY SPARSOMYCIN AND DERIVATIVES

The numbers express the ratio of the ED_{50} value of sparsomycin and the ED_{50} value of the analogue; ED_{50} represents the dose in μM that inhibits growth to 50% of control growth. The ED_{50} values (in μM) for sparsomycin are given in parentheses.

Compound	*polyPhe synthesis*	*Fragment reaction*	*Puromycin reaction on:*	
			ribosomes	*polysomes*
(1)	1 (8.5)	1 (3.2)	1 (0.1)	1 (0.3,0.5)
(1.1)	0.004 [29]	0.003 [29]	0.001 [29]	0.015 [75]
(1.2)	0.004 [29]	0.005 [29]	0.008 [29]	0.069 [75]
(1.3)	0.004 [29]	0.003 [29]	0.002 [29]	0.004 [75]
(1.7)	5.3 [29]	0.053 [29]	0.7 [29]	
(1.8)	14.2 [29]	0.4 [29]	0.8 [29]	
(1.9)	1.06 [16]	0.2 [16]	0.95 [16]	
(2.3)	0.06 [29]	0.7 [29]	0.43 [29]	
(2.6)	4.7 [29]	0.001 [29]	1.3 [29]	
(2.8)				0.17 [34]
(2.9)				0.19 [34]
(2.10)	0.019 [29]	0.4 [29]	0.15 [29]	
(2.11)	0.004 [29]	0.001 [29]	0.001 [29]	
(2.13)				0.067 [34]
(2.14)				0.28 [34]
(2.15)				0.14 [34]
(2.18)				0.03 [34]
(2.19)				0.06 [34]
(2.20)				0.14 [34]
(2.21)				0.04 [34]
(3.4)			0.002 [39]	
(3.5)			0.45 [39]	
(3.6)			0.25 [39]	
(3.8)			0.22 [39]	
(3.11)			0.41 [39]	
(3.12)	0.004 [29]	0.006 [29]	0.02 [29]	
(3.13)	0.004 [29]	0.003 [29]	0.001 [29]	
(3.14)			0.92 [39]	
(3.15)	0.004 [29]	0.004 [29]	0.001 [29]	
(7.2)			0.001 [77]	
(7.3)			0.001 [77]	
(7.4)			0.17 [77]	
(7.5)	0.4 [29]	0.2 [29]	0.27 [29]	
(7.6)	0.017 [29]	0.1 [29]	0.002 [29]	

Sparsomycin – binding to the A-site of the peptidyl transferase centre – interferes more efficiently with the interaction of puromycin with the ribosome than of aminoacyl-tRNA with the ribosome. This difference reflects the difference in the affinity of puromycin and phenylalanyl-tRNA for the ribosome, this being much higher in the case of aminoacyl-tRNA.

The structural features of sparsomycin that are required for an optimal activity as an inhibitor of protein biosynthesis are discussed here.

The configuration of sparsomycin

The presence of two chiral centres in the molecule results in the existence of four stereoisomers, all of which have been synthesized [11]. The necessity of an *S* configuration at the chiral carbon atom [17,39,73–75] for optimal biological activity is unequivocally demonstrated by comparison of the values presented in *Table 6.9* for compounds (1) *vs.* (1.3), (3.4) *vs.* (3.5) and (3.12) *vs.* (3.13), respectively. Compounds having an *R* configuration at the chiral carbon atom have a significantly lower activity than those having the *S* configuration.

A comparison of the activity of (1) and of the analogues (1.2), (2.10) and (2.11) demonstrates clearly the importance of an *R* configuration of the sulphoxide sulphur atom [17,29,69,75]; compounds (1.2) and (2.11) are considerably less active than (1) and (2.10), respectively, from which they differ only by having the opposite configuration at the sulphoxide sulphur atom. In summary, of the four possible stereoisomers, sparsomycin having the S_cR_s configuration is the strongest inhibitor in all of the systems tested.

The presence and oxidation state of the sulphur atoms

The importance of the presence of an oxygen atom on S(α) can be derived from a comparison of the activity of compounds (1) and (1.2) on the one hand, and (3.12) on the other. This conclusion is in accordance with the findings of Lin and Dubois [73], who demonstrated that the biological activity, if any, of the synthetically more accessible monosulphide analogues (R = *S*-alkyl-substituted cysteinol) is invariably lower than that of the corresponding sulphoxide analogues (R = *S*(*O*)-alkyl-substituted cysteinol).

The importance of the proper positioning of the sulphoxide moiety in the cysteinol side-chain of (1), S(α) *vs.* S(β), is demonstrated by comparing the activity of (1) and (3.15); when the positions of the sulphoxide function and the sulphur atom are reversed, the biological activity is reduced markedly. In addition, analogue (3.15) possesses a diminished activity compared with that of the corresponding *S*-deoxo analogue (3.12), suggesting an adverse effect of

a sulphoxide function at the S(β) position. Comparison of the activity of (3.15) with that of (1) and (1.2) shows that the monoxodithioacetal moiety, as such, does not determine the biological activity. A bivalent β-sulphur atom is apparently preferable for an optimal activity.

The correct oxidation state of sulphur in the compound was shown by Flynn and Ash [69] to be necessary to observe a 'preincubation effect' [34,38,39,69,75]. The corresponding sulphide and sulphone analogues had no effect on preincubation of ribosomes with the compound, whereas sparsomycin exhibited a larger effect on peptidyl-puromycin synthesis after preincubation with ribosomes (see section 'Molecular basis of sparsomycin action').

Lee and Vince [39b] showed that the racemic *S*-deoxo derivative (3.14) was almost as active as sparsomycin itself, which appeared to be in conflict with the results obtained by Flynn and Ash [69]. It was suggested that the sulphoxide moiety is not involved in initial, reversible binding of the antibiotic to the ribosome. The presence of the sulphoxide function could enable the molecule to undergo an additional reaction at the binding site which is characteristic of irreversible inhibition; the so-called 'preincubation effect' (see section 'Inhibition of protein synthesis').

Hydrophobic side-chain

A considerable number of sparsomycin analogues carrying a modification at the sulphur-containing end of the molecule have been synthesized (*Tables 6.1–6.4*). These analogues permitted the significance of this part of the molecule in relation to the activity of the drug to be studied. In these derivatives aromatic groups or hydrocarbon chains of different lengths take the place of either the SMe function or the methyl group in this function. Some of the analogues containing a hydrophobic group show a very high activity when tested in the polymerization assay. Benzylsparsomycin (1.8) is about 14-fold, whilst octylsparsomycin (1.7) and *S*-oxo-*S*-decylsparsomycin (2.6) are both about 5-fold more active than the unmodified drug. It is of interest that these three compounds have an activity in the puromycin reaction which is similar to that of sparsomycin, suggesting that the modification increases the capacity of the derivatives to interfere with the aminoacyl-tRNA without increasing their effect on the puromycin reaction. These results seem to indicate that the hydrophobic group introduced in the molecule interacts with a ribosomal region that forms part of the peptidyl transferase centre and that is involved in the binding of the aminoacyl-tRNA molecule, but which is not implicated in the puromycin-binding site.

It is noteworthy that compounds (1.7), (1.8) and (2.6) show very low

inhibitory activities when tested in the fragment reaction. This is probably due to the presence of ethanol in the reaction mixture. The alteration of the solvent characteristics might affect the interaction of these hydrophobic derivatives with the ribosome [29]. Care is necessary, therefore, when using this peptide bond formation test to compare analogues with different physicochemical properties.

The high activity of *S*-oxo-*S*-decylsparsomycin (2.6) and octylsparsomycin (1.7) indicates also that the partial loss of activity caused by removal of the bivalent sulphur atom, (1) *vs.* (2.3), can be compensated for by the addition of aliphatic chains of appropriate length. Even in the case of a deoxygenated compound (3.9), the presence of the *S*-decyl side-chain partially restores the activity of the compound [73].

A marked increase in biological activity can be seen in the series of phenyl-analogues in the order (2.13) < (2.15) < (1.8). This difference in activity can be attributed to an increase in hydrophobicity in this series. An alternative explanation is, however, possible which is based on the consideration that there has to be a minimal distance between the uracil ring and a hydrophobic moiety; in sparsomycin the hydrophobic moiety is the CH_2SMe function (see also section 'Molecular basis of action'). An increase in activity in the order (2.13) < 2.15) < (1.8) supports this hypothesis, which might be a consequence of an increase in length of the moiety linking the sulphinyl and the phenyl group, i.e., RS(O)Ph, $RS(O)CH_2Ph$ and $RS(O)CH_2SCH_2Ph$, respectively. If this hypothesis is correct, an extension of the chain linking the aromatic group to the RS(O) part of the molecule in derivatives (2.13)–(2.21) may render them more active.

Complete removal of the $S(O)CH_2SMe$ moiety eliminates the ribosomal binding affinity of the analogue [39]. Replacement of this moiety by other lipophilic side-chains (*Table 6.4*) causes a substantial decrease in activity. Among these analogues the bromophenyl derivatives (4.4), (4.8) and (4.19) impart the largest inhibitory activity in a protein synthesis assay which is based on incorporation of [^{75}Se]selenomethionine [76]. This might be due to the high lipophilicity and electron-withdrawing character of the bromo substituent.

Hydroxymethylene group

The hydroxyl function of sparsomycin has been blocked by acetylation (1.9) [29]. This compound shows an activity similar to that of sparsomycin itself, indicating that the hydroxyl group is not essential for the activity. To investigate this effect further, the *O*-methylated derivative (1.10), as well as the analogues (1.11) and (1.12) in which the hydroxyl group has been replaced by hydrogen, have been prepared [29], but have not as yet been tested.

Pyrimidinyl ethylene moiety

The C(6)-methyl group and the *trans* geometry of the alkene function are important for an optimal activity as determined by the low inhibitory activity of compounds (7.5) and (7.6) in the peptide bond formation assays (*Table 6.9*). Analogue (7.6) lacking the C(6)-methyl group shows low activity, whilst compound (7.5) having a *cis* alkene function is inactive.

A hybrid antibiotic called sparsophenicol (7.4) was constructed by combination of the pyrimidinyl acrylic acid moiety of sparsomycin and the nitrobenzyl part of chloramphenicol [77]. This new antibiotic inhibits the reaction of *N*-acetyl-Phe-tRNA with puromycin in a competitive manner, showing a considerable activity (*Table 6.9*). It is an interesting fact that the amide (7.2) and the ester (7.3) of the pyrimidinyl acrylic acid, although poor inhibitors, also exhibit a clear competitive inhibition pattern.

These results suggest that the uracil ring plays an important rôle in the inhibition of pepide bond formation by sparsomycin. The pyrimidine moiety probably binds to a ribosomal site which totally or partially overlaps with the puromycin-binding site. The molecular basis of the mechanism of action of sparsomycin and related compounds is discussed in greater detail in the following section, 'Molecular basis of action'.

MOLECULAR BASIS OF ACTION

The true molecular mechanism by which the drug produces its effects is not yet fully understood. Some models for the mode of action of sparsomycin have been proposed which, considering the available data, elicit a general theory and experimentally verifiable hypotheses.

A mechanistic model of peptidyl transfer inhibition

Sparsomycin has structural similarities with the 3′ end of the acceptor tRNA molecule (*Figure 6.1*). The binding of sparsomycin to a site on the peptidyl transferase centre that partially overlaps with the interaction site for the aminoacyladenine moiety of the aminoacyl-tRNA is the most acceptable mechanism of action. The bound drug will totally block the interaction of small-size substrates, such as the 3′ terminal fragments and puromycin. It is also able to disturb the correct positioning of the 3′ end of the large aminoacyl-tRNA molecule. In both cases, the formation of the peptide bond will be inhibited.

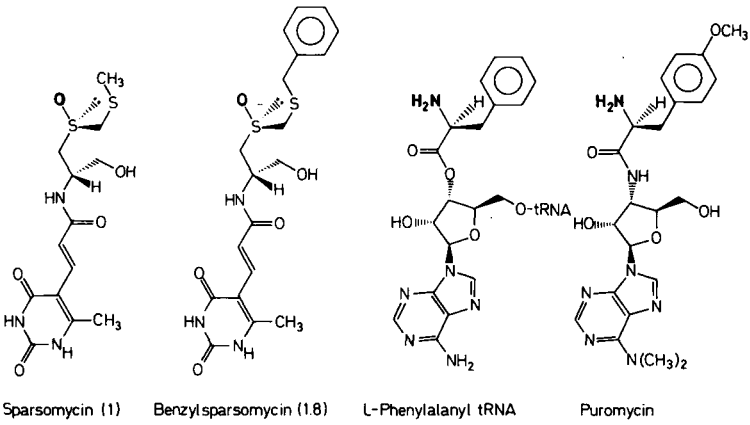


Figure 6.1.

This hypothesis of overlapping binding sites agrees with the competitive nature of this puromycin reaction inhibition by sparsomycin (see section 'Inhibition of protein synthesis'). It is compatible with a high sensitivity of this reaction to the drug ($ED_{50} \approx 0.1\ \mu M$), considering the low affinity of puromycin for the ribosome ($K_a \approx 100\ \mu M$) [62]. In the case of the amino-acid polymerization, the higher affinity of aminoacyl-tRNA for ribosomes ($K_a \approx 1\ \mu M$) explains the lower sensitivity of this reaction towards sparsomycin ($ED_{50} \approx 1\ \mu M$).

The results obtained using the more hydrophobic derivatives of the drug (see e.g. (1.8) in *Figure 6.1*), support the view that the binding sites of the aminoacyl moiety of aminoacyl-tRNA and sparsomycin overlap at the peptidyl transferase.

The high sensitivity of phenylalanine polymerization to sparsomycin analogues carrying a hydrophobic group at the sulphur-containing end of the molecule (*Table 6.9*), suggests that these compounds interact with a region on the peptidyl transferase that probably functions as the recognition site for the lateral chains of the hydrophobic amino acids during protein synthesis. The existence of such a region at the peptidyl transferase centre is supported by data obtained using different aminoacyl-A-C fragments as acceptor substrates. It has been reported that Phe-A-C, probably the most hydrophobic of these substrates, shows the lowest K_M(app) in the peptide bond forming reaction, suggesting that it has the highest affinity for the ribosome [78]. The postulated interference of sparsomycin with the aminoacyl residue of aminoacyl-tRNA

also agrees with the different sensitivity of polymerization systems, which depend on synthetic mRNAs of different nucleotide composition, found earlier in studies on sparsomycin [21,26]. The sensitivity of these systems to the drug in the proposed model will depend, amongst other parameters, on the respective affinities of the aminoacyl residues for the peptidyl transferase centre.

The distance between the hydrophobic region and the pyrimidine base – another significant site for interaction with the peptidyl transferase centre – is important, since, as discussed in the previous section, 'Analogues as inhibitors of protein synthesis', the link joining the aromatic residue to the drug requires a minimum size. Moreover, it is noteworthy in this respect that the distance between the aromatic group on the one side and the amino and sulphoxide functions in L-phenylalanyl-tRNA and benzylsparsomycin (1.8) respectively on the other, differ considerably, being larger for the sparsomycin analogue (*Figure 6.1*).

A chemical rationale for possible irreversible inactivation of ribosomes by sparsomycin

There is evidence that the 'preincubation effect' of sparsomycin on ribosomes – an indication of irreversible inactivation – could be related to the sulphoxide group present in the molecule. Direct evidence relating this preincubation effect to a direct inhibition of the peptidyl transferase is currently unavailable. This point needs to be demonstrated unambiguously, since the possibility exists that inactivation might be due to a secondary effect on the small ribosomal subunit. This possibility and the preincubation effect are discussed in the section 'Inhibition of protein synthesis'.

Irreversible inactivation is concordant with some of the available evidence. Flynn and Ash [69] have presented a mechanism resulting in covalent binding of the drug to the nascent peptide chain. It was suggested that sparsomycin might act as an enzyme-activated inhibitor of peptidyl transferase (*Figure 6.2*). It was proposed that the sulphoxide moiety of the drug might be *O*-acylated by an acylimidazole intermediate of the peptide bond forming reaction. A Pummerer process [79] can be thus initiated resulting in covalent binding of the molecule to the nascent peptide chain. The structural similarity of the drug and the 3′ end of the aminoacyl-tRNA was stressed (*Figure 6.1*). However, the same authors prepared a tritium-labelled sparsomycin derivative and found that at least a portion of this derivative was associated with ribosomal structures and not – as stated above – to the nascent peptide chain. This finding

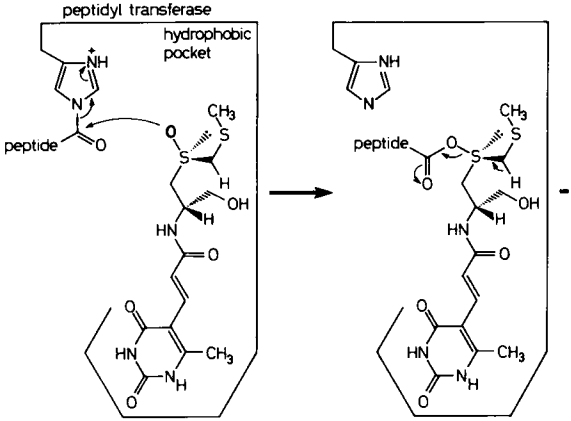


Figure 6.2. Mechanism of action of sparsomycin [69,75].

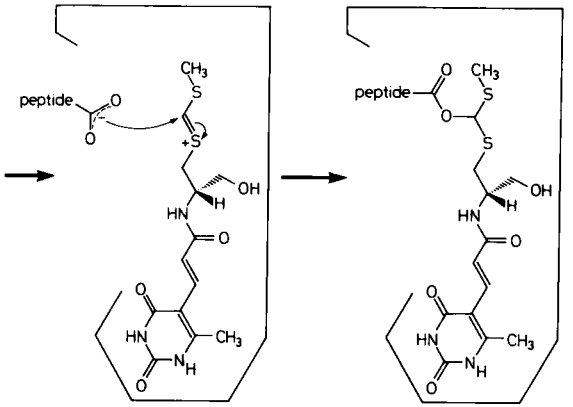
might explain the irreversibility of the binding of sparsomycin to the peptidyl transferase.

The above-mentioned rationale seems to be in conflict with evidence indicating that the sulphoxide group is not involved in the binding of sparsomycin [39]. However, it is probable that the irreversible inactivation of the ribosome is the result of a reaction caused by the drug after binding. In this event, the irreversible reaction must be independent of the inhibition process itself, which is caused by the initial and reversible binding of the drug.

The model presented by Flynn and Ash is, however, of great interest. It not only suggests an elegant mechanism for an irreversible action of sparsomycin but in addition, if confirmed, increases the insight into the peptide bond forming reaction. It is therefore essential to produce experimental evidence in support of it. It would be most logical to demonstrate the formation of a covalent bond between sparsomycin and the ribosome. This point has not yet been demonstrated unambiguously.

CYTOTOXICITY

Sparsomycin, being an inhibitor of protein synthesis, must be intrinsically toxic to cells permeable to the drug. It has been reported for growing cultures of *E. coli* B [18] that the action of the antibiotic is mainly bacteriostatic and not bactericidal. Likewise, Chinese hamster fibroblasts incubated for 1 h with drug concentrations inhibiting 99% of their protein synthesis recover totally after removal of the drug [68]. The cells do not recover their synthetic capabilities



completely after exposure periods exceeding 4 h. Their protein synthesis mechanisms have then, presumably, been damaged too extensively. The duration of drug exposure is apparently important for cytotoxicity; deprivation of cells from vital enzymes and proteins, as a consequence of lasting protein synthesis inhibition, may have caused the observed cytotoxicity. Here, the data available on procaryotic and eucaryotic cells are discussed subsequently.

Effect on procaryotic cells

Initial studies [1] showed that sparsomycin is moderately active against a variety of Gram-negative and Gram-positive bacteria, the former appeared to be slightly more sensitive to sparsomycin. Subsequent efforts were concentrated on the mechanism of inhibition of the protein synthesis machinery, which was considered to be the main effect of sparsomycin. Unfortunately, other cellular alterations were disregarded. These alterations must have occurred, since it was found that the inhibition of bacterial growth by sparsomycin was accompanied by the release of several free L-amino acids from the cells into the growth medium [18,19]. The amount of amino acids released was dependent upon the extent of growth inhibition.

The effect of sparsomycin and several analogues on the growth of several bacteria in the solid phase as well as in liquid medium has been studied recently [29,34]. The results of this study, which are summarized in *Table 6.10*, enable the following conclusions to be drawn.

(1) Only a partial correlation exists between the observed structure-activity

relationship in whole-cell systems and that observed in the cell-free peptide bond formation assays. The relationship between the chirality at the carbon and the sulphur atom and the antibacterial activity correlates, for all species so far tested, to that observed in the peptide bond formation assays; compare the activities shown in *Tables 6.9* and *6.10* of compounds (1), (1.1)–(1.3), (2.10), (2.11), (3.12) and (3.13).

(2) In most of the bacteria tested there is no correlation between the activity of a given drug in peptide bond formation assays and the antibacterial activity. The hydrophobic analogues (1.8) and (2.6) show high activity in the peptide bond formation assays (*Table 6.9*), but are inactive against *E. coli* AB301 and *S. typhimurium*. This inactivity might be the result of permeability barriers.

It was reported [69] that the correct oxidation state of the sulphur atom is important for the entry of analogues of sparsomycin into bacteria with normal permeability barriers, i.e., *E. coli* Es39; sulphide- and sulphone-containing derivatives failed to inhibit the growth of these bacteria. Growth of *E. coli* Es31, which is freely permeable to a variety of antibiotics, was inhibited by all three analogues (sulphide, sulphoxide, sulphone). Care is necessary, however, in extrapolating the results of structure-activity relationship studies – obtained within one species – to another species.

(3) Conversely, compounds (1.2), (2.10) and (3.15), which show a low activity in the peptide bond formation assays, are surprisingly active against, for example, *E. coli* MRE600 and N281. One might interpret this result on the basis of changes in the permeability properties of these derivatives, facilitating their penetration through the cell membranes. However, this explanation is unlikely, since the growth inhibition observed would then have required too high a concentration of the analogue inside the cell to be realistic. On the basis of the above-mentioned results and considering the results mentioned previously on amino-acid release by sparsomycin, it is questionable whether alterations in cell membrane permeability are associated with cell growth inhibition. This problem has been studied using another protein synthesis inhibitor, i.e., streptomycin [80]. It was found that alterations in membrane permeability were associated with cell growth inhibition for this antibiotic. The protein synthesis machinery was, however, found to be the main target of streptomycin; this was demonstrated unambiguously by the isolation of mutants that possessed an altered ribosome structure and which were resistant to streptomycin. Inhibition of protein synthesis by streptomycin causes, therefore, a change in membrane permeability by a process not yet thoroughly understood. In the case of sparsomycin, information in this respect is lacking, but an action of the drug directly on membrane permeability cannot be excluded.

Table 6.10. INHIBITION OF BACTERIAL GROWTH BY SPARSOMYCIN AND DERIVATIVES

The diameter of the inhibition zone on agar plates was used as a measure for the activity of each drug. The numbers given express the ratio of the diameter measured for a drug and the diameter measured for sparsomycin. The diameter (in mm) of the inhibition zone for sparsomycin is given in parentheses.

Compound	E. coli *MRE600 liquid*[a]	E. coli *MRE600*	E. coli *AB301*	E. coli *N281*	E. coli *W3110*	Bacillus cereus	Bacillus subtilis	Sarcina lutea	Salmonella typhimurium	*Reference*
(1)	1 (11)	1 (14)	1 (14)	1 (18)	1 (14)	1 (10)	1 (7)	1 (10)	1 (6)	
(1.1)	0.01	0	0	0	0	0	0	0	0	[29]
(1.2)	0.92	1	0.6	0.8	0.6	0.3	0	0.3		[29]
(1.3)	0.32	0.5	0.2	0.1	0.1	0.1	0	0		[29]
(1.7)	2.5	0.7	0	0	0	0.1	0.1	1.2		[29]
(1.8)	1	1.2	0	0.3	0.1	0.9	0	3.3	0	[29]
(1.9)	0.27	0.7	0.7	0.9	0.6	0.6	0.6	0.5	trace	[16]
(2.3)	0.30	0.7	1	1	0.7	1	0.6	1.2	0.9	[29]
(2.6)	3.6	1.1	0	0	0	1.9	2.4	3.6	0	[29]
(2.8)	0.26									[34]
(2.9)	0.31									[34]
(2.10)	0.55	0.8	1.1	1	0.7	2	1.2	1.2	1.2	[29]
(2.11)	0.01	0	0	0	0	0	0	0	0	[29]
(2.13)	0.59									[34]
(2.14)	0.23									[34]
(2.15)	0.22									[34]
(2.18)	0.01									[34]
(2.19)	0.07									[34]
(2.20)	0.12									[34]
(2.21)	0.08									[34]
(3.12)	1.8	1.5	0.4	0.6	0.6	0.6	0.9	0.6		[29]
(3.13)	0.01	0	0	0	0	trace	0	0		[29]
(3.15)	0.40	0.6	0.2	0.4	0.2	trace	0	0		[29]
(7.5)	0.47	0.7	0.7	0.8	0.8	0.7	0.4	0.8		[29]
(7.6)	0.27		0.9	0.5	0.7	0.3	trace	0	0	[29]

[a] The numbers express the ratio of the ED_{50} value of sparsomycin and the ED_{50} value of the analogue; ED_{50} represents the dose in μM that inhibits growth to 50% of control growth.

(4) The antibacterial activity of analogues (1.7) and (2.6) depends on the species as well as on the strain used. The strain-dependency of the antibacterial activity for the hydrophobic derivatives (1.7) and (2.6) is illustrated by the fact that of the four *E. coli* strains tested, only the growth of strain MRE600 is inhibited by these derivatives. The other three *E. coli* strains are completely resistant to these compounds. At present, ignorance of the permeability mechanisms for sparsomycin as well as of the detailed cell wall structures of the different bacterial species and strains precludes interpretation of these data.

In summary, changing the structure of sparsomycin influences the antibacterial activity of the drug. This correlation is governed firstly by the ability of the drug to penetrate the cell and secondly by the ability of the drug to inhibit protein synthesis. Analogues that have an optimal combination of these two properties are expected to exert an optimal antibacterial activity. In addition, the antibacterial activity of a given drug, for example, (1.8), varies depending on the bacteria used. This species- and strain-dependent difference in sensitivity might be explained by differences in permeability of the bacterial membrane.

Effect on eucaryotic cells

The effect of sparsomycin on Chinese hamster fibroblasts is reversible after 1 h period of incubation [68] (*vide supra*). Exposure of the cells to the drug for longer periods causes cell death. The fibroblasts are sensitive to sparsomycin only during the S phase of the cell cycle. This suggests that inhibition of histone synthesis may be responsible for the observed cell death.

The permeability of eucaryotic cells to sparsomycin, as in the case of bacterial cells, is also an important parameter when analyzing the toxicity of the drug. The eucaryotic cellular envelopes are simpler in structure than those of bacteria, but still show a differential permeability. Sparsomycin was found to have no effect on protein synthesis in intact rabbit reticulocytes *in vitro*. In a cell-free system of these reticulocytes, however, sparsomycin inhibited protein synthesis markedly; it was concluded that the antibiotic did not penetrate the intact reticulocyte [23].

Sparsomycin itself has a pronounced cytotoxic effect on HeLa cells *in vitro*. The data on analogues (2.8), (2.9), (2.13)–(2.15) and (2.18)–(2.21) shown in *Table 6.11* indicate that modification of the hydrophobic sulphoxide substituent on sparsomycin decreases the toxicity of the molecule for mammalian cells by several hundred-fold. This decrease in toxicity on HeLa cells is not due to decreased activity in the protein synthesis assay. Several of the non-toxic compounds showed a marked affinity for the ribosome when

tested in a cell-free system for peptidyl-puromycin synthesis on polysomes from HeLa cells [34].

Compound (2.15) was active against *E. coli* Es31, but inactive against HeLa cells [34]. This differential effect in activity was due to a decreased uptake of the compound by HeLa cells. For this study, a tritium-radiolabelled analogue of (2.15) was prepared [34]. Sparsomycin has, as was initially reported [1], a distinct cytotoxic effect on KB human epidermoid carcinoma cells in tissue culture and has therefore been evaluated *in vivo* as an antitumour agent (see

Table 6.11. INHIBITION OF MAMMALIAN CELL GROWTH BY SPARSOMYCIN AND ANALOGUES

The numbers express the ratio of the ED_{50} value of sparsomycin and the ED_{50} value of the analogue; ED_{50} represents the dose in μM that inhibits growth to 50% of control growth. The ED_{50} values (in μM) for sparsomycin are given in parentheses.

Compound	*KB*	*L1210*	*HeLa*	*Reference*
(1)	1 (0.2)	1 (0.4)	1 (1.2)	
(1.1)		0.001		[17]
(1.2)		0.07		[17]
(1.3)		0.001		[17]
(1.7)		4.0		[17]
(1.8)		3.6		[29]
(1.9)		1.1		[16]
(2.2)	0.04			[73]
(2.3)		0.53		[29]
(2.5)	0.06			[73]
(2.6)		2.9		[29]
(2.8)			0.06	[34]
(2.9)			0.03	[34]
(2.10)		1.4		[29]
(2.11)		0.013		[29]
(2.12)	0.03			[73]
(2.13)			0.03	[34]
(2.14)			0.02	[34]
(2.15)			0.01	[34]
(2.18)			0.008	[34]
(2.19)			0.009	[34]
(2.20)			0.01	[34]
(2.21)			0.01	[34]
(3.12)		0.03		[17]
(3.13)		0.001		[17]
(3.15)		0.002		[17]
(3.16)		0.001		[17]
(7.5)		0.47		[29]
(7.6)		0.03		[29]

section 'Preclinical and clinical studies'). The high activity of sparsomycin against KB cells reported in 1962 [1] was not confirmed, however, in 1975 [81a]. Three analogues (2.2), (2.5) and (2.12) showed a substantially lower activity than the unmodified drug against these carcinoma cells [73]. This result indicates that substitution of the CH_2SMe moiety by an Me, $(CH_2)_3Me$ or $CH_2S(O)Me$ group decreases the activity of the drug.

The *in vitro* activity of sparsomycin and several analogues against leukaemia L1210 cells [17,29] or against Ehrlich ascites carcinoma cells [74,82] has been reported. These assays were used to select compounds having a higher antitumour activity than the unmodified drug itself (see also section 'Antitumour activity'). For the L1210 system, good correlation was found between the *in vitro* and the *in vivo* antitumour activity of the tested drug [17]. Thus, the *in vitro* system is of predictive value for the L1210 *in vivo* system in the mouse.

None of the compounds (3.1), (3.2), (4.10)–(4.15) or (7.1) showed – contrary to sparsomycin – lytic action on Ehrlich ascites carcinoma cells [74]. These analogues have modifications in the $S(O)CH_2SMe$ moiety of sparsomycin. The results on these derivatives stress the importance of this entity for the activity of the drug.

As is shown in *Table 6.11*, compounds (1.7), (1.8) and (2.6) are stronger inhibitors of L1210 colony formation than the unmodified drug. Two derivatives, octylsparsomycin (1.7) and benzylsparsomycin (1.8), were studied in greater detail against L1210 leukaemia in mice (see section 'Antitumour activity').

The *in vitro* activity of sparsomycin against L1210 cells depends strongly on the time of incubation [83]. The drug dose causing 50% inhibition of L1210 colony formation relative to untreated control cells (ID_{50}) decreases from 33.8 μM after 1 h of incubation to 5.5 μM after 6 h of incubation. Continuous incubation for 8 days results in a further decrease of the ID_{50} to 0.8 μM. This result may be related to the earlier reported S-phase specificity of sparsomycin [68] (see section 'Inhibition of protein synthesis').

In general, the data from the peptide bond formation assays (*Table 6.9*) and those on inhibition of cell growth (*Table 6.10*) correlate well in the case of eucaryotic cells. This correlation – not found with procaryotic cells – suggests that in eucaryotic cells inhibition of peptidyl transferase is the main effect of sparsomycin and its derivatives. Some results support this, whereas others are contradictory. Support is found by the isolation of a mouse mammary carcinoma cell line resistant to the antibiotic blasticidin S [67]. This mutant cell line – altered in the 60 S ribosomal subunit – shows some cross-resistance to sparsomycin.

Contradictory results have been observed. Sparsomycin was found to be

particularly toxic to liver cells [22,84–88]. When administered intraperitoneally to mice, sparsomycin induced marked disaggregation of hepatic membrane-bound polyribosomes and inhibited incorporation of [^{14}C]leucine into hepatic proteins by 90%. On the contrary, the addition of sparsomycin *in vitro* to a cell-free amino acid incorporating system did not cause disaggregation of polyribosomes, yet inhibited the incorporation of [^{14}C]leucine into proteins by 80%. These apparently paradoxical results obtained with sparsomycin on hepatic polyribosomes, *in vivo* and *in vitro*, induced Sarma, Murty and Sidransky [85] to propose that sparsomycin does not act *in vivo* as an inhibitor of peptidyl transferase, but, rather, blocks the initiation step of protein synthesis. However, as discussed in the section 'Inhibition of protein synthesis', typical inhibitors of peptidyl transferase such as erythromycin and lincomycin – acting only on the formation of the first peptide bonds – induce disaggregation of polyribosomes.

An investigation to determine whether sparsomycin *in vitro* acts specifically on tumour cells was conducted recently [89]. Sparsomycin was tested, using a clonogenic assay, against some murine tumours, human bone marrow and several human tumours. The results given in *Table 6.12* indicate that at a dose level of 0.1–0.3 μg/ml it is toxic selectively to murine and human tumours, but relatively non-toxic to human bone-marrow cells.

Table 6.12. ACTIVITY OF SPARSOMYCIN (1) *IN VITRO* AGAINST MURINE AND HUMAN TUMOURS AND AGAINST HUMAN BONE MARROW [89]

Cells were exposed continuously for 8 days to the drug. The drug was judged to be active when the number of colonies was less than 30% of the control. The ratio expresses the number of experiments in which the drug was judged to be active versus the total number of experiments.

	Sparsomycin (μg/ml)				
	0.01	*0.03*	*0.1*	*0.3*	*1.0*
Murine tumours[a]	0/3		4/5	5/5	5/5
Human tumours[b]	0/8	1/12	4/17	13/14	14/14
Human bone marrow		0/4	0/5	3/3	3/3

[a] P388, L1210, B16, LL, C38.

[b] Lung carcinoma (epithelial/adeno-), lung carcinoma (small cell), colon carcinoma, melanoma, stomach carcinoma, breast carcinoma, mesothelioma, thyroid carcinoma.

PRECLINICAL AND CLINICAL STUDIES

TOXICITY

Acute toxicity of sparsomycin and analogues

The acute toxicity has been determined in mice, dogs and rats. The 50% and 10% lethal doses (LD_{50} and LD_{10}, respectively) were determined after single intraperitoneal or intravenous injections. The results are shown in *Table 6.13*. Sparsomycin given orally to the mouse is probably not active, having a LD_{50} value greater than 20 mg/kg [81c]. The data of *Table 6.13* indicate that modification of the hydrophobic sulphoxide substituent decreases the acute toxicity by several factors of 10- and up to a 1000-fold. It was observed [90] that the LD_{50} values of (1), (1.7) and (1.8) in mice were subject to change when different mouse strains, solutes, or when suspensions were used.

Table 6.13. LD_{50} AND LD_{10} VALUES OF SPARSOMYCIN AND ANALOGUES
LD_{50} and LD_{10} are the 50% and 10% lethal doses, respectively.

Drug	*Species*	*LD_{50} (mg/kg)*	*LD_{10} (mg/kg)*	*Reference*
(1)	dog	0.5–1.0 i.v.		[81c]
(1)	rat	2.25 i.v.	1.07 i.v.	[81c]
(1)	mouse	4.32 i.v.	1.67 i.v.	[81c]
(1)	mouse	2.4 i.p.		[1]
(1)	mouse	4.2 i.p.	2.2 i.p.	[90]
(1.7)[a]	mouse	30–50 i.p.		[90]
(1.8)[a]	mouse	30–50 i.p.		[90]
2.8	mouse	134 i.p.		[34]
2.9	mouse	229 i.p.		[34]
2.14	mouse	630 i.p.		[34]
2.15	mouse	1600 i.p.		[34]
2.21	mouse	1114 i.p.		[34]

[a] These analogues are poorly soluble in water; therefore, vehicles other than water were used. The LD_{50} values, however, fluctuate depending on the vehicle used.

Toxicity to man, phase I clinical study

A phase I clinical study on sparsomycin was initiated in 1964, 2 years after the first description of the drug [91]. Five patients suffering from far-advanced carcinoma took part in this study. The patients were treated with increasing

daily doses of the drug. Two patients – Nos. 2 and 3 – developed ring scotomata, one after 13 days of therapy (total dose 0.24 mg/kg) and the other one after 15 days of therapy (total dose 0.15 mg/kg); this toxicity was later defined as sparsomycin-related retinopathy [92]. The phase I clinical study was discontinued.

It would be interesting to discuss these data in retrospect. The use of incremental daily doses has now been abandoned, because of the high risk of drug accumulation and toxicity [93]. The two patients that developed retinopathy had very low initial body weights – both 50 kg – suggesting poor general condition. Patient No. 5 was treated for 17 consecutive days and received a total dose of 0.154 mg/kg but, in contrast to patients Nos. 2 and 3, developed no retinopathy. This patient's initial body weight, 92 kg, suggested a better clinical condition.

On the basis of these considerations, the sparsomycin-related retinopathy is suggestive of a poor general condition and inappropriate drug schedules. This conclusion is supported by the results discussed in the following paragraph.

Organ toxicity

Of the animals studied, mice and rats are the most susceptible to sparsomycin-induced organ damage [81c]. Dogs and monkeys show hardly any gross or microscopic pathology at toxic doses of sparsomycin. Monkeys had less weight-loss than dogs after prolonged treatment with sparsomycin. No organ toxicity was found in humans after treatment with sparsomycin, apart from the observed eye toxicity.

Sparsomycin induces emaciation and cachexia in mice and rats [81c]. The animals suffer from diarrhoea (sometimes bloody) and haematuria. Occasionally, mice develop orbital bleeding [94,95,96a]. The retinopathy observed in man is, however, not due to this orbital bleeding. Microscopic examination of organs frequently discloses abnormalities of the pancreas and male reproductive systems; this was observed in rats only.

Sparsomycin induces emesis, bloody diarrhoea, petechies, dehydration, renal insufficiency and hypothermia in dogs [81c]. Haematological and biochemical studies reveal marked leucocytosis, mainly granulocytosis, elongation of prothrombine time and marked increase in SGOT and SGPT plasma activity. There is no evidence of bone marrow depression. The platelet counts remain normal. On occasions the number of reticulocytes decreases; emesis, diarrhoea and anorexia are probably the cause of dehydration and renal insufficiency. There is no evidence that sparsomycin damages the kidneys directly. All haematological and biochemical disturbances cease several days after drug discontinuation in monkeys as well as in dogs.

There were no observable pathological changes in the retinas of any animal treated with toxic doses of sparsomycin [81c]. Electroretinograms failed to show any drug-related functional disturbances in rats and monkeys. Such sparsomycin-induced disturbances were observed only in very sick animals shortly prior to death [81d]. These data suggest that sparsomycin, being water-soluble, does not pass through the blood-retina barrier in healthy animals. This integrity of the blood-retina barrier may be destroyed, however, in pathological conditions such as: post-radiation, previous treatment with cytotoxic drugs, infection or malnutrition. We propose that only under these conditions does sparsomycin reach the retina, inducing toxicity.

It has been described above that sparsomycin is particularly toxic to the liver [22,84–88]. The liver polysomes, affected by sparsomycin, disaggregate, making mRNA translation impossible (see section 'Cytotoxicity'). The liver compensates for protein loss due to the tumour by increased protein synthesis during cancer cachexia [88]. The inhibition of this compensatory mechanism may result in serious drug-induced toxicity.

The inhibition of the biosynthesis of liver-dependent blood coagulation factors may explain the side-effects observed in animals. It is interesting that blood coagulation disorders were rare or non-observable following treatment with benzylsparsomycin (1.8) or octylsparsomycin (1.7), respectively [90].

ANTITUMOUR ACTIVITY

Activity of sparsomycin against animal tumours

The *in vivo* activity of sparsomycin against several animal tumours was reported first in 1962 [1]. The drug was active against 11 out of 20 tumours tested. At present, only one of the tumours employed is used in the screening panel. The Lewis lung carcinoma, the one remaining tumour, was unfortunately not sensitive to sparsomycin. The treatment schedules were considered to be successful even when they caused one or two toxic deaths. The present general criterion used for activity of a drug against solid tumours is that $T/C \leq 42\%$ without obvious drug-related lethality [81b]. This criterion was not fulfilled by sparsomycin in any tumour system investigated.

Sparsomycin was reinvestigated during the course of studies on the antitumour properties of sparsomycin analogues. In *Table 6.14* a review of available data on the antitumour activity of sparsomycin is presented [81,90]. Sparsomycin was adequately tested against four tumours. Complete cure of tumour-bearing animals was not observed. In general, multiple dose schedules were more effective than single ones. Sparsomycin nearly reached the DN 2

Table 6.14. ANTITUMOUR ACTIVITY OF SPARSOMYCIN (1)
For ascitic tumours L1210, B16 and W256, the median survival times and for the solid tumour W256 mean tumour weights were determined. *T/C* represents the ratio of the median survival times or mean tumour weights of the treated animals over those of the control animals expressed as a percentage.

Tumour	*Schedule*	*Dose per injection (mg/kg)*	T/C *(%)*	*Reference*
P388 leukaemia	Q01D × 09	0.08	145 ± 6	[81a]
	Q01D × 09	0.15	136 ± 16	[81a]
	Q01D × 09	0.30	145 ± 12	[81a]
	Q01D × 09	0.60	156 ± 8	[81a]
	Q04D × 02	3.13	166	[81a]
	Q04D × 03	1.44	144	[81a]
	Q04D × 03	2.0	132,166	[81a]
L1210 leukaemia	Q01D × 05	1.0	131	[81a]
	Q01D × 09	0.25	121	[90]
	Q01D × 09	0.30	116,132	[81a]
	Q01D × 09	0.50	118 ± 4	[81a]
	Q01D × 09	0.50	125	[90]
	Q04D × 02	1.56	110	[81a]
	Q04D × 02	3.13	130	[81a]
	Q03D × 03	2.0	127	[81a]
	Q04D × 03	2.0	128	[81a]
	Q04D × 03	2.0	116	[90]
	Q04D × 03	3.0	116	[90]
B16 melanoma	Q01D × 09	0.15	112	[81a]
	Q01D × 09	0.25	107 ± 9	[81a]
	Q01D × 09	0.30	108	[81a]
	Q01D × 09	0.50	102	[81a]
Walker 256	Q01D × 04	0.20	52 ± 1	[81a]
carcinosarcoma	Q01D × 04	0.40	54 ± 4	[81a]
(solid)	Q01D × 04	0.75	34 ± 21	[81a]
(ascitic)	Q01D × 04	0.40	112,162	[81a]
	Q01D × 04	0.40	130,219	[81a]

activity criterion [81b], i.e. $T/C \geq 175\%$ when tested against P388 lymphocytic leukaemia and showed borderline activity (DN 1 activity criterion [81b], $T/C \geq 125\%$) against L1210 lymphoid leukaemia. It was active against Walker carcinosarcoma 256 but inactive against B16 melanoma.

The *in vivo* antitumour activity of analogues of sparsomycin was evaluated for only a limited number of compounds. Derivatives (6.1)–(6.6) were tested [97] against Walker carcinosarcoma 256, but even at high dose levels

Table 6.15. ANTITUMOUR ACTIVITY OF SPARSOMYCIN ANALOGUES
For ascitic tumours L1210 and P388 the median survival times and for the solid tumour W256 mean tumour weights were determined. *T/C* represents the ratio of the median survival times or mean tumour weights of the treated animals over those of the control animals expressed as a percentage.

Tumour/ Drug	*Schedule*	*Dose per injection (mg/kg)*	T/C *(%)*	*Reference*
L1210 leukaemia				
(1.8)	Q01D × 05	5.0	135,135	[90]
(1.8)	Q01D × 05	10.0	172	[90]
(1.8)	Q01D × 09	2.5	130	[90]
(1.8)	Q01D × 09	5.0	135	[90]
(1.8)	Q01D × 09	10.0	217	[90]
(1.8)	Q03D × 03	40.0	139	[90]
(1.8)	Q04D × 03	20.0	122	[90]
L1210 leukaemia				
(1.7)	Q01D × 05	3.0	116	[90]
(1.7)	Q01D × 05	12.0	142	[90]
(1.7)	Q01D × 09	3.0	121,135	[90]
(1.7)	Q01D × 09	12.0	153	[90]
(1.7)	Q02D × 03	15.0	126	[90]
(1.7)	Q03D × 03	10.0	145	[90]
(1.7)	Q04D × 03	10.0	120	[90]
Walker 256 (solid)				
(6.1)	QD3-6	50	85	[97]
(6.2)	QD3-6	160	64	[97]
(6.3)	QD3-6	40	55	[97]
(6.4)	QD3-6	80	96	[97]
(6.5)	QD3-6	120	49	[97]
(6.6)	QD3-6	50	107	[97]
P388 leukaemia				
(2.2)	Q01D × 09	256	137	[73]
(2.5)	Q01D × 09	5.0	161	[73]
(2.7)	Q01D × 09	6.25	153	[73]
(2.12)	Q01D × 09	5.0	137	[73]
(3.9)	Q01D × 09	100	155	[73]

(50–200-fold higher than used for sparsomycin) none of these analogues exhibited tumour inhibition sufficient to meet the criterion for antitumour activity. Compounds (2.2), (2.5), (2.7), (2.12) and (3.9) were tested against P388 leukaemia [73]. Although these derivatives exhibited some antitumour activity at dose levels 20- to 300-fold higher than found for sparsomycin, antitumour properties superior to the parent compound were not found.

The first analogues more active than sparsomycin are benzylsparsomycin (1.8) and octylsparsomycin (1.7) [17,29]. The results obtained with these derivatives are presented in *Table 6.15*. Compound (1.8) was active against L1210 in all schedules tested, reaching the DN 2 activity criterion at a dose level of 10.0 mg/kg. Analogue (1.7) was also active against this tumour in nearly all schedules studied; in some cases, maximal tolerable doses have not yet been reached. The increased antitumour activity of these two derivatives compared with sparsomycin correlates well with their high activity in cell-free protein-synthesizing systems (see *Table 6.9*) and in the L1210 clonogenic assay (see *Table 6.11*). Thus, the *in vitro* L1210 system is of predictive value for the *in vivo* L1210 system in the mouse.

Combination with cisplatin

There is sufficient evidence to indicate that protein synthesis inhibitors may potentiate the activity of other cytotoxic drugs [98–101]. This potentiating effect has also been observed with sparsomycin [96b]. Preincubation of Chinese Hamster Ovary cells (CHO) growing *in vitro* with high sparsomycin

Table 6.16. MODULATION OF CISPLATIN CYTOTOXICITY AGAINST CHO CELLS *IN VITRO* BY SPARSOMYCIN (1) [96b]

Chinese hamster ovary cells were incubated with sparsomycin prior to the administration of cisplatin. Subsequently, cells were incubated with different cisplatin concentrations (1–10 μg/ml) for 1 h. D_{50} is the difference in concentration of cisplatin required for 50% reduction of cell survival relative to control cells untreated with sparsomycin. Wilcoxon's two-sample test compares two survival curves at the level of 5 μg/ml cisplatin. Statistical significance, $P \leqslant 0.05$. Area under the survival curve (AUC) is expressed as a $T/C \times 100\%$ ratio.

Sparsomycin preincubation time (h)	*Sparsomycin concentration (μg/ml)*	D_{50} *difference (μg/ml)*	*Wilcoxon test*	*AUC* T/C *(%)*
3	1.0	+0.8	0.118	143
5	1.0	+3.9	0.005	158
3	5.0	−0.8	0.250	86
3	10.0	−2.5	0.004	36

concentrations prior to administration of cisplatin rendered these cells more sensitive to cisplatin (*Table 6.16*). Protection against cisplatin-induced cytotoxicity was observed when low sparsomycin concentrations were used. Simul-

Table 6.17. MODULATION OF CISPLATIN *IN VIVO* AGAINST L1210 LEUKAEMIA BY SPARSOMYCIN (1) [96c]

CDF_1 mice were injected i.p. on day 0 with 10^5 L1210 cells; mice were treated on days 1, 5 and 9. The time interval is that between the injection of sparsomycin and that of cisplatin; – 3 h means that sparsomycin was given 3 h prior to the administration of cisplatin. The first drug was given 24 h after tumour implantation. *T/C* is the ratio of the median survival times of the test animals over those of the control animals expressed as a percentage. 'Significance' is the significance of the difference between the survival curves of treated and untreated animals according to the Mantel-Haenszel procedure. Weight loss is the difference between the means of treated and untreated groups of mice. For 'cures' animals surviving longer than 60 days were considered to be cured. n.s., not significant.

Sparsomycin dose per injection (mg/kg)	*Cisplatin dose per injection (mg/kg)*	*Time interval (h)*	T/C (%)	*Significance*	*Weight loss (g)*	*Cures*
1.5	–	–	110	–	+0.1	0
–	2.0	–	170	–	+0.1	0
–	3.0	–	205	–	−0.9	0
–	5.0	–	372	–	−1.9	0
–	7.5	–	216	–	−4.4	0
–	10.0	–	122	–	−4.4	0
1.5	2.0	−12	185	n.s.	−0.3	0
1.5	2.0	−6	270	$p < 0.001$	−0.8	0
1.5	2.0	−3	260	$p < 0.001$	−0.5	0
1.5	2.0	0	190	n.s.	+0.4	0
1.5	2.0	+3	180	n.s.	+0.1	0
0.5	3.0	−3		$p < 0.05$	−0.7	4/6
1.0	3.0	−3		$p < 0.001$	−1.7	4/6
1.5	3.0	−3		$p < 0.001$	−1.8	4/6
2.0	3.0	−3	230	n.s.	−1.7	1/6
3.0	3.0	−3	100	n.s.	−3.9	0
1.5	1.0	−3	155	n.s.	+0.1	0
1.5	2.0	−3	260	$p < 0.001$	−0.5	0
1.5	3.0	−3		$p < 0.001$	−1.8	4/6
1.5	5.0	−3		n.s.	−1.2	4/6
1.5	7.5	−3		$p < 0.05$	−1.8	4/6
1.5	10.0	−3	125	n.s.	−3.1	2/6

taneous or post-incubation with sparsomycin did not have any potentiating effect on the cytotoxicity of cisplatin against CHO cells. This effect could be explained as follows. Administration of cisplatin causes the production of antagonistic proteins against this drug; sparsomycin, being a protein synthesis inhibitor, could block the formation of these antagonists.

Sparsomycin showed the same potentiating effect *in vivo* when given prior to cisplatin (*Table 6.17*) [96c]. It prolonged animal survival significantly in several groups of mice bearing L1210 leukaemia. In the most optimal schedules, 66% cures were found. Normally, weight-loss is observed when animals are treated with cisplatin. Protection against this weight-loss was observed when sparsomycin was incorporated into the treatment schedule. There is currently no explanation of this phenomenon.

PHARMACOKINETICS

Little is known about the pharmacokinetics of sparsomycin. An HPLC method for the determination of sparsomycin has been described (see also Ref. 102). This method has been improved recently; the detection limit of the method is 10 ng/ml of plasma and 20 ng/ml of urine [103a]. The elimination $t_{1/2}$ in dogs of sparsomycin given in a non-toxic dose was 1.1 h [90,103a]. Most of the drug is excreted in the urine [90]. This is in contradiction to the results reported earlier [103a]. A lethal sparsomycin dose (1.4 mg/kg) given to the dog, resulted in non-linear kinetics and an increase of $t_{1/2}$ to 2.3 h [103b]. These results suggest that renal clearance is of primary importance for elimination of the drug.

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