PROGRESS IN MEDICINAL CHEMISTRY 22

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Preface

We have pleasure in presenting seven reviews in this volume. Important advances in the chemistry and biology of *C*-nucleosides, particularly in relation to microbiologically derived *C*-arylglycoside antibiotics, are surveyed in Chapter 1. The amino acid, 4-aminobutanoic acid, is present in every region of the mammalian central nervous system and appears to function as an inhibitory neurotransmitter. Chapter 2 covers this interesting topic.

The discovery of the β-blocking properties of propanolol has stimulated research into the effect of molecular modification and of the balance between cardioactivity and antihypertensive action; recent work is reviewed in Chapter 3.

The story of thalidomide is a tragic episode in the progress of drug therapy. Chapter 4 describes the use of this compound in leprosy and in the treatment of skin lesions. An account of drugs discovered recently in India forms the basis of Chapter 5.

A discussion of the controversial issue of the relationship between the parasympathetic nervous system and the tissue mast cell system is given in Chapter 6, and the volume ends with a review of new approaches to bronchodilator and antiallergic drug therapy.

We thank our authors for their work, the owners of copyright for permission to reproduce material in this volume and the staff of our publishers for their constant help and encouragement.

December 1984

G.P. Ellis
G.B. West

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Contents

Progress in Medicinal Chemistry – Vol. 22, edited by G.P. Ellis and G.B. West

1 The Chemistry and Biochemistry of *C*-Nucleosides and *C*-Arylglycosides

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INTRODUCTION

Since our 1976 review [1], many important advances have occurred in the chemistry and biology of *C*-nucleosides, which include development of new strategies for synthesis of *C*-nucleosides, discovery of the new *C*-nucleoside antibiotic, ezomycin B_1 [2], and the preparation of synthetic *C*-nucleosides which possess impressive anticancer and antiviral properties. Heightened interest in *C*-nucleoside research has led to several reviews [3–11] of selected aspects of this rapidly growing literature.

The goals of the present chapter are (a) to review selectively recent advances in the chemistry and biochemistry of *C*-nucleosides, (b) to review similarly the literature concerning *C*-arylglycoside (i.e., non-nitrogen heterocyclic *C*-nucleoside) antibiotics, and (c) to highlight recent significant advances in synthesis of *C*-nucleosides and *C*-glycosides.

Our earlier work [1] was restricted to nitrogen heterocyclic *C*-nucleosides and specifically excluded *C*-glycosides because the majority of known *C*-glycosides were of plant origin and their biological functions and effects were unknown. This is still the case, and we have again decided not to include plant *C*-glycosides in our discussion; however, a significant number of microbiologically derived *C*-arylglycoside antibiotics have been isolated, identified and studied in biological systems and we have expanded the scope of our survey

to include them. To our knowledge, this literature has not been reviewed previously.

We have included a significant amount of biological test data and have been especially careful to present data which are relevant to structure-activity relationships. Our literature review was concluded in June 1984; some more recent material has been made available to us in manuscript form and has been included.

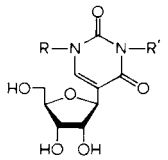
CHEMISTRY AND BIOCHEMISTRY OF *C*-NUCLEOSIDES

PSEUDOURIDINE AND RELATED PYRIMIDINE *C*-NUCLEOSIDES

Pseudouridine

The chemistry and biology of pseudouridine (1) have been reviewed previously [1]. The important rôle of the pseudouridine modification of tRNAs continues to attract interest. It has, for example, been shown that a mutant of *Salmonella typhimurium* lacking pseudouridine synthetase, an enzyme that catalyzes the pseudouridine modification in the anticodon region of tRNAs, has depressed levels of certain amino-acid-synthesizing enzymes [12]. In another interesting investigation, a mutant $tRNA^{Tyr}$ ochre suppressor gene (from which the 14-basepair intervening sequence containing pseudouridine is deleted) was constructed [13].

The suppressor activity of the mutant was reduced relative to that of the unaltered gene, probably due to the absence of pseudouridine [14]. A crystallographic refinement of the structure of $tRNA^{Phe}$ [15,16] shows that the pseudouridine on the anticodon stem pairs with an adenosine on the complementary stem via the N_3H and $C_2{=}O$ sites (that is, in the *anti* conformation). This led to the speculation [17] that N^1H of the pseudouridine residue is part of a recognition or binding site for a regulator involved in the modulation of the expression of amino acid operons, and disfavours the idea [18] that pseudouridine would pair in the *syn* conformation with adenosine. Several new syntheses of pseudouridine (1) have been reported [19–23].



(1) : R = R' = H
(1a): R = CH_3, R' = H

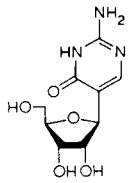
1-Methylpseudouridine

1-Methylpseudouridine (1a) has been isolated from *Streptomyces platensis* [24] and from *Hallococcus morrhuae* tRNA [25]. Chemical synthesis from pseudouridine [26,27] or from 4,5′-anhydro-2′,3′-*O*-isopropylidenepseudouridine [27] provides unambiguous evidence for the structure of (1a).

SYNTHETIC PYRIMIDINE *C*-NUCLEOSIDES

More synthetic pyrimidine *C*-nucleosides have been prepared since 1976 [19, 23, 28–53] (see *Table 1.1* for a summary), but only rarely have biological test results been reported. However, four 5′-deoxy-5′-halopseudouridine derivatives, probably synthesized as potential antimetabolites, were found to be inactive *in vivo* and *in vitro* in L1210 mouse leukaemia screens [34]. Of the 2′-deoxypseudouridine derivatives synthesized, the 1-methyl analogue exhibited activity *in vitro* against P815 cells (ID_{50} value 4.9 μg/ml), whereas the non-methylated and 1,3-dimethylated analogues were considered inactive [45]. 1-Methyl-2′-deoxypseudouridine also showed activity against *Streptococcus faecium* var. duran [45].

A general synthesis of 2′-deoxy-*C*-nucleosides has been reported [31]. Various *C*-nucleosides afforded 3′,5′-tetraisopropyldisiloxanyl derivatives on treatment with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane in pyridine, and the free 2′-hydroxyl was then converted to an imidazol-1-ylthiocarbonyloxy group. Reduction of this derivative with n-Bu_3SnH (sometimes after initial silylation of the aglycone) afforded a protected 2′-deoxy-*C*-nucleoside which was deprotected conveniently with n-Bu_4NF. Alternatively, 2′-deoxypseudouridine can be prepared in about 50% yield from pseudouridine (1) by treatment with α-acetoxyisobutyryl chloride followed by reduction of the resulting mixture of chloro-sugar derivatives and deblocking [28,29,45]. This reaction produces 3′-deoxypseudouridine as a by-product [29] and not α-2′-deoxypseudouridine as originally suggested [45].



(2)

Table 1.1. SYNTHETIC *C*-NUCLEOSIDES RELATED TO PSEUDOURIDINE[a]

Compound	*References*
Uracil, 5-(β-D-2′-deoxyribofuranosyl)-	28–31
5-(β-D-3′-deoxyribofuranosyl)-	29
5-(β-D-arabinofuranosyl)-	32
6-(β-D-ribofuranosyl)-	33
5-(β-D-5′-deoxyribofuranosyl)-	34
5-(β-D-5′-deoxy-5′-haloribofuranosyl)-[b]	34
4,2′-anhydro-(β-D-arabinofuranosyl)-	35
5-β-hamamelofuranosyl-	36
5-tetrahydrofuranyl-[c]	37–39
3-Methylpseudouridine	40
2′-Deoxy-1-methylpseudouridine	30,31
2′-Deoxy-3-methylpseudouridine	40
2-Thiouracil, 5-β-D-ribofuranosyl-	
(2-thiopseudouridine)	19,23,41
6-β-D-ribofuranosyl-	33
Cytosine, 5-β-D-ribofuranosyl-	
(pseudocytidine)	6,23
5-β-D-arabinofuranosyl-	35
6-β-D-ribofuranosyl-	33
2-Amino-4-hydroxypyrimidine,	
5-β-D-ribofuranosyl-	
(pseudoisocytidine)	19,23,42–44
5-β-D-2′-deoxyribofuranosyl-	30,31,45
5-β-D-arabinofuranosyl-	46
4,2′-anhydro-β-D-arabinofuranosyl-	46
2,4-Diaminopyrimidine, 5-β-D-ribofuranosyl-	41
2-Thiocytosine, 5-β-D-ribofuranosyl-	41
4-*S*-Methyl-4-thiouracil, 6-β-D-ribofuranosyl-	33
3,4-Dihydro-4-oxo-3*H*-1,3-thiazine-2-thione,	33
6-β-D-ribofuranosyl-	33

[a] In addition to the compounds listed here, a number of racemic C-5′, C-4′ and C-2′ alkylated or arylated, C-1′-dialkylated, and C-4′ hydroxymethylated pyrimidine ribo-*C*-nucleosides have been prepared [47–53].

[b] halo = F, Cl, Br, I.

[c] The synthesis afforded a racemic mixture.

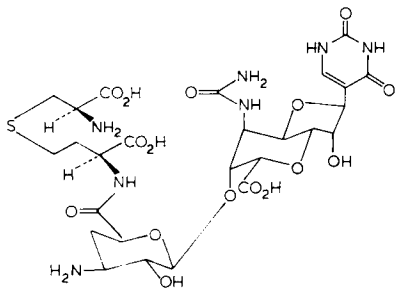
Several preparations of pseudoisocytidine (2) have been reported [19,23,42–44]. Compound (2) is most easily prepared (in 60% yield) from 1,3-dimethylpseudouridine by treatment with guanidine [42,44]. A total synthesis of (2) based on Noyori, Sato and Hayakawa's cycloaddition strategy

[23] is elegant but less effective. Pseudoisocytidine shows very interesting biological activities [6]. Most importantly, it is inhibitory, both *in vitro* and *in vivo*, to growth of mouse and human leukaemia cells which are resistant to 1-β-D-arabinofuranosylcytosine [53]. In fact, treatment of mice bearing P815 leukaemias with (2) proved therapeutically effective [55]. The antileukaemic effects of (2) are probably due to the incorporation of phosphorylated (2) or 2′-deoxy-(2) into nucleic acids [55,56]. *C*-Nucleoside (2) is not deaminated by cytidine deaminase; it inhibits this enzyme competitively [57]. It has been demonstrated that (2) and other cytidine analogues containing a modification in the 5-position of the pyrimidine ring inhibit DNA methylation [58–60] and that (2) is weakly mutagenic [61]. Unfortunately, hepatic toxicity due to rapid accumulation of (2) in the liver proved dose-limiting in a phase I clinical trial and no therapeutic effects were observed [62].

The arabinofuranosyl epimers of pseudouridine, pseudocytidine and pseudoisocytidine were found to be inactive *in vitro* against L1210 cells [35].

THE EZOMYCINS

Ezomycin B_1, $[\alpha]_D^{22} - 5.5$ (*c* 0.83, H_2O) was isolated in the early seventies [63,64] from a strain of *Streptomyces*. Other ezomycins isolated are the *N*-nucleosides, ezomycin A_1 and A_2, and the *C*-nucleosides, ezomycin B_2, C_1, C_2, D_1 and D_2 [65–68]. However, the A_2, C_1, C_2, D_1 and D_2 derivatives seem to be artefacts resulting from hydrolysis and rearrangement reactions. Ezomycin B_1 inhibits growth of the phytopathogenic fungi *Sclerotinia* and *Botrytis* sp. [64,65]. The structural elucidation of the ezomycins has been reviewed previously [5,7,69].

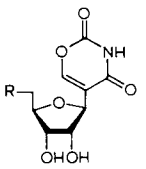


Enzomycin B_1

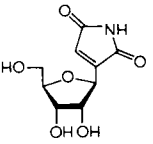
OXAZINOMYCIN AND RELATED *C*-NUCLEOSIDES

Oxazinomycin (minimycin), (3), has been reviewed previously [1,3–5,7]. The structure of (3) has been confirmed by a multistep total synthesis starting with an anomeric mixture of 2′,3′-*O*-isopropylidene-5′-*O*-trityl-D-ribofuranosyl-acetonitriles [70]. The biosynthesis of (3) was elucidated in elegant studies [71–73] which led to the following conclusions: (a) the ribofuranosyl moiety of (3) arises from D-ribose [71,72]; (b) asymmetric incorporation of carbons C-5, C-4 and C-3 of glutamate results in carbons C-4, C-5 and C-6, respectively, of the oxazine ring [72,73] and (c) the origin of C-2 of the oxazine ring is carbon dioxide [71]. New data on the antitumour activity of (3) have been published [74]; it had an ID_{50} of 3×10^{-7} M for L1210 cells and 5×10^{-7} M for HeLa cells, and showed about 5% inhibition of RNA, DNA and protein synthesis after 24 h of incubation at a concentration of 3×10^{-7} M. The growth-inhibitory effect of (3) on L1210 cells was reversed by uridine and cytidine.

Four oxazinomycin derivatives, 5′-deoxyoxazinomycin (3a), disodium oxazinomycin 5′-phosphate (3b), O^4,2′-anhydrooxazinomycin and 3′-*O*-acetyl-2′-deoxyoxazinomycin, which were synthesized from (3), were found to be inactive against L1210 and P388 cells [74]. Unfortunately, it was not possible to deprotect the 3′-*O*-acetyl-2′-deoxy derivative without accompanying ring opening of the oxazinedione ring.



(3) R = OH
(3a) R = H
(3b) R = OPO_3Na_2



(4)

SHOWDOMYCIN

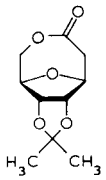
Showdomycin (4) is a naturally occurring *C*-nucleoside with well-established antibacterial and antitumour activities, and enzyme inhibitory properties [1,3–5,7,58]. Showdomycin is frequently used as a biochemical tool due to its ability to bind covalently with sulphydryl groups in enzymes [76–79]. However, the view that the inhibitory activity of (4) on uridine phosphorylase (UrdPase) is due to formation of a covalent bond to a thiol group of the enzyme [79] has been challenged [80].

Showdomycin preferentially inhibits translation in a number of encephalomyocarditis-infected cells [81]. Also, (4), in contrast to maleimide, is twice as toxic for murine L1210 leukaemia cells as for murine bone marrow cells, and the selectivity can be increased 2-fold by pretreatment of the cells with cytidine [82,83]. This increased selectivity is due to a more favourable competition of cytidine (which is nontoxic) for saturable transport carriers in normal cells than in leukaemia cells. Experimental evidence indicates that the cytotoxic action of (4) is related directly to the rate of showdomycin transport into the cell, where it supposedly reacts with sulphydryl groups on the plasma membrane [84].

It has been stated that (4) is not subjected to phosphorylation [7,75]. However, recent results seem to indicate that (4) is converted to the corresponding ribosyl triphosphate in mouse lymphoma cells [85]. The enzyme in *Streptomyces* sp. No. 383 that converts (4) into the biologically inactive isomer, isoshowdomycin, has been partially purified and found to have an approximate molecular weight of 125,000 [86].

The conformational behaviour of (4) has been investigated using PILCO computations. The results indicate that the preferred conformation for isolated showdomycin is *anti* with respect to the glycosidic bond and *gauche-gauche* about the C-4′–C-5′ bond [87]. The crystallographic conformation of (4) is *syn* [88]. The difference between observed and calculated conformations seems to be due to intermolecular hydrogen bonding in the crystal.

Showdomycin (4) has been a popular target for synthetic chemists and several total syntheses have been reported since 1979: (a) (4) was prepared in 8% yield from D-ribose by a route involving a palladium(II)-catalyzed carbonylation of a protected D-ribofuranosylethyne as the key step [89]; (b) (4) was synthesized from bicyclic lactone (5), which was obtained by cycloaddition followed by optical resolution [23] or by synthesis from D-ribose [90]; (c) Kane and Mann [91] utilized the addition of ethoxycarbonylethylidenetriphenylphosphorane to a protected D-ribose derivative followed by selenium chemistry as important elements in their formal total synthesis of (4); (d) enzymatic enantioselective hydrolysis of the Diels-Alder adduct of furan and dimethyl acetylenedicarboxylate gave a chiral intermediate which was converted to (4) in eight steps [92,93]; (e) the most efficient synthetic approach reported to date gives (4) in 70% yield based on commercially available 1-acetyl-2,3,5-tribenzoyl-β-D-ribofuranose using 1,2-bis(trimethylsiloxy)cyclobut-1-ene for the construction of the maleimide unit [94]. Recently, two syntheses of racemic (4) starting with Diels-Alder adducts were reported [95,96].



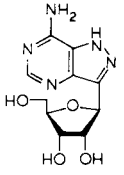
(5)

Showdomycin analogues

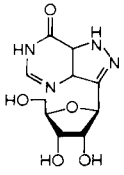
2-α-D-Ribofuranosylmaleimide, α-(4), has been synthesized from a protected D-ribofuranosylethyne derivative, but showed no significant biological activity when tested against bacteria and viruses [97]. The synthesis of the racemate [98] as well as the β-D-ribo enantiomer [99] of 2′-deoxy showdomycin has been reported, but no biological data seem to be available for these interesting compounds. Similarly, no biological data have been reported for the racemic arabino analogue of (4) [95], for the two 2,3-dihydro diastereomers of (4) [100], or for the 5′-deoxy-5′-halo derivatives of (4) synthesized by Earl and Townsend [101]. Two total syntheses of the racemic carbocylic analogue of showdomycin have been reported [102,103]. This compound seems to be devoid of antibacterial and antiviral activity [102]. The biological activity of 2-[(β-D-ribofuranosyl)methyl]maleimide, i.e., homo-showdomycin, has not been reported [104,105]. An *N*-methoxycarbonyl-protected pyrrolidine derivative of (4) has also been synthesized [106].

THE FORMYCINS

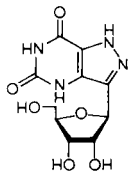
Formycin (6) is a naturally occurring *C*-nucleoside which has demonstrated antitumour, antiviral and antifungal activity. Formycin B (7), the deamination metabolite of (6), shows less biological activity, and the oxidation metabolite of (7), oxyformycin B (8), seems biologically inert. The biology of these three *C*-nucleosides has been reviewed extensively [1,3–5,7], and only very recent findings will be discussed here.



Formycin
(6)



Formycin B
(7)



Oxyformycin
(8)

Formycin seems to be a low-potency adenosine-receptor agonist in human fibroblasts [107]. Formycin (6) and formycin B (7) are vasoactive in the arterio-arterial vascular bed of the trout gill, which probably contains specific vascular purinergic receptors [108].

These compounds, (6) and (7), also cause significant haemodynamic effects in rats, which may arise from competition with adenosine for the nucleoside transport system [109,110]. Formycin acts both as a substrate and as a low-potency irreversible inhibitor for *S*-adenosyl-L-homocysteine (SAH) hydrolase in intact lymphocytes [111,112]. It is possible that formycin, by being metabolized to an SAH analogue, interferes with cellular methylation reactions [113].

Formycin inhibits morphogenetic development in *D. discoideum*, presumably by being incorporated into RNA, but formycin B, which also inhibits total RNA synthesis, has no apparent influence on morphogenesis in this species [114]. Analysis of nuclear RNA from L1210 cells showed that the inhibitory activity of formycin is due to inhibition of base methylation, and not to 2′-*O*-methylation [115].

2′-Deoxycoformycin inhibits adenosine deaminase, the enzyme responsible for formation of formycin B from formycin. A good correlation was found between 2′-deoxycoformycin-enhanced cytocidal effects of formycin in human colon carcinoma cells *in vitro* and the corresponding enhancement of [^{3}H]formycin incorporation into DNA, suggesting that substitutions of formycin into DNA may be responsible for its cytotoxic effects [116]. It is assumed that formycin is converted to the corresponding triphosphate before being incorporated into RNA or DNA, and the cytotoxicity of formycin has actually been found to correlate well with its anabolism to nucleotide metabolites [117]. The sometimes confusing results obtained in investigations of the precise mechanism of action of formycin may be due to species differences [117].

Attempts to increase the therapeutic index of formycin have been made; co-administration of isocoformycin, a new adenosine deaminase inhibitor, increased the toxicity of formycin, but when adequate doses were given, the two drugs in combination increased the life-span of L1210 mice as compared with formycin given alone [118].

Formycin B seems to be of potential use in the therapy of leishmaniasis [119] and Chagas disease [120]. In infected macrophages, its mechanism of action is as follows: first, formycin B is converted to the 5′-monophosphate by a nucleoside phosphotransferase [121] and then aminated by adenylosuccinate synthetase to formycin monophosphate [122]. Further phosphorylation by AMP kinase and nucleoside diphosphokinase leads to the formation of formycin triphosphate, which is incorporated into DNA [123]. In contrast to earlier belief, non-infected macrophages also metabolize formycin B to formycin

monophosphate, but the amount of metabolite formed is much lower than in infected macrophages and a good therapeutic index seems to exist for formycin B [119].

Formycin inhibits purine nucleoside phosphorylase (PNP) in a competitive manner [124,125], but its potential in simulating lymphocyte PNP deficiency is uncertain due to its effects on other enzyme systems [126].

The preferred conformation of formycin in D_2O, ND_3 and dimethylsulphoxide seems to be *S* in the ribose ring, *syn* around the *C*-glycosyl bond and *gauche-gauche* around the C-4′–C-5′ bond [127–129]. In the crystal, the conformation around the *C*-glycosyl bond in formycin B is *anti*, whereas in both formycin and oxoformycin the conformations are *syn* [130].

Formycin tautomerization has been carefully studied. There is a temperature-dependent, very rapid equilibrium between N^1-H and N^2-H forms, with the N^1-H form predominating [131]. The tautomeric interconversion is catalyzed by acid or base [132]. Luminescence studies of formycin indicate that protonation occurs predominantly on N^4 [133]. Both formycin B and oxoformycin form strongly fluorescent monoanions and have the same p*K* values. The ionization of each compound is ascribed to dissociation of the proton from N^1 [134].

The biosynthesis of formycin has been reviewed [7]. Recently, two multistep total syntheses of formycin were reported [135–137]. Kalvoda used a 1,3-dipolar addition of diazomethane to an ethyl 3-cyano-2-propenoate derivative for constructing an important pyrazole intermediate in his synthesis [135]. He also prepared formycin B by this methodology. The other total synthesis utilized, as the key step, cine substitution by cyanide ion, at the 1-nitro group in a *C*-glycosidic 1,4-dinitropyrazole [136,137].

Synthetic C-nucleosides related to the formycins

N-*Methylated analogues*

The finding that N^2,5′-anhydroformycin, but not N^4,5′-anhydroformycin, is deaminated by adenosine deaminase lent support to the hypothesis that formycin (6) is a substrate for this enzyme only in the *anti* conformation [138]. The synthetic availability of all five *N*-methylformycins [139,140] allowed this hypothesis to be tested. 1-Methyl-, 4-methyl- and 6-methylformycin are inactive as substrates for human erythrocyte adenosine deaminase, whereas the 2-methyl- and N^7-methyl derivatives are substrates for this enzyme [140,141]. The 2-methyl derivative is a *syn*-nucleoside and the 4-methyl analogue is clearly an *anti*-nucleoside. Thus, the above-mentioned hypothesis seems to be an oversimplification and it has been suggested that other steric as well as

electronic factors must be considered when discussing these enzyme activities [140]. It should be pointed out that a conflicting report on the deamination of 1-methyl- and 2-methylformycin has appeared [141]. In this latter study, 1-methylformycin is reported to be deaminated much more quickly than the 2-methyl analogue by Takadiastase and calf intestinal adenosine deaminase [140]. Also, 1-isopropyl- and 2-isopropylformycin were synthesized and found not to be deaminated by these enzymes [142]. 1-Methyl-, 2-methyl- and N^7-methylformycin are potent cytotoxic agents against L1210 cells *in vitro* [141]. The cytotoxic activity of 1-methylformycin, which seems to be comparable with that of formycin, and its stability toward deamination make this formycin analogue very interesting. In addition to the *N*-methylformycins, 2′-*O*-methyl- and 3′-*O*-methylformycin and 1-methyl-, 4-methyl- and 6-methylformycin B have been synthesized [132,133].

Modifications of the aglycone

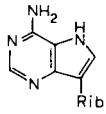
Thioformycin B has been found to be a potent anti-myxoviral agent *in vitro* [143]. This led to the synthesis of 3-β-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidine-7-carboxamide. However, this compound showed only moderate antiviral activity against vaccinia virus [143]. In the same investigation, the 7-(methylthio) analogue showed significant antiviral activity against this cell line.

A number of aglycone-modified formycin and formycin B analogues have been synthesized [6,144–155], typical examples of which are presented as formulae (9)–(22) (Rib = β-D-ribofuranosyl). Of these compounds, APTR (10), OPTR (17), 9-deazaadenosine (9), 9-deazainosine (18a) and (19) have demonstrated interesting biological activities.

APTR (10) shows 10-times higher potency than formycin against L1210, P815 and L5178y leukaemic cells *in vitro*. It is also antileukaemic in mice with L1210 leukaemia, and in mice with vincristine-resistant P315 leukaemia. Deamination of APTR seems to occur more slowly in L1210 cell homogenates than with formycin, but the antileukaemic effects of APTR *in vitro* are potentiated by deaminase inhibitors [6].

Furthermore, OPTR (17) [144] as well as 8-(β-D-ribofuranosyl)-4-thioxo-3*H*-pyrazolo[1,5-*a*][1,3,5]triazine, 4-methylthio- and 4-hydroxyamino-8-(β-D-ribofuranosyl)pyrazolo[1,5-*a*][1,3,5]triazine [147] show inhibitory activity against mouse leukaemia cell lines L1210 and P815; the 4-methylthio derivative is even more potent than APTR in this respect [147].

9-Deazainosine (18a) [149] is an antiprotozoal agent *in vitro* with a remarkably low toxicity toward L-cells. The ID_{50} values for *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma gambiensi* are 1, 2–10, and 20 μM, respectively [156]. 9-Deazaadenosine (9) [150], on the other hand, is quite toxic to



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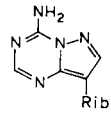
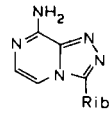
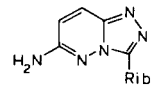


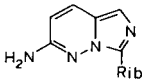
APTR (10)



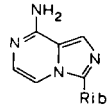
(11)



(12)



(13)



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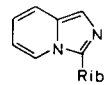

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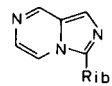

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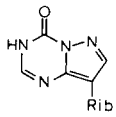
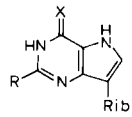
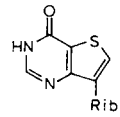


OPTR (17)



(18a) : R = H, X = O
(18b) : R = NH_2, X = O
(18c) : R = H, X = S



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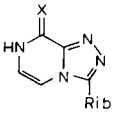
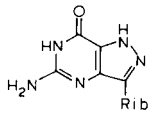


(20a) : X = O
(20b) : X = S



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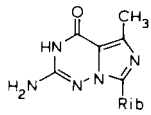


(22)

human cancer cells. It is a potent inhibitor of nine different human tumour cells *in vitro* (ID_{50} values ranged from 1.1×10^{-8} to 8.5×10^{-8} M) and of pancreatic carcinoma in ATS immunosuppressed mice [157]. These effects of 9-deazaadenosine are potentiated by coadministration of the nucleoside transport inhibitor, *p*-nitrobenzyl-6-thioinosine, which seems to protect normal cells selectively. 9-Deazaadenosine (9) is a poor substrate for adenosine deaminase *in vitro*; only in one out of nine tumour cell lines (ovarian) did coadministration of the adenosine deaminase inhibitor 2-deoxycoformycin potentiate the antitumour activity of 9-deazaadenosine [157]. Preliminary experiments indicate that 9-deazaadenosine is converted to the triphosphate in human pancreatic

carcinoma cells [157]. In this cell line, 9-deazaadenosine inhibits the incorporation of [^{3}H]thymidine into DNA and of [^{3}H]uridine into DNA and RNA. Another recent investigation [158] indicates that 9-deazaadenosine is the most effective nucleoside analogue examined so far in human colon carcinoma cells. The results obtained suggest that the effects of 9-deazaadenosine in this cell line are related to inhibition of initiation of translation via its incorporation into RNA [158]. Also, 9-deazaadenosine inhibits lymphocyte-mediated cytolysis, apparently due to metabolism to 9-deazaadenosine 5′-triphosphate and a resulting decrease in cellular ATP [159].

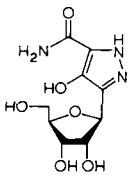
7-Thia-7,9-dideazainosine (19) [152] is a potent inhibitor of growth in P815 and L1210 leukaemic mouse cells *in vitro* (ID_{50} values = 2.5 and 3.3 μg/ml). It also has moderate *in vitro* activity against *Trypanosoma cruzi* and *Trypanosoma gambiense* [156].

Formycin analogues modified in the sugar moiety

The D-arabinofuranosyl analogue of formycin has been prepared by total synthesis [160]. 5′-Chloro- and 5′-iodo-5′-deoxyformycins B were prepared as potential inhibitors of purine nucleoside phosphorylase [161]. The association constant of the 5′-iodo derivative for purine nucleoside phosphorylase was found to be 10-times higher than that of formycin B [161].

PYRAZOFURIN (PYRAZOMYCIN)

Pyrazofurin (23) is a naturally occurring *C*-nucleoside with antitumour and antiviral activities [1,3–6,10]. It is bioactivated by conversion to the 5′-phosphate, which is a potent competitive inhibitor of orotidine-5′-phosphate decarboxylase [162–165]. Thus, pyrazofurin 5′-phosphate inhibits *de novo* pyrimidine biosynthesis. It is also an inhibitor of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl-5′-monophosphate formyltransferase [166]. However, this inhibition of *de novo* purine synthesis is less pronounced than that of pyrimidine synthesis [166] and does not seem to be important for the biological activity of pyrazofurin [167].



Pyrazofurin (23)

Although pyrazofurin has demonstrated biochemical effects on tumours *in vitro* and *in vivo* [168,169], it has shown disappointingly little activity in clinical trials [163,170–178]. This may be due to one or a combination of three factors [167]: (a) the tumour cells may be capable of utilizing preformed nucleosides by salvage pathways; (b) there may be less adenosine kinase activity in tumour cells than in sensitive normal cells; (c) even in the presence of pyrazofurin, some uridylate synthesis may occur, thus rendering treatment ineffective. At present, cancer therapy using pyrazofurin in combination with other drugs looks more promising. For example, synergistic responses have been suggested and observed in tumours as the result of combinations of pyrazofurin and fluorinated pyrimidines [167,179,180], lycurim [181,182], galactosamine [183], purine derivatives [184], and 5-azacytidine [164,180,185]. The use of pyrazofurin in the therapy of tumours with overgrowth of 1-β-D-arabinofuranosylcytosine-resistant cells has also been suggested [186].

Pyrazofurin is a powerful broad-spectrum antiviral agent *in vitro*; however, its toxicity seems to prevent it from being a curative antiviral agent *in vivo* [187–189].

Recent experimental evidence indicates that pyrazofurin is biosynthesized from D-ribose and the C-1 to C-4 carbons of L-glutamate [15,190,191].

The conformational properties of pyrazofurin have been studied by proton magnetic resonance [192], circular dichroism [193] and theoretical calculations [194,195]. Results obtained are not conclusive, but indicate that pyrazofurin assumes a *syn* conformation around the glycosidic bond in the absence of intramolecular hydrogen bonding (that is, in water) [195].

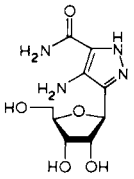
Four total syntheses of pyrazofurin have been accomplished [196–201], one of which gave pyrazofurin in 30% yield as calculated from a protected D-ribose derivative [200,201].

Synthetic pyrazole C-nucleosides

The pyrazofurin analogues 4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide (24) [202] and 3-amino-2-*N*-carbamoyl-4-(β-D-ribofuranosyl)pyrazole (25) [148] have been synthesized and found to be inactive *in vitro* against L1210 and P815 cells, respectively. Also, the carbocyclic analogue of pyrazofurin seems to be devoid of biological activity [203].

New syntheses have been developed for previously reported pyrazole *C*-nucleosides [204–207]. Among new synthetic *C*-nucleosides in this class are 3-(β-D-arabinofuranosyl)- and 3-(β-D-xylofuranosyl)pyrazole [208,209], 5-phenyl-3-(β-D-ribofuranosyl)pyrazole [210], and ethyl 4,5-dihydro-5-(β-D-ribofuranosyl)pyrazole-5-carboxylate [211]. The protected *C*-nucleoside (26)

has been prepared from an isoxazole *C*-nucleoside as part of an elegant synthetic sequence [212].



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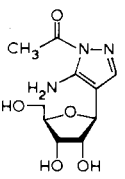

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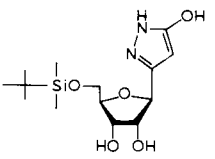
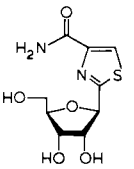


(26)

THIAZOLE, TRIAZOLE AND RELATED HETEROAROMATIC *C*-NUCLEOSIDES

In addition to the pyrazole derivatives presented above, several other five-membered ring heterocyclic *C*-nucleosides have been synthesized (see *Table 1.2*). As concluded from investigations in which biological studies are included, most of these compounds possess only marginal, if any, biological activity. However, there are a few exceptions.

Thiazole *C*-nucleoside (27) and its 2′,3′,5′-triacetate possess significant antiviral activity *in vitro* [217]. When tested *in vivo* against type 1 parainfluenza virus in mice, compound (27) increased survivor numbers but proved much less effective than ribovirin [217]. However, in contrast to ribovirin, (27) is a potent antitumour agent [227]. It has a remarkable ability to increase life-spans of mice inoculated intravenously with Lewis lung carcinoma [227], which is a test system for lung tumours and metastases. Compound (27) also shows notable activity *in vivo* against L1210 and P388 leukaemias [227]. In addition, (27) inhibits growth of T-cell leukaemia, cutaneous T-cell lymphoma and B-cell leukaemia cell lines *in vitro* [228].



(27)

Attempts to elucidate the mechanism of action of (27) resulted in the conclusion that (27) is being bioactivated to an anabolite(s) which is a powerful inhibitor of inosine monophosphate dehydrogenase [216]. This would be consistent with the observation that (27) inhibits guanine nucleotide synthesis

Table 1.2. THIAZOLE, TRIAZOLE AND RELATED SYNTHETIC *C*-NUCLEOSIDES[a]

Ring system, substituents	*Sugar positions*[b]	*References*
Thiazole	5	213
Thiazole, 4-methyl	2[c]	214,215
4-bromomethyl	2[c]	215
4-chloromethyl	2[c]	215
4-carboxylic acid	2	216
4-carboxamide (27)	2[d]	214,217
4-thiocarboxamide	2	217
5-carboxamide	2	214
4,5-dicarboxamide	2	214
4,5-dicarboxylic acid, diethyl ester	2	214
2-methyl	5	213
2-benzyl	5	213
2-amino	5	213
2-carboxamide	5[e]	218
4-methyl-5-bromo	2[c]	215
4-(thiazol-2-yl-4-carboxamide)	2	216
2(3*H*)-Thiazolone	5	213
Selenazole, 4-carboxamide (29)	2	219
Imidazole, 4-carboxamide-5-hydroxy	2[f]	220
4-ethoxycarbonyl-5-hydroxy	2	220
1,2,4-Oxadiazole, 5-carboxamide	3	216
1,3,4-Oxadiazole, 5-phenyl	2	220
1,2,4-Triazole, 5-cyano	3	220
5-carboxamide	3[g]	220–223
5-carboxylic acid, ethyl ester	3	222
5-hydrazinocarbonyl	3	220
4*H*-1,2,4-Triazole, 5-amino	3	220
1*H*,4*H*-1,2,4-Triazole, 5-thione	3	220
1*H*-1,2,4-Triazole, 1-methyl-5-carboxamide	3	220
2-Tetrazole	5[h]	223

[a] The table contains only deprotected *C*-nucleosides. Various protected 5-(β-D-ribofuranosyl)-3,5-disubstituted thiazoles have been reported [225].

[b] The sugar moiety is β-D-ribofuranosyl.

[c] In addition, the tetrahydropyran-2-yl derivative has been reported [215].

[d] In addition, the 5′-deoxy- and 5′-deoxy-5′-iodo-β-D-ribofuranosyl derivatives have been reported [217].

[e] In addition, the D-ribo-1,2,3,4-tetrahydroxybutyl derivative has been reported [218].

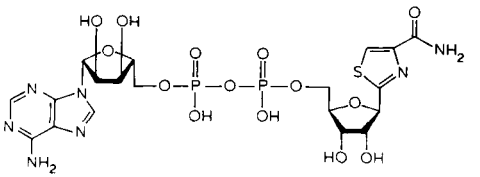
[f] In addition, the β-D-arabino derivative has been reported together with several other imidazole *C*-nucleosides [226].

[g] In addition, the 2′-deoxy-β-D-ribofuranosyl- and β-D-arabinofuranosyl derivatives have been reported [223].

[h] The sugar moiety is β-D-arabinofuranosyl.

[217], since this enzyme converts inosine monophosphate to xanthosine monophosphate, which is a guanosine precursor. Two *in vitro* and *in vivo* metabolites of (27), the 5′-monophosphate [216] and the NAD analogue, (28) [228,229], have been identified and synthesized. The formation of (28) has been proposed to involve initial formation of the 5′-phosphate of (27) (catalyzed by a kinase), followed by an NAD-phosphorylase-catalyzed reaction with ATP [228].

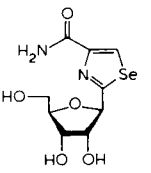
Compound (28) is a very potent inhibitor of inosine monophosphate dehydrogenase [228,230] and probably inhibits the enzyme by binding to the NAD cofactor site with the thiazole-4-carboxamide moiety occupying the pocket normally filled by the nicotinamide ring [231].



(28)

It has been suggested that the actual inhibition of cell growth resulting from treatment with (27) may be a result of perturbations of guanosine triphosphate-dependent processes such as mRNA, nRNA or DNA synthesis [228].

Several structural analogues of (27) have been synthesized, but even minor structural changes result in loss of activity [216,217]. For example, the 2′-deoxy analogue of (27) [231,232] is biologically inactive. However, isosteric substitution of sulphur for selenium, which gives compound (29), seems to increase activity; compound (29) is 5-times more potent than (27) against L1210 and P388 cells and (29) is also a curative agent in mice with Lewis lung carcinoma [219].



(29)

CHEMISTRY AND BIOCHEMISTRY OF *C*-ARYLGLYCOSIDES

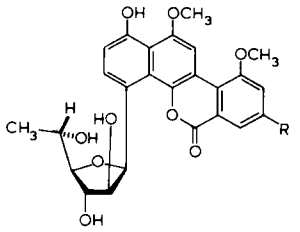
C-Glycosides in great number and structural diversity are known to be present in nature. In the 1960's, Haynes [233] reviewed the early *C*-glycoside literature, which consists almost entirely of plant *C*-glycoside isolation studies. More recently, the chemistry, occurrence and biosynthesis of plant *C*-glycosides have been reviewed [234]; reviews of *C*-glycosyl flavonoids are also available [235,236]. Plant *C*-glycosides are not included in the present survey because, as noted in the Introduction, the available information consists almost entirely of isolation and structural studies; information concerning their biological roles and physiological effects has not appeared.

The review is limited in a second way. We have adopted as a working definition: a *C*-arylglycoside is a molecule (not nitrogen heterocyclic) which incorporates a carbohydrate moiety through one carbon-carbon bond linkage involving C-1 of the carbohydrate, i.e., the 'anomeric' carbon. Use of this definition led to the exclusion of important compounds, e.g., palytoxin [237] and aurodox (antibiotic X-5108) [238], which incorporate carbohydrate moieties through two carbon-carbon bond linkages.

BENZO[*d*]NAPHTHO[1,2-*b*]PYRAN-6-ONE *C*-GLYCOSIDES

The gilvocarcins (toromycins)

Gilvocarcin M, [4-(6-deoxy-α-galactofuranosyl)-10,12-dimethyl-1-hydroxy-8-methyl-6*H*-benzo[*d*]naphtho[1,2-*b*]pyran-6-one, antibiotic 2064B, (30), m.p. 252–256°C, $[\alpha]_D^{20}$ −209° (*c* 0.2, Me_2SO)], and gilvocarcin V, [4-(6-deoxy-α-galactofuranosyl)-10,12-dimethoxy-8-ethenyl-1-hydroxy-6*H*-benzo[*d*]naphtho[1,2-*b*]pyran-6-one, toromycin, antibiotic 2064A, (31), m.p. 264–267°C (dec.), $[\alpha]_D^{20}$ −216° [*c* 0.16, Me_2SO)] have been isolated from *Streptomyces gilvotanareus* [239], *Streptomyces arenae* 2064 [240], *Streptomyces collinus* subspecies *Albescens* [241] *Streptomyces anandii* [242] and from an unnamed *Streptomyces* species designated AAC-324 [243]. A third analogue, gilvocarcin E, [4-(6-deoxy-α-galactofuranosyl)-10,12-dimethoxy-8-ethyl-1-hydroxy-6*H*-benzo[*d*]naphtho[1,2-*b*]pyran-6-one, dihydrotoromycin (32), m.p. 218–220°C (dec.), $[\alpha]_D^{22}$ −200° (*c* 0.5, Me_2SO)] has also sometimes been detected [242,243]. The gilvocarcins have been characterized spectroscopically [240,242–246] and by X-ray crystallography [242,247] and their biosynthesis has been studied using ^{13}C-labelled acetate and propionate as precursors [246].



(30) : R = CH_3
(31) : R = $CH{=}CH_2$
(32) : R = CH_2CH_3

Gilvocarcin V (31) is active against Gram-positive bacteria, mycobacteria, mycoplasma, trichomonad, DNA viruses such as Vaccinia virus and Herpes simplex, inhibits the plaque formation of λ, ϕ170, T_1, T_3 and T_5 phages, but is not active against Gram-negative bacteria, Newcastle disease virus or Q_β, ϕ 174, T_2 and T_4 phages [239,241] or fungi [242]. Gilvocarcin M (30) is 10–100-fold less active [239,241]. Gilvocarcin V is active against several isolates of anaerobic bacteria [248]. Gilvocarcin V exhibits a broad spectrum of activity against neoplasia in mice; the 8-methyl analogue, gilvocarcin M (30), has not shown antitumour activity [242,249]. Gilvocarcin V is more effective against Ehrlich ascites carcinoma than mitomycin C, producing a 200% increase in life-span, with four out of five mice surviving 60 days [249]. Good results were obtained using gilvocarcin V (31) for treatment of other mouse tumours including Meth 1 fibrosarcoma, MH134 hepatoma and lymphocytic leukaemia P388; it was less effective against B16 melanoma and Lewis lung carcinoma [242,244] and exhibited no activity against sarcoma 180 upon oral administration [249]. The safety margin using gilvocarcin V appears to be very high, since the compound is remarkably non-toxic to mice (LD_{50} values 1000 mg/kg intraperitoneally and 375 mg/kg intravenously) [242,249].

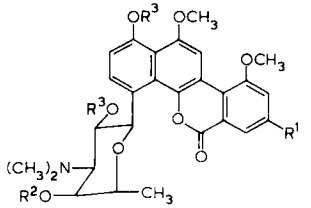
Gilvocarcin V (31) is a potent inhibitor of DNA synthesis [250], causing strand breaks which are more frequent upon irradiation of gilvocarcin V-DNA complexes [251], but does not affect directly either RNA or protein biosynthesis [250]. Visible light activates gilvocarcin V to induce bacteriophage lambda in *Escherichia coli* by a DNA-dependent mechanism [252].

Little chemistry is available for the gilvocarcins. The vinyl group at C-8 of (31) has been reduced [243,245] or oxidized to the corresponding aldehyde [244,250]; tetraacetyl derivatives have been prepared to aid in characterization [242,244,245] or isolation [243]. Jain, Simolike and Jackman [243] elucidated an acid-catalyzed rearrangement process whereby gilvocarcin V (31) is converted to an equilibrium mixture of four *C*-glycosides in the ratio of 1

(unchanged gilvocarcin V):1 (the corresponding α-anomer):2.5 (the β-fucopyranosyl isomer):1 (unidentified isomer). A number of degradation products of gilvocarcin V (31) were prepared and characterized in connection with structural studies [245]. The 8-aldehydo derivative did not interact with *B. subtilis* DNA and exhibited only weak antibacterial activity [250].

Ravidomycin

Ravidomycin, [4-(4-*O*-acetyl-3,6-dideoxy-3-dimethylamino-α-altropyranosyl)-10,12-dimethoxy-8-ethenyl-1-hydroxy-6*H*-benzo[*d*]naphtho[1,2-*b*]pyran-6-one (33), m.p. 248–250°C, $[\alpha]_D^{26}$ −105.5 (*c* 0.2, $CHCl_3$)], an antitumour *C*-glycoside antibiotic with the same aglycone as the gilvocarcins ((30)–(32)), was isolated from the fermentation broth of a newly recognized Streptomycete strain named *Streptomyces ravidus* [253]. Structure (33) was assigned to ravidomycin based on spectrometric studies of the compound, its diacetyl derivative, the alkali-fusion-derived aglycone, the 8-ethyl (vinyl reduction) analogue and a number of degradation products [254,255]. The biosynthesis of ravidomycin (33) has been studied using ^{13}C-labelled acetate and propionate [256].



(33) : $R^1 = CH{=}CH_2$, $R^2 = Ac$, $R^3 = H$
(34) : $R^1 = CH{=}CH_2$, $R^2 = R^3 = H$
(35) : $R^1 = CH{=}CH_2$, $R^2 = R^3 = Ac$
(36) : $R^1 = C_2H_5$, $R^2 = Ac$, $R^3 = H$
(37) : $R^1 = C_2H_5$, $R^2 = R^3 = H$
(38) : $R^1 = C_2H_5$, $R^2 = R^3 = Ac$

Ravidomycin (33) is markedly active against Gram-positive bacteria, weakly active against Gram-negative bacteria, and inactive against fungi [253]. Several modifications have been made to the ravidomycin structure (33) to probe the structural features necessary for antimicrobial and antitumour activities [257]. Comparative biological data (*Table 1.3*) were obtained for (33), the deacetyl (34) and diacetyl (35) derivatives, the 8-ethyl analogue (36) and corresponding deacetyl (37) and diacetyl (38) compounds. This limited study of structure-

Table 1.3. BIOLOGICAL ACTIVITIES OF RAVIDOMYCIN AND DERIVATIVES

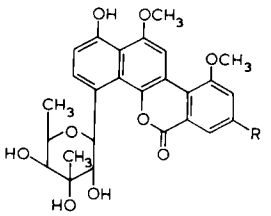
	Compound					
	(33)	*(34)*	*(35)*	*(36)*	*(37)*	*(38)*
Antitumour						
P388 mouse leukaemia, survival (%T/C)[a]						
200 mg/kg	205[d, e]		inact.	toxic		inact.
50	140	205		137	140	
3.1		165				
Colon 38 mouse tumour, tumour weight (%T/C)[b]						
400 mg/kg	0					
200	13					
100	30					
Cd8F1 rat mammary tumour, tumour weight (%T/C)[b]						
50 mg/kg	0					
25	51					
Antimicrobial[a, c]						
min. inhib. concn. (μg/ml)						
Staphylococcus pyogenes[d]	6.4	3.2	inact.	12.5	inact.	inact.
Streptococcus faecalis	3.2	1.6	inact	3.2	12.5	inact.
Bacillus subtilis	2.5	inact.	10	2.5	10	5
DNA synthesis inhibition in *B. subtilis* at 0.5 μg/ml (% inhib.)	58	73	10	none	none	

[a] [256].
[b] [253].
[c] None of the compounds exhibited appreciable activity against *Escherichia coli*, *Salmonella pullorum*, *Pseudomonas aeruginosa*, *Proteus micrabilis*, *P. vulgaris* or *Klebsiellia pneumoniae*.
[d] Both penicillin-sensitive and penicillin-resistant strains were equally responsive.
[e] %T/C of 238 at 100 mg/kg was reported [253].

activity relationships shows that (a) antimicrobial and antitumour activities respond similarly to modifications of the ravidomycin structure and correlate with ability to inhibit DNA synthesis [257,258], and (b) masking of polar groups or reduction of the 8-vinyl subsitutent diminishes inhibitory activity [256]. Rakhit, Eng, Baker and Singh note [257] that ravidomycin (33) and its deacetyl derivative (34) exhibit significantly greater biological activity than the gilvocarcins ((30)–(32)) or the chrysomycins (see below) and suggest that the observed differences reflect a key rôle for the aminosugar in determining biological activity.

Chrysomycins A and B

Chrysomycin A, 4-(6-deoxy-3-*C*-methyl-β-D-gulopyranosyl)-10,12-dimethoxy-8-ethenyl-1-hydroxy-6*H*-benzo[*d*]naphtho[1,2-*b*]pyran-6-one (39), and chrysomycin B, 4-(6-deoxy-3-*C*-methyl-β-D-glucopyranosyl)-10,12-dimethoxy-1-hydroxy-8-methyl-6*H*-benzo[*d*]naphtho[1,2-*b*]pyran-6-one (40), were isolated as an unseparated and only partially characterized mixture in 1954 [259] from an unidentified *Streptomyces*. No further report on chrysomycin appeared until 1982, when the chrysomycins (39) and (40) were characterized using samples stored since 1955 [260], as remaining cultures of the *Streptomyces* strain no longer produced the antibiotics. Use of high-pressure liquid chromatography led to facile separation of (39) and (40), which exhibit ultraviolet spectra identical with gilvocarcins V (31) and M (30), respectively, thereby establishing the aglycone structures. The structure of the carbohydrate moiety of (39) and (40) was established by ^{1}H and ^{13}C nuclear magnetic resonance spectrometry and appears to be identical with (or antipodal to) virenose recently obtained as a methyl glycoside by methanolysis of an unidentified antitumour antibiotic virenomycin [261,262]. The structure and stereochemistry of methyl β-D-virenoside were confirmed by synthesis from α-D-galactose [263].



(39) : R = $CH{=}CH_2$
(40) : R = CH_3

In biological studies carried out in 1954 [259], a mixture of chrysomycins A and B (A principally) was active against *Bacillus cereus* phage at 0.01 μg/ml; in disc tests, chrysomycin was active against 31 of 62 bacteriophages tested and was phagocidal against Staphylophage 14, Coliphage T1, *B. cereus* phage, *B. subtilis* phage C.S.C. and Cholera phage C. Antibacterial activity was exhibited against *Micrococcus pyogenes*, *B. subtilis*, *Mycobacterium smegmatis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Eschericha coli* and, at concentrations of 50–100 μg/ml, chrysomycin inhibited the growth of the fungi *Aspergillus niger*, *Chaetomium convuluta*, *Memnoniella chinata*, *Myrothecium*

verrucaria, *Penicillium notatum*, *Phycomyces blakesleeanus*, *Saccharomyces cerevisiae*, *Stemphylium consortiale* and *Trichophyton mentagraphytes*.

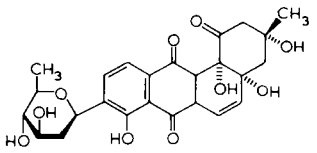
Similar to the gilvocarcins [242,249], the chrysomycins are remarkably non-toxic to mice [259,260]; while no LD_{50} value has been determined, it is estimated as over 1000 mg/kg [258]. A single dose (400 mg/kg) of chrysomycin consisting of 86% of (39) and 14% of (40), administered intraperitoneally to mice 24 h after inoculation with P388 lymphocytic leukaemic cells, produced an increase in the life-span of treated mice of 54% while exhibiting no lethal toxicity [260]. Chrysomycin A (39) was only half as effective as gilvocarcin V (31) in inhibiting DNA synthesis in *Bacillus subtilis* cells during log-phase growth [251]. Gilvocarcin V (31) caused DNA degradation at concentrations of less than 10 μg/ml; in contrast, even at concentrations of chrysomycin A (39) as high as 50 μg/ml, no DNA degradation was observed.

ANTHRACENE AND BENZANTHRACENE C-GLYCOSIDES

Aquayamycin

Aquayamycin [9-(2,6-deoxy-D-*arabino*-hexopyranosyl)-3-methyl-3,4,4a,12b-tetrahydro-3,4a,8,12b-tetrahydroxybenz[*a*]anthracene-1,7,12(2*H*)-trione (41), m.p. 189–190°C (dec.), $[\alpha]_D^{20}$ +160° (*c* 1, dioxan)] is produced by *Streptomyces misawanensis* [264]. Its structure (41) was determined [265] following extensive spectroscopic and degradative studies. Aquayamycin (41) is a strong inhibitor of the enzymes tyrosine hydroxylase [266] and dopamine β-hydroxylase [267].

Chemical transformations of aquayamycin (41) during structural studies [265] have produced a number of analogue *C*-glycosides of interesting structure. Treatment of (41) with barium hydroxide at room temperature or, alternatively, heating of (41) at 200°C *in vacuo*, brought about a double dehydration and rearrangement to yield (42). Ultraviolet irradiation of aquayamycin (41) led to anthracyclinone *C*-glycoside (43) and treatment of (41) with methanolic hydrochloric acid (followed by saponification of the methyl carboxylate formed) produced ring-cleavage product (44). If aquayamycin (41) were first hydrogenated to reduce the 5,6-double bond, treatment with acid brought about simple dehydration and aromatization to yield (45) [265].



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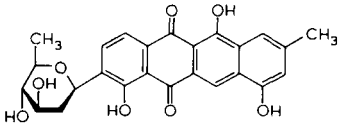

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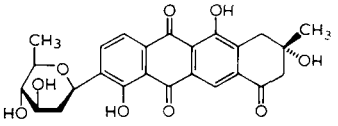

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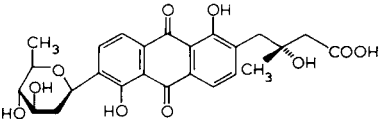

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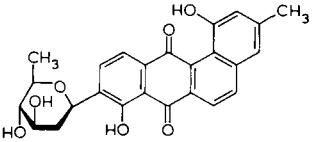


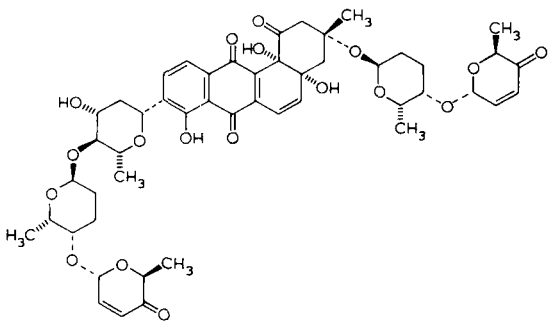
(45)

The vineomycins

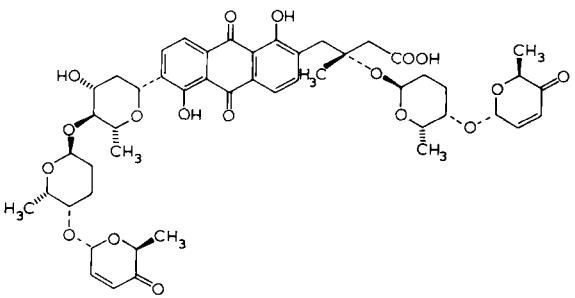
Vineomycin A_1 [antibiotic DS-4742A_1, P-1894B, (46), m.p.162–163°C, $[\alpha]_D^{26}$ +92 (*c* 0.5, $CHCl_3$)] and vineomycin B_2 [antibiotic DS-4742B_2 (47), m.p. 128–131°C, $[\alpha]_D^{26}$ +30.8 (*c* 0.5, $CHCl_3$)] were isolated from the culture broth of *Streptomyces matensis* subsp. *vineus* together with two other, as yet uncharac-

terized, antibiotics [261]. A collagen proline hydroxylase inhibitor produced by *S. alborgriseolus*, which was termed P-1894B [269] and characterized by X-ray crystallography [270], proved to be identical to vineomycin A_1 (46) [271]. Apparently, *S. albogriseolus* does not produce vineomycin B_2 (47) [272].

Treatment of vineomycin A_1 (46) with dilute hydrochloric acid at room temperature to hydrolyse *O*-glycosidic bonds produced aquayamycin (41); more vigorous acid hydrolysis (90° C) of (46) yielded a ring-cleaved *C*-glycoside (44) identical with that obtained from aquayamycin (41) under these conditions [265]. Finally, acidic (room temperature) de-*O*-glycosidation of vineomycin B_2 (46) also yielded (44) [272]. That vineomycin B_2 (47) is not a processing artefact was shown by direct chromatographic examination of fermentation broth of *S. matensis* subsp. *vineus* [272]. The identity of the sugars of (46) and (47) released upon hydrolysis was established by gas chromatography-mass spectrometry; that the sugar sequences were the same was shown by nuclear magnetic resonance [272].



(46)



(47)

Biosynthetic studies of the vineomycins in *S. matensis* subsp. *vineus* [273] led to the recognition that the organism also produces rabelomycin [274], the benzo[*a*]anthraquinone aglycone common to vineomycin A_1 (46) and aquayamycin (41). This benz[*a*]anthraquinone system is biosynthesized from a decaacetate metabolite decarboxylated at the carboxyl end [273]. Vineomycin B_2 (47) arises biosynthetically from vineomycin A_1 (46) [273]. Danishefsky, Uang and Qualich [275] have recently synthesized the *C*-glycoside core (44) of vineomycin B_2.

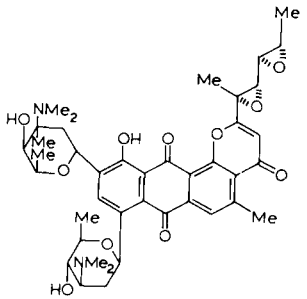
Vineomycin A_1 (46) is active against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* PCI 219, *B. cereus* T, *Sarina leutea* PCI 1001), *Piricularia oryzae* and *Microsporum gypseum* and against the sarcoma 180 solid tumour in mice [268]. The acute toxicity (LD_{50} values, intraperitoneally) of vineomycin A_1 in mice was estimated to be 100–150 mg/kg [268] and 100–200 mg/kg (but over 1000 mg/kg orally) in rats [269]. Vineomycin B_2 (47) exhibits a similar pattern of antimicrobial activities, but appears to be somewhat less active. Vineomycin B_2 (47) was not tested in pure form against the sarcoma 180 solid tumour; a mixture of (47) and a closely related, but uncharacterized, co-occurring antibiotic exhibited activity similar to that observed for vineomycin A_1 (46) [268].

Vineomycin A_1 (46) inhibited chick embryo prolyl hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2), which catalyzes the conversion of specific proline residues in the peptide precursor to collagen to 4-hydroxyproline, exhibiting 50% inhibition at 2 μg/ml [269,276]. The inhibition was only marginally sensitive to added ferrous ion or ascorbic acid (enzyme cofactors), indicating that vineomycin A_1 (46) does not function by chelating ferrous ion or as an antioxidant toward ascorbate [269,276]. Collagen biosynthesis in the uterus of immature rats, stimulated by oestradiol administration, was markedly inhibited by vineomycin A_1 at a dose of 0.15 mg/kg [276].

Hedamycin

Hedamycin [2-(1,2:3,4-diepoxy-1-methylpentyl)-11-hydroxy-5-methyl-8-[3-(dimethylamino)-2,3,6-trideoxy-β-D-*arabino*-hexopyranosyl]-10-[3-(dimethylamino)-3-*C*-methyl-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl]-4*H*-anthra[1,2-*b*]pyran-4,7,12-trione, (48), m.p. 243–245°C (dec.)] was detected in a fermentation broth of *Streptomyces griseoruber* inhibitory to the sarcoma S180 mouse tumour [277]. The structure of hedamycin was determined [278–282] using X-ray crystallography [280], spectroscopic investigations and synthesis of model compounds to elucidate the relative configurations and stereochemistries

of aglycone side-chains [281]. A notable point concerning the crystal structure of hedamycin (48) is the almost axial position of the aglycone with respect to the tetrahydropyranyl (carbohydrate) ring at C-10 [280]. That a bulky aglycone would assume an axial position was not appreciated during early studies. Nuclear magnetic resonance results, which were interpreted assuming an equatorial position for the aglycone, led to the suggestion that, in solution, hedamycin (48) assumed a flexible, non-chair conformation [282]. This study and an X-ray structure of a bisquaternary ammonium derivative of the related antibiotic, kidamycin (49, see below), in which the amino sugar at C-10 adopts a boat conformation [283,284], has led to the probably erroneous formulation of the glycosyl moiety at C-10 in a boat conformation for hedamycin (48), kidamycin (49) and the pluramycins (50) and (51). It has even been suggested [283] that a boat conformation of this ring is responsible for the observed biological activity.



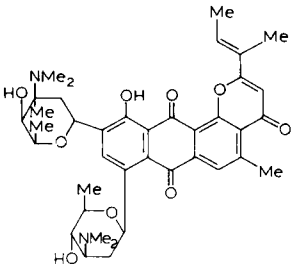
(48)

Hedamycin (48) is inhibitory to Gram-positive bacteria, yeasts (e.g., *Kloekera brevis*) and protozoa (e.g., *Tetrahymena pyriformis*); it induces lysogenic *Escherichia coli* W1709 (λ) at a minimum level of 0.0125 μg/ml and has antiphage activity at 0.015 μg/ml [285]. Hedamycin (48) is highly toxic to HeLa cells and inhibits Walker 256 tumour in rats and duodenal adenocarcinoma in hamsters; it inhibits slightly mouse sarcoma 180 but does not affect lymphatic leukaemia L1210, myeloid leukaemia C1498 or adenocarcinoma 755 [281]. In Swiss mice the LD_{50} value of hedamycin (48) is 0.3 mg/kg (intraperitoneally) [281].

Hedamycin (48) interacts strongly with DNA [285–289] and inhibits the synthesis of bacterial RNA and DNA [282,283] suggesting [282] that its mechanism of biological action involves intracellular binding to DNA with consequent inhibition of strand separation and prevention of DNA synthesis.

Kidamycin

Kidamycin [2-(1-methyl-1-propenyl)-11-hydroxy-5-methyl-8-[3-(dimethylamino)-2,3,6-trideoxy-β-D-*arabino*-hexopyranosyl]-10-[3-(dimethylamino)-3-*C*-methyl-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl]-4*H*-anthra[1,2-*b*]pyran-4,7,12-trione (49), m.p. 214–217°C, $[\alpha]_D^{20}$ +457 (*c* 1.5, $CHCl_3$)], is a metabolite of *Streptomyces phaeoverticillatus* var. *takatsukiense* produced when anthraquinone-2,7-disulphonic acid is added to the culture medium [290,291]. The structure of kidamycin (49) was elucidated by chemical and spectroscopic studies [279,282,288] and X-ray crystallography [283,284]. As noted (see hedamycin), X-ray crystallography revealed that the *C*-glycosyl moiety at C-10 adopts a boat conformation in the bis(trimethylammonium) derivative of kidamycin [283,284]. Further study [292,293] showed that a similar bis-quaternary ammonium derivative of isokidamycin, which is formed by acid-catalyzed isomerization of the anomeric carbon of the C-10 glycosyl unit [283], adopts a chair conformation for this carbohydrate unit. These data suggest [293] that the greater stability of isokidamycin as compared with kidamycin (49) and the unexpected carbohydrate conformations observed for kidamycin (49) and hedamycin (48) [280] arise from severe 1,3-diaxial interactions in the C-10 carbohydrate unit.



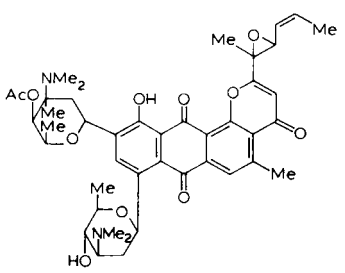
(49)

A number of kidamycin derivatives have been prepared. Thus, kidamycin (49) readily forms a triacetate [283,294] and, following prior reduction of the quinone system, a pentaacetate [283]. Partial hydrolysis of kidamycin triacetate permitted isolation of two different diacetyl kidamycins [283]. A derivative was prepared by Cope elimination of an intermediate di-*N*-oxide prepared by treatment of kidamycin triacetate with perbenzoic acid [283]. Similar manipulation of isokidamycin (see above) produced a corresponding series of iso derivatives [283]. Hauser and Rhee [295,296] have synthesized the methyl ether of kidamycinone, the aglycone.

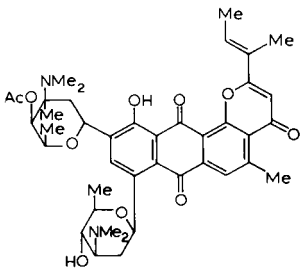
Kidamycin (49) and triacetylkidamycin are active against Gram-positive bacteria but not Gram-negative bacteria or fungi [291,294,297]. The antimicrobial activity of kidamycin (49) was diminished only slightly (less than 5-fold) by acetylation; in contrast, the acute toxicities of the two compounds in mice show somewhat greater differences. The LD_{50} value of kidamycin (49) in mice is 12.5–20 mg/kg (intravenous or intraperitoneal) [291] while LD_{50} values of triacetylkidamycin are 200 mg/kg (intravenous), 50 mg/kg (intraperitoneal) and 600 mg/kg (oral) [294]. Both kidamycin (49) and triacetylkidamycin exhibit growth inhibition of animal tumours, including Ehrlich ascites, mouse leukaemias SN36 and L1210 and solid tumours sarcoma 180 and Ehrlich carcinoma [291,294,297].

Pluramycin A and neopluramycin

Pluramycin A [2-(1,2-epoxy-1-methyl-3-(2)pentenyl)-11-hydroxy-5-methyl-8-[3-(dimethylamino)-2,3,6-trideoxy-β-D-*arabino*-hexopyranosyl]-10-[3-(dimethylamino)-3-*C*-methyl-2,3,6-trideoxy-4-*O*-acetyl-α-L-*lyxo*-hexopyranosyl]-4*H*-anthra[1,2-*b*]pyran-4,7,12-trione (50)] produced by *Streptomyces pluricolorescens* Okami et Umezawa [298], is closely related structurally [299] to hedamycin (48), differing only in the absence of a 3,4-epoxy group and in the *cis* orientation of aliphatic side-chain carbon atoms [281]. Neopluramycin [2-(1-methyl-1-propenyl)-11-hydroxy-5-methyl-8-[3-(dimethylamino)-2,3,6-trideoxy-β-D-*arabino*-hexopyranosyl]-10-[3-(dimethylamino)-3-*C*-methyl-2,3,6-trideoxy-4-*O*-acetyl-α-L-*lyxo*-hexopyranosyl]-4*H*-anthra[1,2-*b*]pyran-4,7,12-trione (51)] is derived from another strain of *S. pluricolorescens* [300] and has been identified as a monoacetyl derivative of kidamycin (49) by spectroscopic comparisons [299].



(50)



(51)

At the time of its discovery (1956) [298,301], preliminary biological evaluation of pluramycin A (50) established activity against Gram-positive bacteria [298] and activity against murine Ehrlich carcinoma [301]; no further biological studies are available. Neopluramycin (51) exhibited somewhat lower antimicrobial activity than did pluramycin A (50) [300]. For leukaemia L1210 in mice, intraperitoneal injection of doses of neopluramycin (51) of 3.12, 1.56 and 0.78 mg/day for 10 days resulted in increased survival times of 190, 167 and 161%, respectively [300]. Neopluramycin (51) added in concentrations of 0.1, 0.02 and 0.004 mg/ml inhibited the multiplication of Yoshida rat sarcoma cells in cultures by 86, 62 and 23%. Mice dosed with neopluramycin (51) at 25 mg/kg died after 7–11 days; the LD_{50} value in mice was estimated as 12.5–25 mg/kg [300].

Nogalamycin

Nogalamycin [11-[(6-deoxy-3-*C*-methyl-2,3,4-tri-*O*-methyl-α-L-mannopyranosyl)oxy]-4-(dimethylamino)-3,4,5,6,9,11,12,13,14,16-decahydro-3,5,8,10,13-pentahydroxy-6,13-dimethyl-9,16-dioxo-2,6-epoxy-2*H*-naphthaceno[1,2-*b*]-oxocin-14-carboxylic acid, methyl ester (52), m.p.195–196°C (dec.), $[\alpha]_D^{25}$ +479 ($CHCl_3$)] is produced by *Streptomyces nogalater* [302]. Structural studies were initiated in the mid-1960's [303], but a complete structural assignment has only recently been completed using chemical degradation and spectroscopic data [304,305] and X-ray crystallography [306]. Wiley has recently reviewed nogalamycin research [307].

Nogalamycin (52) is active against several animal tumours [302,308] in animals but it has exhibited toxic effects which precluded clinical investigations. Extensive studies of biological and biochemical properties of nogalamycin (52) have been carried out [302,308–329], leading to the conclusion [323,324] that its biological activity is probably due to complex formation with DNA and RNA involving nogalamycin intercalation.

A large number of nogalamycin analogues and derivatives have been prepared during structural studies [303,304] and to permit study of structure-activity relationships [316,327,328]. In *Table 1.4* are presented data for selected nogalamycin derivatives which permit conclusions to be drawn concerning structure-activity relationships in the mouse leukaemia P388 system. A study of L1210 leukaemia *in vitro* and *in vivo* produced similar results [313]. It is evident from the data in *Table 1.4* that (a) numerous derivatives, although less potent than nogalamycin (52), are more effective in prolonging life, (b) neither the methoxycarbonyl group at C-10 nor the *O*-glycosyl at C-7 is essential for activity, (c) modification of the *C*-glycosyl at C-2 decreases activity and (d) 7-

Table 1.4. ACTIVITY OF NOGALAMYCIN AND DERIVATIVES AGAINST MOUSE LEUKAEMIA P388[a, b]

Compound	*Optimal dose (mg/kg per day)*	*Increase in life-span (%)*
Nogalamycin (52)	1	49
Diacetyl- (53)	6.25	3
N-Demethyl- (54)	25	59
N-Formyl- (55)	25	12
N-Formyldisnogamycin (56)	50	0
Disnogamycin (57)	5	93
7-Deoxynagalarol (58)	50	27
7-Deoxynogalarolic acid (59)	400	−4
7-Deoxynogarol (60)	100	21
7-con-*O*-Methylnogarol (61)	12.5	197
7-con-*O*-Ethylnogarol (62)	6.5	63
7-con-*O*-Methylnogarol,6-M deriv. (63)	20	25
7-Acetoxynogarol (64)	10	87
7-Aminonogarol (65)	40	47
7-Methylaminonogarol (66)	25	58
7-Dimethylaminonogarol (67)	50	54
7-Methylthionogarol (68)	8	37
7-con-*O*-Methylnogarol, *N*-formyl deriv. (69)	1.25	73
7-dis-*O*-Methylnogalarol (70)	25	60
7-con-*O*-Methylnogalarol (71)	25	98
7-dis-*O*-Methylnogarol (72)	12.5	76
Nogalarene (73)	70	50
Nogarene (74)	12.5	17

[a] Data taken from Refs. 315 and 326.

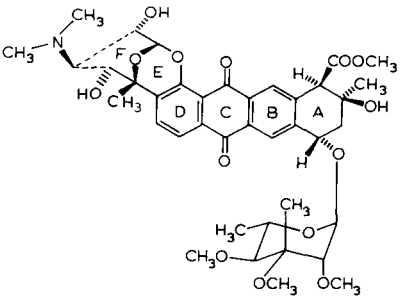
[b] 10^6 leukaemia cells injected intraperitoneally on day 0; drug injected daily on days 1–9 or on days 1, 5 and 9.

O-alkyl derivatives with unnatural stereochemistry are more active than corresponding derivatives with the stereochemistry of nogalamycin at C-7.

The activity of 7-con-*O*-methylnogarol (61) is particularly striking and a clinical trial is planned [328]. This compound is active against P388, L1210, Colon 26, Lewis lung (marginally) B 16 and Colon 38 mouse tumours [316,328,329]. In paired tests with adriamycin, 7-con-*O*-methylnogarol (61) compared favourably in activity, although adriamycin is about 10-times more potent [316]. Adriamycin is significantly more cardiotoxic than 7-con-*O*-methylnogarol [316].

Numerous studies of the effects of nogalamycin (52) and its derivatives on nucleic acid synthesis suggest a key rôle for nogalamycin complex formation with DNA and RNA in the expression of its biological activity [324]. 7-con-*O*-Methylnogarol (61) also interacts with nucleic acid, inhibiting RNA and DNA synthesis. However, when added to cultures of L1210 leukaemic cells at 0.1 μg/ml, 7-con-*O*-methylnogarol inhibited over 90% of cell growth, although at 25 μg/ml only 30% inhibition of DNA and RNA syntheses occurred. These results suggest [313] that the biological activity of 7-con-*O*-methylnogarol (61) is mediated through a mechanism other than interaction with DNA.

No synthesis of nogalamycin (52) has appeared. Methods for construction of the benzoxocin system, i.e., the *C*-glycosyl-functionalized terminal aryl ring in both racemic [330,331] and chiral forms [332], have been reported recently.



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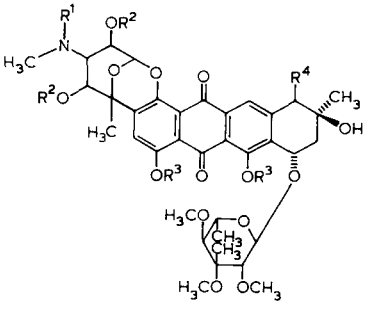


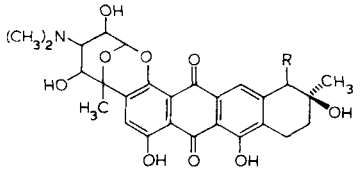
(53) : $R^1 = CH_3$, R^2 = Ac, R^3 = H, $R^4 = COOCH_3$

(54) : R^1, R^2, R^3 = H, $R^4 = COOCH_3$

(55) : R^1 = CHO, R^2, R^3 = H, $R^4 = COOCH_3$

(56) : R^1 = CHO, R^2, R^3, R^4 = H

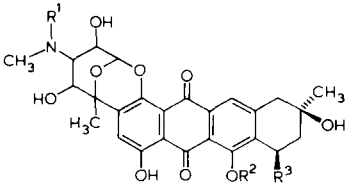
(57) : $R^1 = CH_3$, R^2, R^3, R^4 = H



(58) : R = $COOCH_3$

(59) : R = COOH

(60) : R = H



(61) : R^1 = CH_3, R^2 = H, R^3 = OCH_3

(62) : R^1 = CH_3, R^2 = H, R^3 = OCH_2CH_3

(63) : R^1 = R^2 = CH_3, R^3 = OCH_3

(64) : R^1 = CH_3, R^2 = H, R^3 = OAc

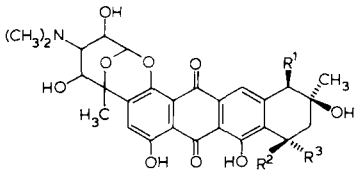
(65) : R^1 = CH_3, R^2 = H, R^3 = NH_2

(66) : R^1 = CH_3, R^2 = H, R^3 = $NHCH_3$

(67) : R^1 = CH_3, R^2 = H, R^3 = $N(CH_3)_2$

(68) : R^1 = CH_3, R^2 = H, R^3 = SCH_3

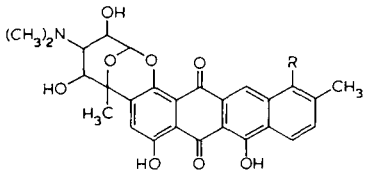
(69) : R^1 = CHO, R^2 = H, R^3 = OCH_3



(70) : R^1 = $COOCH_3$, R^2 = H, R^3 = OCH_3

(71) : R^1 = $COOCH_3$, R^2 = OCH_3, R^3 = H

(72) : R^1 = R^2 = H, R^3 = OCH_3



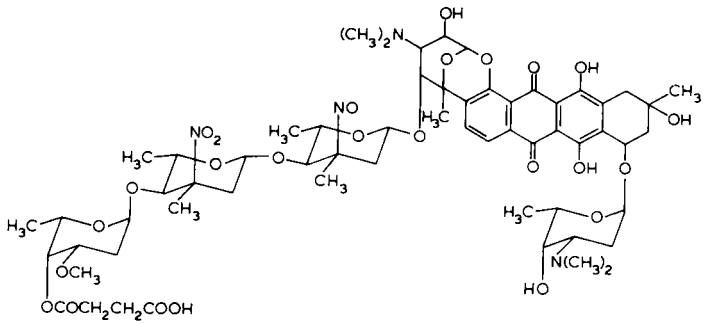
(73) : R = $COOCH_3$

(74) : R = H

Decilorubicin

Decilorubicin (m.p. 170–174°C (dec.), $[\alpha]_D^{25}$ +460° (*c* 0.05, $CHCl_3$-MeOH, 1:1)) was isolated from the culture of *Streptomyces virginiae* MF266-g4 [333] and tentatively assigned structure (75) based on spectroscopic studies and chemical degradation. Hydrogenolysis of decilorubicin methyl ester released the 3-aminosugar, L-rhodosamine [334]. Methanolysis of decilorubicin yielded two other constituent sugars as the corresponding methylglycosides [335]. One of the sugars obtained in this way was identified as methyl 4-*O*-succinyl-L--diginoside methyl ester; the other was a previously unrecognized nitrosugar, named decilonitrose, which was identified by spectroscopic analysis and synthesized [335].

Decilorubicin exhibits significant activity against Gram-positive bacteria and, when injected into L1210 leukaemic CDF mice for 10 days (2.5 mg/kg per day), increased the life-span over untreated mice by 177% [333]. The LD_{50} value of decilorubicin dihydrochloride in mice is 50–100 mg/kg (intravenous or intraperitoneal).



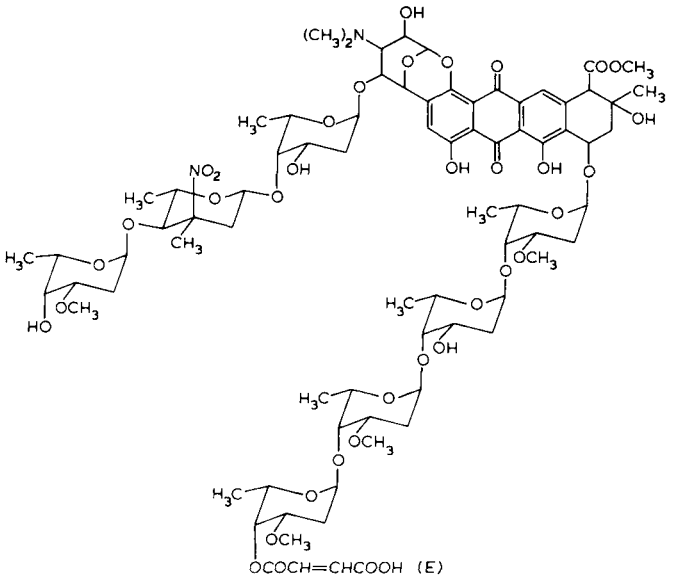
(75)

Arugomycin

Arugomycin (m.p. 208–212°C (dec.), $[\alpha]_D^{25}$ +112° (*c* 0.1, $CHCl_3$-MeOH, 9:1)) was isolated from *Streptomyces violochromagenes*, strain 1098AV2 [336] and tentatively assigned structure (76). Mild acid hydrolysis of arugomycin permitted isolation of nogalarol [304], which identified the *C*-glycosyl-containing aglycone. Also released and identified were the sugars 2-deoxyfucose, diginose and decilonitrose [335].

Arugomycin inhibited growth of Gram-positive, but not Gram-negative, bacteria. Intraperitoneal injections of arugomycin into mice with Ehrlich

carcinoma (0.5 mg/kg on days 1,3 and 5) resulted in an increase in life-span of 189%. The LD_{50} value of arugomycin in mice was 1.75 mg/kg [336].

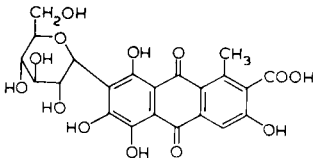


(76)

Carminic acid

Carminic acid (7-α-D-glucopyranosyl-9,10-dihydro-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-2-anthracenecarboxylic acid (77)) was isolated from the scale insect *Coccus cacti* L., *Homoptera* (cochineal) in the 1890's and first characterized in 1920 [337]. The recognition that carminic acid was a *C*-glycoside [338] and complete structure assignment [339] was accomplished more recently.

Carminic acid (77) has long been used as a red dye in foods and elsewhere.



(77)

Viriplanin

Viriplanin (m.p. 210–215°C (dec.)) is produced by fermentation of *Ampullariella regularis* SE 47 [340]. The molecular weight of viriplanin was estimated by gel filtration to be about 2,000. Acid-catalyzed methanolysis of viriplanin produced the *C*-glycosyl aglycones 7-*O*-methylnogalarol (70, 71) and nogalarene (73); acid or alkaline hydrolysis yielded 2-deoxy-2-fucose and mesaconic acid.

Viriplanin inhibited the growth of Gram-positive bacteria and viruses, including herpesvirus [340].

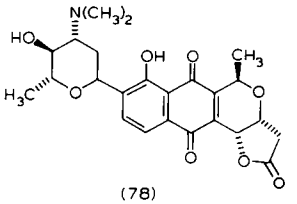
Other anthracene C-glycosides

A number of antibiotics which have been isolated and partially characterized are presumed to be anthracene *C*-glycosides. Included in this group are antibiotic DC-14 [341], indomycins A, B and C [342], griseorubins A–H [343,344], the iyomycins [345] and rubiflavin [346].

OTHER *C*-GLYCOSIDE ANTIBIOTICS

Medermycin

Medermycin (9-hydroxy-1-methyl-8-[3-hydroxy-2-methyl-4-(*N,N*-dimethylamino)-2*H*-tetrahydropyran-6-yl-1*H*,3*H*,4*H*-5,10-dihydro-2-oxaanthracene-5,10-dioxo-3-acetic acid 12,4-lactone (78)) was isolated from culture broths of a *Streptomyces* species (K73) and identified by chemical and spectroscopic studies [347].



(78)

The papulacandins

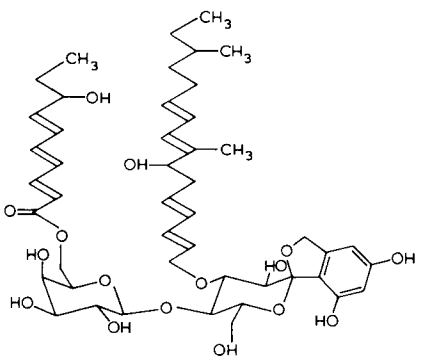
Papulacandins A, B, C and D ((79)–(82)) are a family of *C*-glycoside antibiotics produced by a deuteromycete, *Papularia sphaerosperma* (Pers.), Hoehnel [348]. The structure of the principal component of the antibiotic complex, papulacan-

din B (80) was elucidated by degradation and spectrometric studies [349]. Extension of these studies has yielded structures for papulacandins A, C and D, (79), (81) and (82) [350].

Studies which led to the isolation and characterization of the papulacandins were stimulated by the observation that these antibiotics strongly inhibit the growth of *Candida albicans* [348]. The papulacandins are inhibitory to a number of yeasts. They are largely inactive against filamentous fungi, are slightly active against Gram-positive bacteria, but are otherwise ineffective against bacteria and exhibit no antiprotozoal activity [348].

Papulacandin B (80) caused a 50% inhibition of glucan synthesis in spheroplasts of *Saccharomyces cerevisiae* and *Candida albicans* when present at concentrations of 0.16 μg/ml and 0.03 μg/ml, respectively [351]. It was concluded [351] that papulacandin B inhibits glucan synthesis during cell-wall construction, causing lysis of cells by osmotic rupture. In the fungus *Geotrichum lactis*, papulacandin B (80) inhibits the enzyme 1,3-β-D-glucansynthase but does not counteract the stimulatory effect of guanosine triphosphate on the enzyme [352].

A recent study [353] has provided some insight into the structural requirements for inhibition of glucan synthesis in *S. cerevisiae* and *C. albicans*. The long fatty acid residue is an absolute requirement for activity; neither the shorter fatty acid residue nor the galactosyl moiety is essential for biological activity. Hydrogenation of fatty acid double bonds leads to loss of activity.



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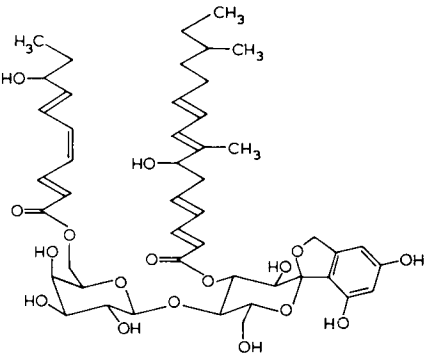

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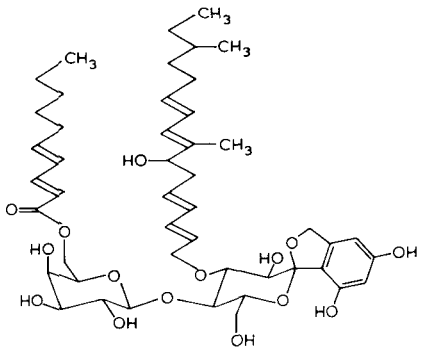

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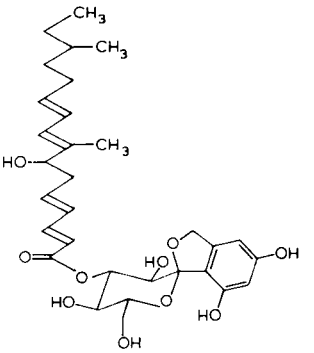


(82)

MODERN METHODS FOR SYNTHESIS OF *C*-NUCLEOSIDES AND *C*-ARYLGLYCOSIDES

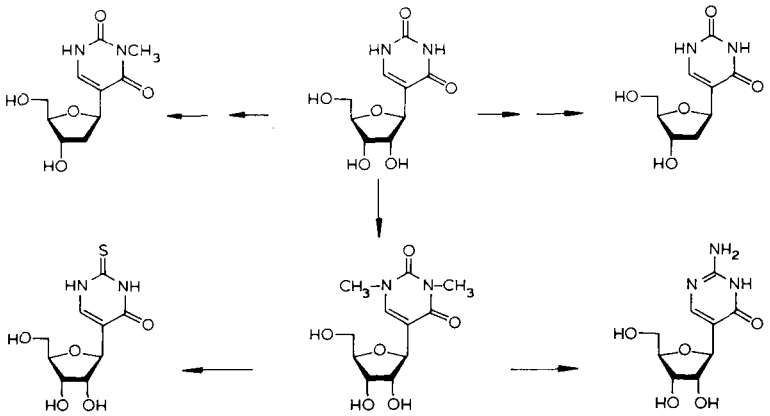
Several recent reviews of methods for *C*-nucleoside synthesis are available [1,3,6–8,10,354,355]. The present review is selective. We have attempted to provide an overview of available synthetic methods with minimum discussion of previously reviewed work. Emphasis has been placed on newer synthetic strategies which are, in our opinion, conceptually innovative and/or have potential for broad utility. Because synthetic methods leading to *C*-arylglycosides are much less well developed than methods for *C*-nucleoside synthesis, we have discussed somewhat more fully recent work in this area.

SYNTHESIS OF *C*-NUCLEOSIDES

Modification of naturally occurring C-nucleosides

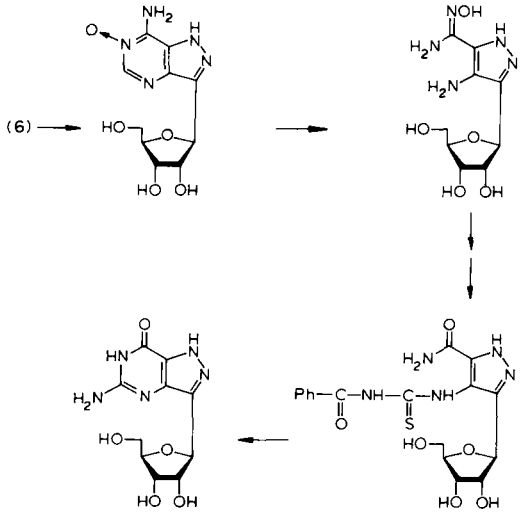
Modification of readily available natural *C*-nucleosides is an attractive route to new *C*-nucleoside analogues and derivatives, since one starting material often possesses much of the desired functionality and chiral properties. This approach is illustrated with two examples.

The Sloan-Kettering group has used the commercially available pseudouridine (1) as starting material for the preparation of several other *C*-nucleosides [27,30,31,40,42,44]. The procedures indicated in *Scheme 1.1* have been optimized and are quite efficient.



Scheme 1.1.

In a similar way, formycin (6) has been modified to produce other purine nucleoside analogues. For example, (*Scheme 1.2*), Lewis and Townsend [153] reported the conversion of (6) to the corresponding guanosine analogue 5-aminoformycin B (21). Among other accomplishements using the natural *C*-nucleoside modification strategy is the conversion of oxazinomycin (3) into related analogues and derivatives [74].



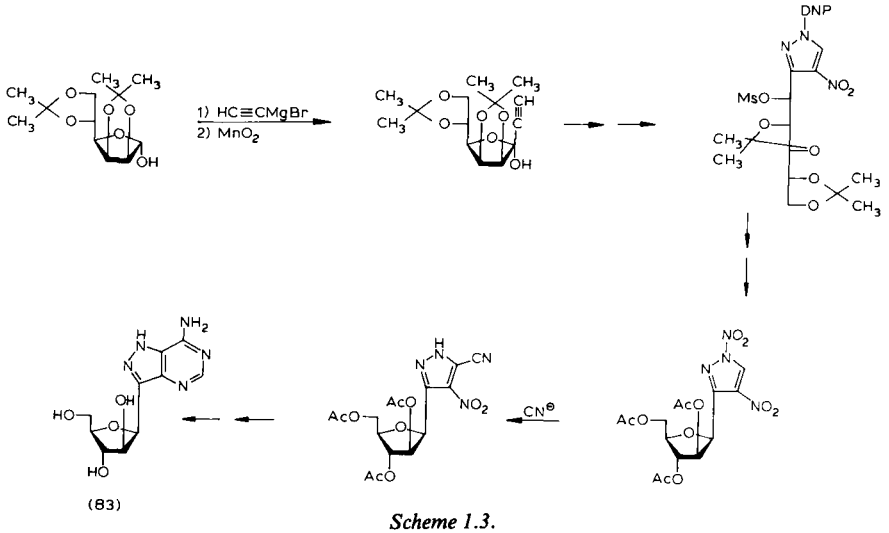
(21)

Scheme 1.2.

Construction of an aglycone from a C-1-functionalized carbohydrate

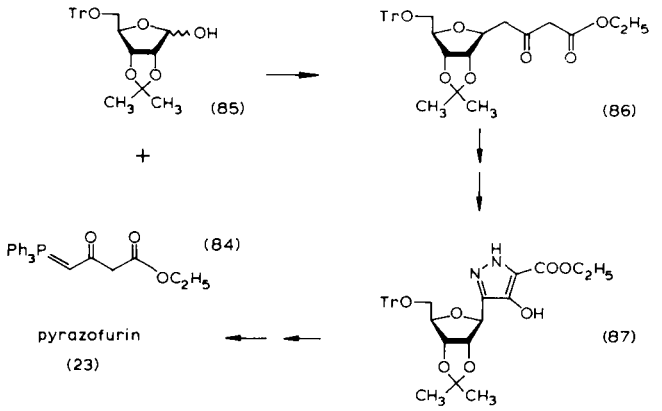
The most frequently used strategy for *C*-nucleoside synthesis involves construction of a heterocyclic aglycone from the C-1 substitutent of a functionalized sugar intermediate. We have chosen six examples to illustrate the scope and current trends in this important area.

Carbohydrates bearing ethynyl functionality at C-1 are versatile synthetic intermediates. Buchanan and co-workers have used 1-ethynyl sugars for synthesis of formycin (6) [136,137], the unnatural 3-β-D-arabinofuranosyl analogue (83) of formycin [160] (*Scheme 1.3*) and several other *C*-nucleosides.



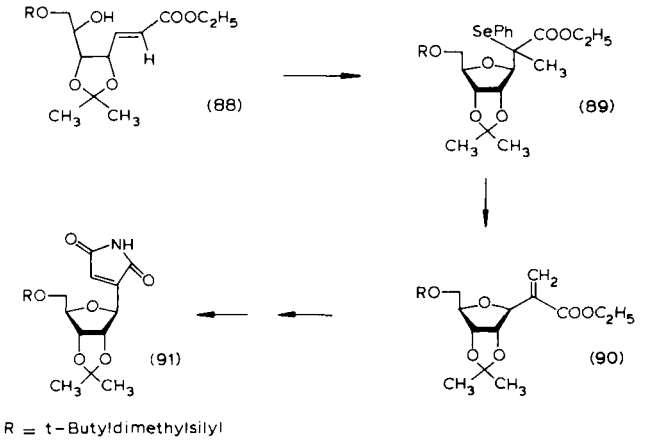
Scheme 1.3.

James [10] has provided an excellent account of the use of reactions of phosphorus ylids with protected ribose derivatives for *C*-nucleoside synthesis. Recently, this strategy was used in a new synthesis of pyrazofurin (23) (*Scheme 1.4*) [200,201,356]. Wittig reaction of phosphorane (84) with the protected D-ribose aldol (85) yielded the C-1-functionalized ribose derivative (86) and the corresponding α-anomer in a 1 : 1 ratio. Treatment of the mixture with *p*-toluenesulphonyl azide gave the corresponding azo derivatives which were cyclized using sodium hydride in 1,2-dimethoxyethane to afford a mixture of pyrazole derivative (87) and the α-anomer. After isolation, (87) was treated with ammonia and deprotected to afford pyrazofurin (23) in 30% overall yield from (85). This reaction sequence compares favourably with other syntheses of pyrazofurin (23) [196–199].



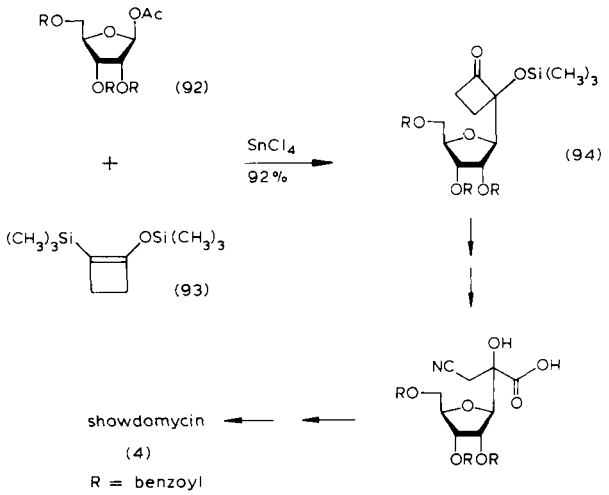
Scheme 1.4.

Kane and Mann [91] devised a new synthesis of showdomycin (4) using a combination of Wittig and selenium chemistry (*Scheme 1.5*). The Wittig product (88) was treated with phenylselenyl chloride to give the isomer (89) stereospecifically. Elimination of phenylselenic acid following hydrogen peroxide oxidation afforded α-methylene ester (90) in high yield. To complete the formal total synthesis of showdomycin (4), compound (90) was ozonized, the resulting α-ketoester was reacted with carbamoyl methylenetriphenylphosphorane and the resulting product was cyclized using mild acid to produce the protected showdomycin derivative (91).



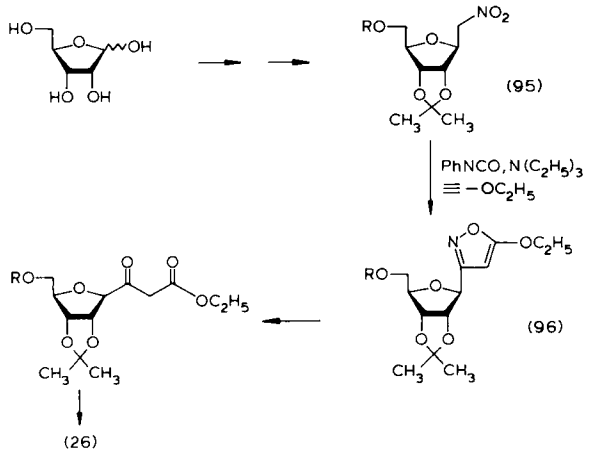
Scheme 1.5.

The most efficient synthesis of showdomycin (4) reported to date was accomplished by Inoue and Kuwajima [94]. These workers synthesized showdomycin (4) in 70% overall yield from β-D-ribose derivative (92) using as the key step a stereoselective addition of 1,2-bis(trimethylsiloxy)cyclobut-1-ene (93) (*Scheme 1.6*). The stereochemistry of this reaction is most likely controlled by participation of the neighbouring C-2′-benzoyloxy group. Adduct (94) contains the four carbons of the maleimide unit of (4) and was easily converted to the target compound.

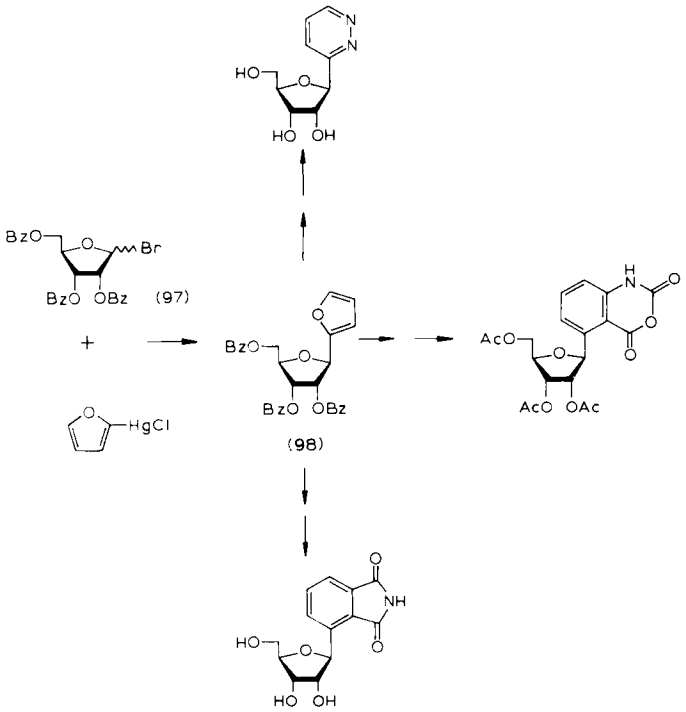


Scheme 1.6.

Cycloaddition of ethoxyacetylene with a nitrile oxide prepared *in situ* gives access to isoxazole *C*-nucleosides which serve as useful synthetic intermediates [212] (*Scheme 1.7*). Thus, D-ribofuranosylnitromethane derivative (95) was converted to (96) without apparent epimerization. Ring-opening of (96) followed by addition of hydrazine gave pyrazole derivative (26) in 70% yield from (95) [212].



Scheme 1.7.

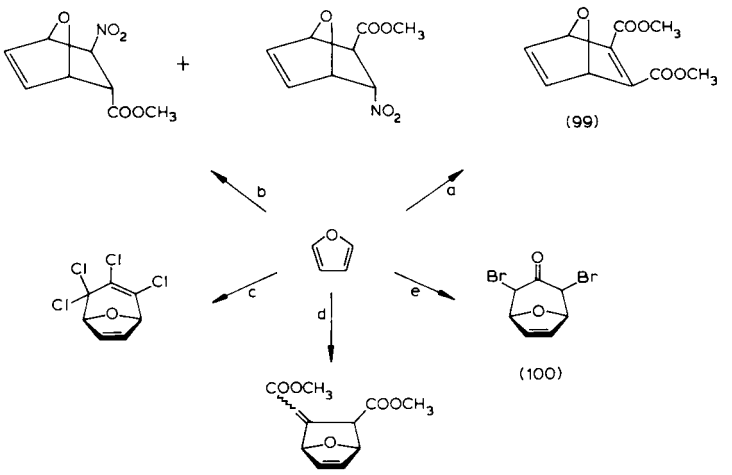


Scheme 1.8.

Recently, the interesting furanosyl *C*-nucleoside, (98), was prepared by reaction of 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl bromide (97) with 2-(chloromercuri)furan [357] (*Scheme 1.8*). The reaction gave (98) and the α-anomer in a 1;6 ratio. However, equilibration of this mixture using trifluoroacetic acid gave mainly the β-isomer (the β:α ratio was approximately 9:1). Compound (98) serves as a useful intermediate for synthesis of a variety of *C*-nucleosides and *C*-arylglycosides [357,358].

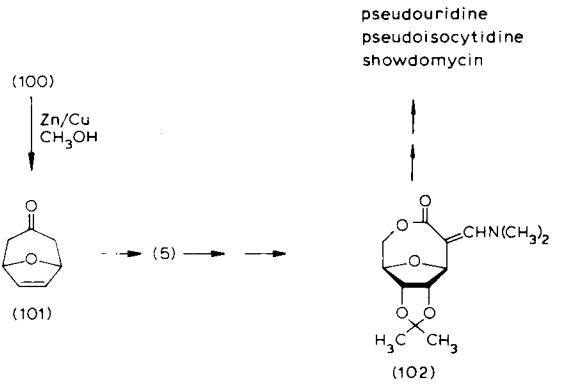
Construction of a sugar moiety from furan

Cycloaddition as a strategy in *C*-nucleoside synthesis has been reviewed previously [10,355] and will therefore be discussed only briefly here. Construction of a *C*-nucleoside furanosyl moiety by cycloaddition of a suitable dienophile to furan was first accomplished by Just and Martel [359].



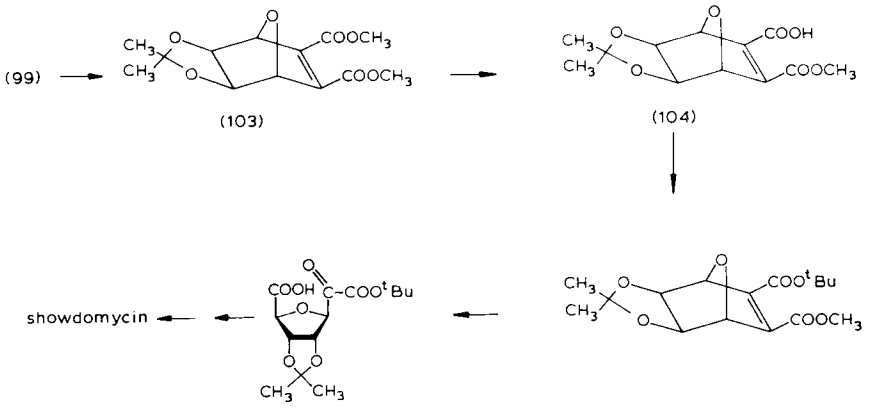
Scheme 1.9.

The cycloaddition adducts shown in *Scheme 1.9*, which have all been converted into *C*-nucleosides, were prepared from furan and (a) dimethyl acetylenedicarboxylate [359], (b) methyl β-nitroacrylate [360], (c) tetrachloropropene [361], (d) 1,3-dicarbomethoxy allene using aluminium chloride catalysis [96] and (e) α,α,α′,α′-tetrabromoacetone in an $Fe_2(CO)_9$-promoted reaction [23]. Debromination of compound (100) gave bicyclic ketone (101) which was further converted to racemic lactone (5) [23] (*Scheme 1.10*). Optical resolution of (5) was accomplished by fractional crystallization of the cinchonidine salts



Scheme 1.10.

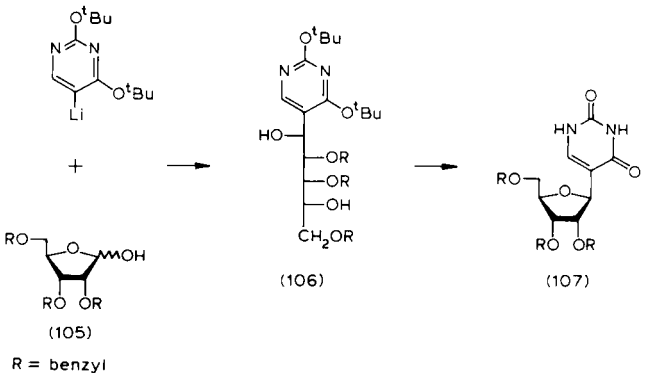
of the hydrolysed enantiomers, followed by reclosure of the lactone ring. Reaction of (+)-(5) with *N,N*-dimethylformamide dimethylacetal produced (102), which served as a common intermediate for synthesis of several natural *C*-nucleosides and related analogues and derivatives [22]. A disadvantage of the furan cycloaddition route to *C*-nucleosides is the requirement for optical resolution. However, the use of enzymatic, enantioselective ester hydrolysis has partially overcome this obstacle [92,93]. Using pig liver esterase, diester (103) was converted to half-ester (104) of high, but not complete, optical purity which was subsequently converted into optically pure showdomycin (4) [92,93] (*Scheme 1.11*).



Scheme 1.11.

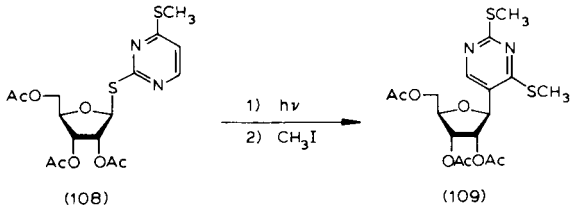
C–Nucleosides from preformed heterocycles

Addition of lithiated heterocycles to protected carbohydrate carbonyl derivatives has been reviewed [1,10]. One recent example of this approach to *C*-nucleoside synthesis involves addition of the protected 5-lithiopyrimidine (104) to 2,3,5-tri-*O*-benzyl-D-ribofuranose (105), which yielded a mixture of (106) and the D-*allo*-epimer (*Scheme 1.12*). This mixture of polyols was cyclized stereospecifically using ethanolic hydrogen chloride to afford (107) and its α-anomer. Subsequent deprotection and isomer separation gave pseudouridine (1) in moderate yield [21].



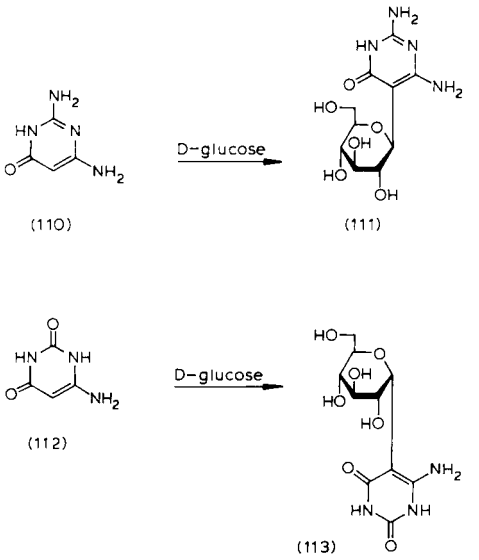
Scheme 1.12.

Certain *S*-nucleosides undergo a photorearrangement to produce *C*-nucleosides [362]. This reaction has been used in a synthesis of pseudouridine (1) [22]. In the key step of this reaction sequence (*Scheme 1.13*), *S*-nucleoside (108) was rearranged to (109) in 15% yield. *C*-Nucleoside (109) was then converted in three steps to pseudouridine (1) [22].



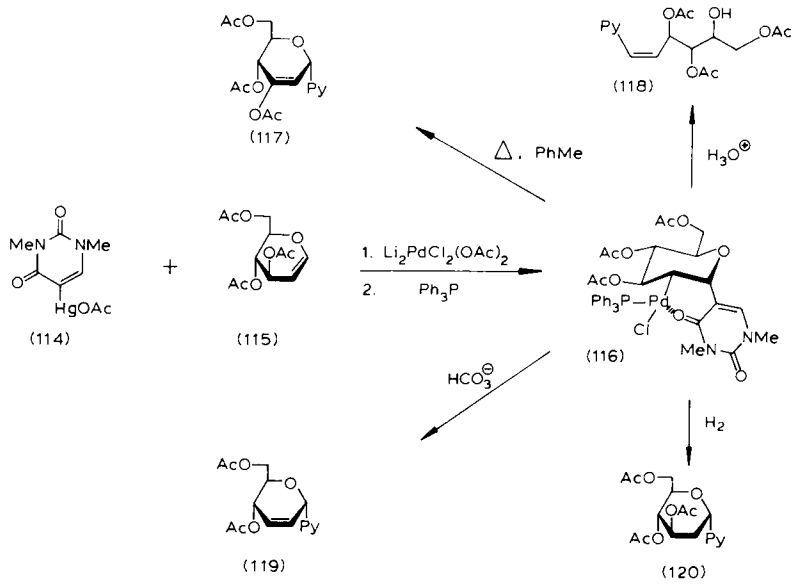
Scheme 1.13.

Some interesting results obtained in acid-catalyzed condensations of 6-aminopyrimidine derivatives with unprotected sugars have appeared [353]. For example (*Scheme 1.14*), diaminopyrimidine (110) was reported to yield β-*C*-nucleoside (111) in 54% yield upon reaction with D-glucose in aqueous ammonium chloride or acetic acid solutions. Somewhat surprisingly, when 6-aminouracil (112) was used instead of (110), the *C*-nucleoside product (113), produced in 31% yield, had the α-configuration [363].



Scheme 1.14.

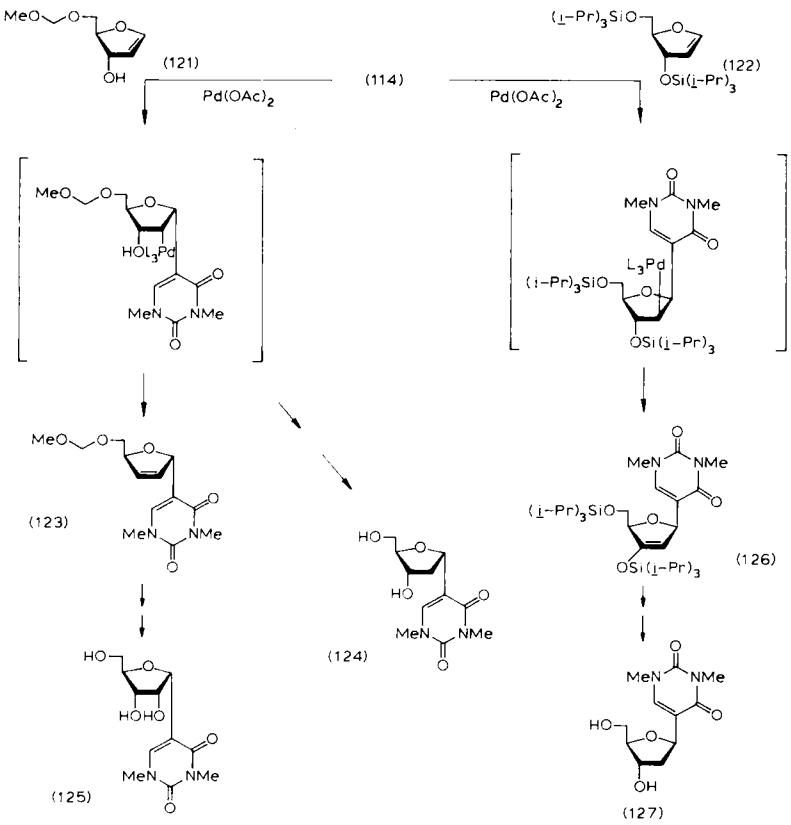
Palladium-mediated reaction of aryl or heterocyclic mercurials with glycals (1,2-unsaturated carbohydrates) has been developed into an effective route for the synthesis of *C*-nucleosides [364–371]. In this *C*-nucleoside synthesis, an aglycone-palladium reagent (aryl or heterocyclic), formed by transmetallation from the corresponding organomercurial, undergoes regiospecific *syn* addition to the *enol* ether double bond of a glycal to form the *C*-glycosidic carbon-carbon bond. Control of the sterochemistry of the key carbon-carbon bond-forming organopalladium addition reaction is effected by manipulation of the relative steric bulks of substituents on the two respective faces of the unsaturated carbohydrate ring [364]. Thus, regio- and stereo-controlled formation of the *C*-glycosidic linkage and control of the decomposition (depalladation) of the intermediate organopalladium adduct [366,371] make available an array of furanosyl and pyranosyl *C*-nucleosides and *C*-arylglycosides.



Py = 1,3-Dimethyl-2,4(1H,3H)-pyrimidinedion-5-yl

Scheme 1.15.

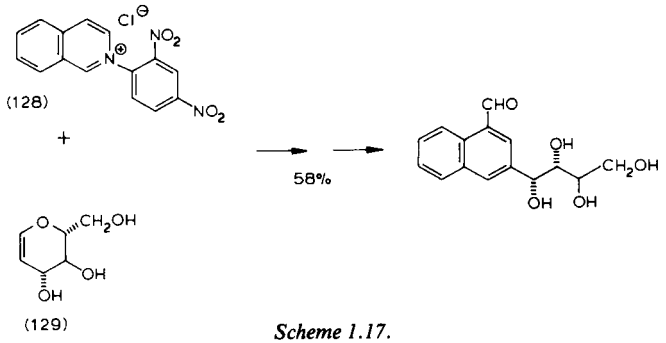
In *Schemes 1.15* and *1.16* several examples of this synthetic strategy are illustrated. Pd-mediated reaction of 1,3-dimethyl-2,4(1*H*,3*H*)-dioxopyrimidin-5-ylmercuric acetate (114) with 3,4,6-tri-*O*-acetyl-D-glucal (115) followed by by triphenylphosphine addition permitted isolation of a single adduct (116) [366]. Controlled decomposition of adduct (116) was accomplished (*Scheme 1.15*), allowing selective preparation of any one of the *C*-nucleosides (117)–(120) in quantitative yield [366]. Similar sequences (*Scheme 1.16*) led to the preparation of furanosyl *C*-glycosides [364]. Palladium-mediated reaction of (114) with furanoid glycal (121) involved adduct formation at the α, least sterically hindered, face of the glycal and led to the α-*C*-nucleosides (124) and (125). In contrast, when glycal (122) was used, the resultant *C*-nucleoside (127) possessed the β-configuration. Significantly, in none of these reactions is a mixture of regio- or stereoisomers formed [364–367].



Scheme 1.16.

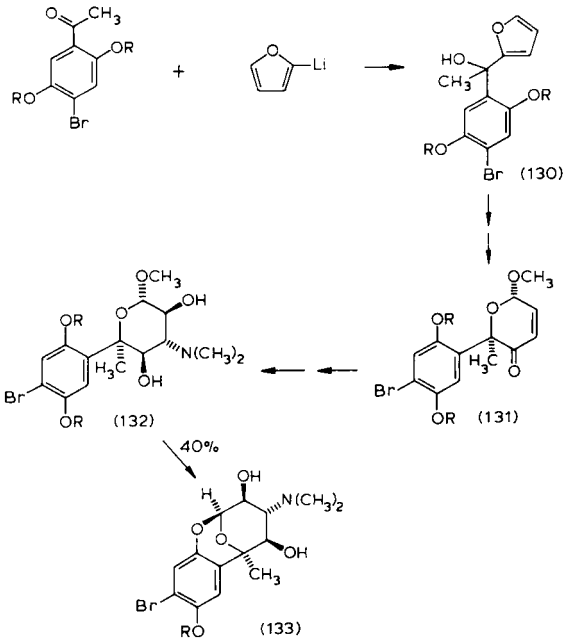
SYNTHESIS OF *C*-ARYLGLYCOSIDES

Glycals, such as (129), form cycloadducts with isoquinolinium salt (128) [372]. Ring opening of the adduct followed by aromatization of the aminoaldehyde thus formed affords *C*-naphthylglycosides (*Scheme 1.17*) [372]. As noted in the previous section, glycals serve effectively as intermediates in palladium-mediated *C*-glycosidic coupling reactions [364–371].



Scheme 1.17.

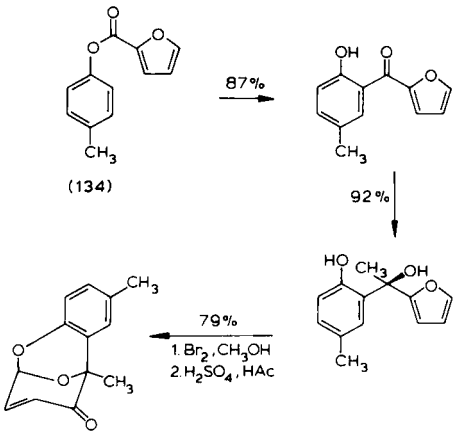
Several syntheses of complex *C*-arylglycosides have been reported recently. Bates and Sammes [330] prepared the racemic *C*-glycosidic fragment of nogalamycin (52) (*Scheme 1.18*). The organolithium addition product (130) was oxidized (*m*-chloroperbenzoic acid) and converted to methylglycosides, and so (131) was obtained by diastereoisomer separation. Functionalization of



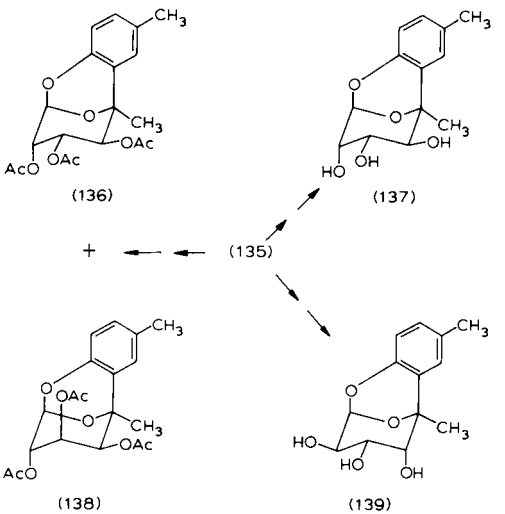
Scheme 1.18.

(131) was achieved in three steps in moderate yield to produce (132) which, upon treatment with trimethylsilyl iodide and trimethylsilyl chloride, gave racemic (133) (*Scheme 1.18*).

A versatile method for synthesis of various sugar analogues of 2,6-epoxy-[1(2*H*)]benzoxocine has been developed [331]. 2,6-Epoxyketobenzoxocine (135) was prepared from *p*-cresol ester (134) in 63% overall yield (*Scheme 1.19*). Compound (135) was then converted to racemic compounds

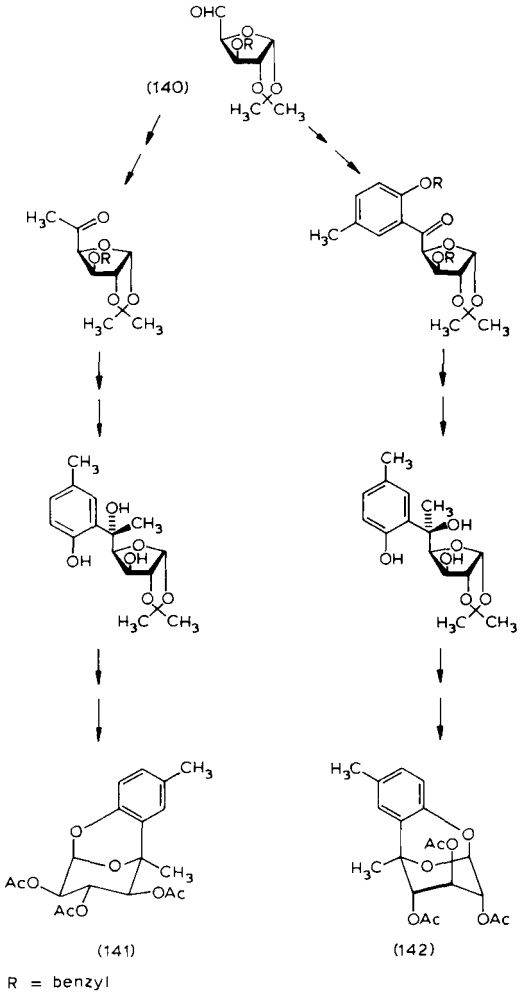


Scheme 1.19.



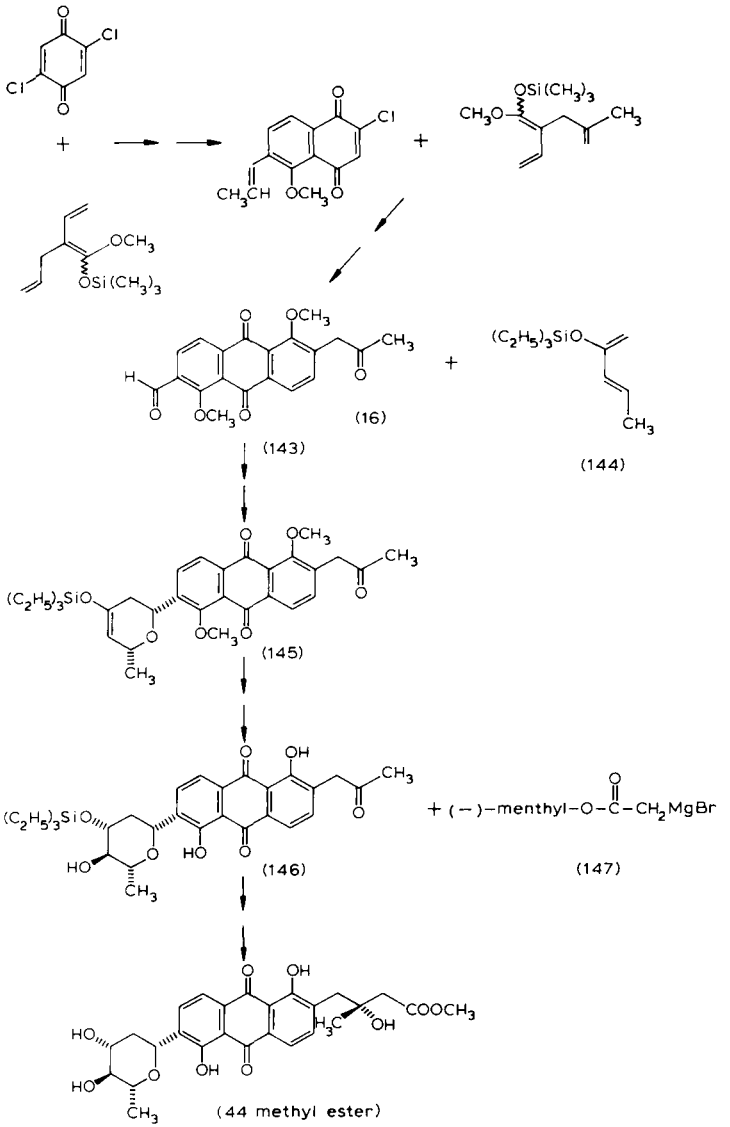
Scheme 1.20.

(136)–(139) of the *talo*, *altro*, *manno* and *galacto* configurations, respectively (*Scheme 1.20*). Moreover, the optically active D-*gluco*- and L-*ido*-2,6-epoxy-2*H*--1-benzoxocines (141) and (142) have been prepared from furanoside (140) by the route shown in *Scheme 1.21* [332]. Thus, the development of effective methods for the construction of rings E and F of nogalamycin (52) and the impressive progress made in anthracycline synthesis (see, for example, Ref. 373 and references cited therein) presage a total synthesis of this complex *C*-arylglycoside.



Scheme 1.21.

Most impressive is the total synthesis of the optically pure vineomycin B_2 aglycone methyl ester ((44) methyl ester) in twelve synthetic steps using racemic starting material [275] (*Scheme 1.22*). The key elements of this elegant



Scheme 1.22.

synthesis are two all-carbon and one hetero Diels-Alder reactions using siloxy dienes [374]. The first two cycloadditions were accomplished in good yield; unfortunately, the preparation of (143) was hampered by a very low yield in the ozonolysis-reduction sequence following the second cycloaddition. Compound (143) was then allowed to react with diene (144) in a lanthanide-catalyzed hetero Diels-Alder cycloaddition to afford (145) in 92% yield. Several other examples of this facile reaction in *C*-arylglycoside synthesis have been reported [375,376]. Hydroboration of the silylenol ether function of (145) produced two more chiral centres of the desired stereochemistry. The fifth chiral centre of ((44) methyl ester) proved to be more difficult to introduce. Reaction of bromomagnesium salt (147) with (146) gave a mixture of four products from which the desired diastereoisomer was separated by high-pressure liquid chromatography. Finally, transesterification yielded the desired product (*Scheme 1.22*).

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2 Heterocyclic Analogues of GABA: Chemistry, Molecular Pharmacology and Therapeutic Aspects

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INTRODUCTION

The neutral amino acid 4-aminobutanoic acid (GABA) is present in every region of the mammalian central nervous system (CNS). Although many aspects of the functions of GABA are still incompletely understood, it is now generally accepted that GABA has a neurotransmitter function, and it fulfils the main criteria established for the identification of an inhibitory neurotransmitter [1–5]. Whilst GABA is considered to be the major inhibitory transmitter in the mammalian brain, glycine probably plays a similar rôle in the spinal cord [1,6].

Figure 2.1 is a schematic illustration of an axo-somatic GABA-operated synapse. The synthesis of GABA from (*S*)-glutamic acid (GLU) is catalyzed by (*S*)-glutamate 1-carboxy-lyase (glutamate decarboxylase, GAD), and GABA is transformed into succinic semialdehyde (SSA) by GABA : 2-oxoglutarate aminotransferase (GABA-T). This initial step of the metabolism of GABA occurs in the presynaptic terminals as well as surrounding glia cells. Activation of the postsynaptic GABA receptor complex (for further details see later section and *Figure 2.2*) results in an opening of the chloride ionophore with subsequent influx of chloride ions and hyperpolarization of the cell membrane, which makes the postsynaptic cell less sensitive to stimulation by excitatory neurotransmitters. The synaptic transmission is terminated by transport of GABA from the synaptic cleft into the nerve terminals and glia cells by high-affinity uptake systems [7, 8]. Extracellular enzymatic degradation of GABA does not play any significant rôle in the termination of the GABA-mediated neurotransmission.

In addition to the direct electrophysiological evidence for a rôle of GABA as a major central inhibitory neurotransmitter [1,2,6], an overwhelming amount of direct evidence has been derived from a variety of experimental models. It has been demonstrated that blockade of the GABA neurotransmission in animals at different levels results in convulsions [9,10]. Thus, inhibition of

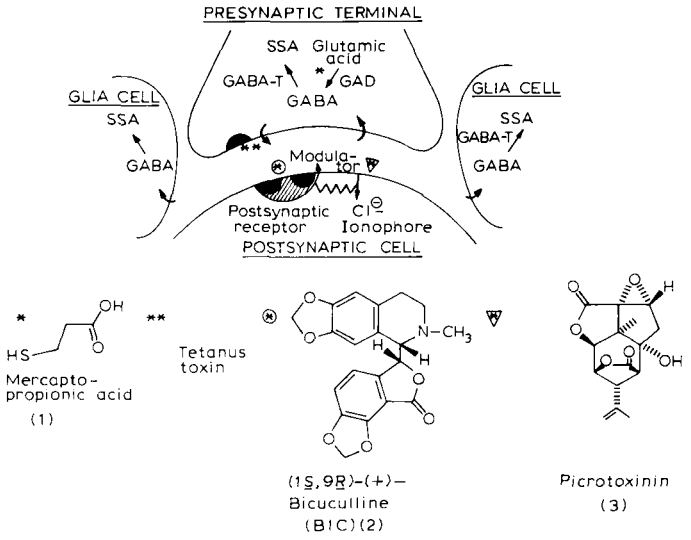


Figure 2.1. The processes and functions at a GABA-operated oxo-somatic synapse. The sites of action of different compounds are indicated.

GAD by 3-mercaptopropanoic acid (1) or blockade of the release mechanism by tetanus toxin provokes severe convulsions. It must, however, be emphasized that tetanus toxin blocks the release of glycine as well as that of GABA [1]. Similarly, blockade of different mechanisms of the postsynaptic GABA receptor complex provokes convulsions, the ionophore antagonist picrotoxinin (3) and the receptor antagonist (1*S*, 9*R*)-bicuculline (BIC, 2) being powerful convulsants [10–12].

GABA is involved in the control of many physiological mechanisms, including the regulation of the secretion of prolactin [13,14] and of other hormones, including the growth hormone [15]. Furthermore, GABA plays a rôle in the regulation of cardiovascular functions, such as blood pressure and heart rate [16–20], and GABA is involved in the sensation of pain [21–25] and anxiety [26–28]. There are strong indications that activation of the GABA systems modifies feeding [29,30] and aggressive behaviour [31] in animals.

GABA DYSFUNCTIONS AND NEUROLOGICAL AND PSYCHIATRIC DISEASES

The growing interest in the pharmacology of GABA has been stimulated by the findings that GABA apparently is involved in the development of certain neurological and psychiatric diseases such as epilepsy, Huntington's chorea, Parkinson's disease, and, perhaps, schizophrenia and dementia of the Alzheimer type. Thus, analyses of brain samples from sites near seizure foci in epileptics and in animals made epileptic have revealed severe impairments of the GABA system [32]. Low levels of GAD and reduced GABA uptake capacity indicate that decreased activity and probably partial degeneration of GABA neurones are factors of importance in epileptic phenomena [33,34]. Low levels of GABA and GAD have been measured in postmortem brain tissue from choreic patients [35,36]. In Parkinson's disease, there is an imbalance between the GABA and dopamine (DA) systems, and in some brain areas of parkinsonian patients, especially the substantia nigra, GAD activity and GABA receptor density are below normal levels [37]. Decreased GABA activities in certain regions of brains from patients dying of schizophrenia suggest that GABA is involved in the pathophysiology of schizophrenia [38–40]. The precise rôle of GABA in schizophrenia, and perhaps in Alzheimer's disease [41], is, however, far from clear. There is some evidence for a GABA-ergic contribution to the symptoms in depressed patients [40] and to the action of antidepressant drugs [42], and remarkably low plasma GABA levels have been measured in alcoholics [40].

STRATEGIES FOR THE DEVELOPMENT OF GABA-STIMULATING THERAPIES

In the development of strategies for pharmacological intervention in the GABA neurotransmission process, the degree of degeneration of the GABA neurones has to be taken into consideration [43]. In stages of the diseases at which GABA neurones are still functioning, but at an abnormally low level, presynaptic mechanisms, including the uptake systems and GABA-T as well as the postsynaptic receptors, are potential pharmacological sites of attack (*Figure 2.1*). In the case of extensive neuronal degeneration, only the postsynaptic GABA receptors have survived, and, deprived of their neurotransmitter, such receptors apparently develop hypersensitivity to GABA agonists [44]. In the advanced stages of epilepsy or Huntington's chorea, where GABA neurones in some brain areas are extensively degenerated, GABA agonist therapies may be particularly pertinent. It must, however, be emphasized that hypersensitive GABA receptors may not be functioning optimally, and, furthermore, it is normally difficult to estimate the degree of degeneration of GABA neurones in patients suffering from the diseases concerned. In any case, the most direct, though probably not the most flexible, approach [45,46] to stimulation of the GABA-mediated neurotransmission appears to be activation of the postsynaptic GABA receptors. Consequently, design and development of specific GABA agonists suitable for pharmacological and clinical studies has been, and continues to be, an active research field [43, 47–53], and GABA agonists have been studied in a variety of animal models [54,55] and in different groups of patients [56,57] (see subsequent sections).

BIOLOGICAL TEST SYSTEMS FOR MEASURING GABA-ERGIC ACTIVITIES

IN VITRO TEST SYSTEMS

GABA receptor binding studies were performed using a modified version [58,59] of a published procedure [60] for the preparation of cerebral cortex membranes from adult rats. The membrane preparation was rapidly frozen at $-70°C$ and kept at $-20°C$ for at least 18 h before use in the receptor binding assay. For the [^{3}H]GABA binding assay procedure, aliquots of synaptic membranes (0.8–1.2 mg of protein) were incubated in triplicate at 4°C for 5 min in 2 ml of 0.05 M Tris-citrate buffer (pH 7.1) containing 5 nM of [^{3}H]-GABA. IC_{50} values were determined using a conventional procedure [58].

The effects of GABA analogues on the neuronal GABA uptake system were determined *in vitro* under different experimental conditions using synaptosomes or mini-slices. A suspension of crude synaptosomes isolated from rat brain was prepared [61], and 0.2 ml of this suspension was preincubated for 10 min at 25°C with phosphate medium containing the inhibitor. Then [^{3}H]GABA was added to give a final GABA concentration of 5×10^{-8} M, and the incubation was continued for a further 10 min. Mini-slices of mouse cerebral cortex were prepared as described, and brain slices corresponding to approximately 0.2 mg of protein were incubated for 5 min at 37°C in the appropriate medium containing [^{3}H]GABA at a concentration of 10^{-6} M and the desired concentration of inhibitor [8,62,63]. These mini-slices were not preincubated in the presence of inhibitor prior to the addition of [^{3}H]GABA. IC_{50} values were calculated as described earlier [62,63].

The effects of GABA analogues on the glial GABA uptake system were determined using cultured astrocytes [64]. Cells corresponding to 0.03–0.05 mg of protein were incubated for 5 min at 37°C in the appropriate medium containing [^{3}H]GABA (1×10^{-6} M) and inhibitor at the desired concentration without preincubation of the cells in the presence of inhibitors, and IC_{50} values were calculated as described earlier [62,63].

In light of the different experimental conditions, the IC_{50} values for inhibition of GABA uptake into synaptosomes are not directly comparable with those measured using mini-slices or cultured astrocytes.

SINGLE-CELL PHARMACOLOGICAL METHODS

The effects of GABA analogues on single dorsal horn interneurones and Renshaw cells were determined using microelectrophoretic techniques on cats anaesthetized with pentobarbitone [65]. Extracellular action potentials were recorded by means of the centre barrel of seven-barrel micropipettes. The compounds tested were administered electrophoretically from the outer barrels of the micropipettes, which contained solutions of the GABA analogues (0.1–0.2 M, adjusted to pH 3–4) or the GABA antagonists BIC (2) hydrochloride or bicuculline methochloride (BMC) (0.01 M in 0.17 M NaCl). Firing of the cat spinal neurones was induced by electrophoretically administered (*RS*)-homocysteic acid (0.2 M, adjusted to pH 7.5). The submaximal depressant effects are expressed relative to that of GABA (– – –) (*Figure 2.5* and *Tables 2.1* (p. 95), *2.2* (p. 100), and *2.3* (p. 106)). The number of symbols indicates greater, equal or lesser activity on an approximately linear scale. Effects of doubtful significance are cited in brackets. The sensitivity of the depressing effects to BIC or BMC or to strychnine, an antagonist of glycine

[1,66], was examined by simultaneous ejection of the GABA analogues and these antagonists.

PHARMACOLOGICAL METHODS

GABA analogues, and in particular GABA agonists, have been studied in a variety of animal models [5,55,67,68], but, so far, no animal model has been shown to give responses specific for GABA-stimulating agents. Local injections of compounds into substantia nigra of rats have been extensively used as an *in vivo* model for studies of GABA agonists, which have been shown to induce contralateral and ipsilateral turning behaviour, respectively, of the animals [69–71]. The effects of GABA analogues on isoniazide-induced convulsions (*Table 2.2*) were determined as described elsewhere [55]. The analgesic effects of GABA analogues have been measured under a variety of different conditions [23–25,72]. The analgesic effects listed in *Table 2.2* (p. 100) were determined according to a published method [72].

GABA ANALOGUES AND THE BLOOD-BRAIN BARRIER

All compounds so far known with specific actions on the GABA synaptic recognition sites have zwitterionic structures, and the relatively early stage of the pharmacology of BMC-sensitive GABA agonists reflects the difficulties in developing such compounds with satisfactory pharmacokinetic properties. Small, and frequently negligible, fractions of neutral amino acids exist as un-ionized molecules in solution, the ratio between the concentrations of ionized and un-ionized molecules (I/U ratio) being a function of the difference between the pK_aI and pK_aII values [49,54,73–76]. A great difference between the two pK_a values is tantamount to high I/U ratios for the compounds. Since the amino acids are likely to penetrate the blood-brain barrier (BBB) in the un-ionized forms, it is of pharmacological interest to develop analogues of GABA with small differences in the pK_a values, and, thus, low I/U ratios as compared with GABA (pK_a 4.0, 10.7; I/U 800,000). The I/U ratios have been calculated for a number of GABA agonists, including piperidine-4-sulphonic acid (P4S, 47) ($pK_a < 1$, 10.3; $I/U > 1{,}000{,}000$), isoguvacine (29) (pK_a 3.6, 9.8; I/U 200,000), 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP, 14) (pK_a 4.4, 8.5; I/U 1,500 or 500, depending on the method of calculation), muscimol (5) (pK_a 4.8, 8.4; I/U 900 or 400, depending on the method of calculation) and thiomuscimol (7) (pK_a 6.1, 8.9; I/U 13) [74,75]. From these data, it is understandable that neither GABA, (47) nor (29) is capable of

penetrating the BBB to any significant extent [76]. On the other hand, approximately 0.1% of doses of (5) or THIP exist as un-ionized molecules in aqueous solutions, and these values can readily explain why these GABA agonists enter the brain after peripheral administration in animals and man [76,77]. The low I/U ratio for (7) suggests that this compound is capable of penetrating the BBB very easily. Detailed studies on this property of (7) must await the availability of radioactive (7), the preparation of which is in progress [78]. In any case, (7) is pharmacologically active after systemic administration in animals.

THE POSTSYNAPTIC GABA RECEPTOR COMPLEX

In *Figure 2.2* our present knowledge of the structure of the postsynaptic GABA receptor complex is summarized [11,49,79]. The chloride channel is regulated by the GABA receptor consisting of two [11] or possibly three GABA-binding sites [59]. The physiological relevance of these multiple binding sites, which can be detected *in vitro* under different experimental conditions, is unclear. The

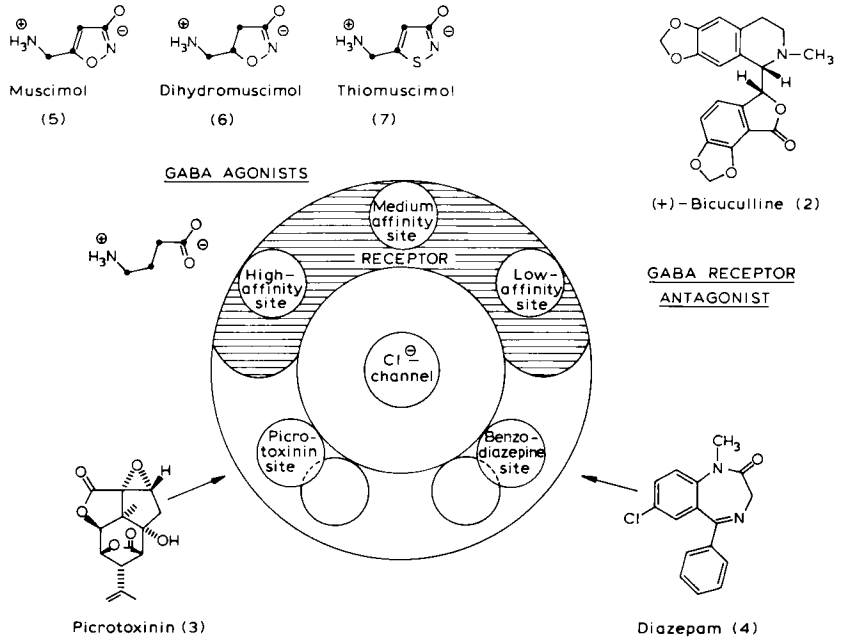
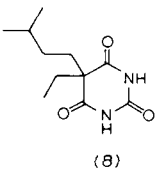


Figure 2.2. A schematic illustration of the postsynaptic GABA receptor complex. The sites of action of some GABA analogues are indicated.

GABA receptor function appears to be modulated by various additional units, which can be detected *in vitro* as distinct binding sites for the benzodiazepines (BZD) [80] and picrotoxinin (3) [81] or the cage convulsant *t*-butyl bicyclophosphothionate (TBPS) [82]. There is some evidence of heterogeneity of both of these binding sites [11] and of the existence of a distinct binding site at the GABA receptor complex for the avermectines, including avermectin B_{1a} (13) [83–85]. The physiological relevance of these additional sites (pharmacological receptors) of the GABA receptor complex is unknown, but the intimate contact and allosteric interactions between these sites, as detected *in vitro*, may reflect certain aspects of the dynamic properties of the GABA receptors.

There is strong evidence that the picrotoxinin-binding sites at the postsynaptic GABA receptor complex represent the pharmacological receptors for the



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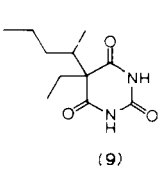

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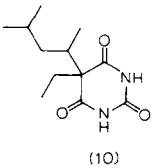

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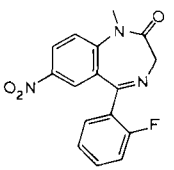

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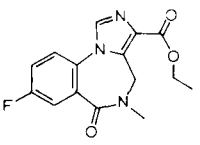

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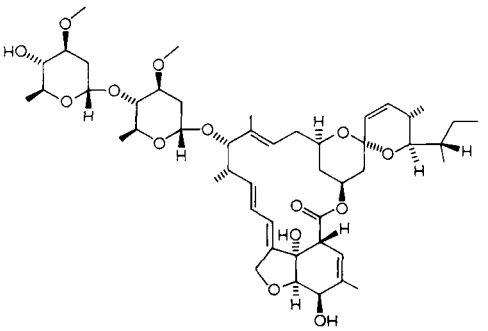


Avermectin B_{1a} (13)

barbiturates [11,81,82,86]. Not only the sedative, hypnotic and anticonvulsant barbiturates, such as amylobarbitone (amobarbital, 8) and pentobarbitone (pentobarbital, 9), interact with the picrotoxinin binding sites, but also the convulsant barbiturates, such as 5-ethyl-5-(1,3-dimethylbutyl)barbituric acid (10), are inhibitors of the binding of radioactive dihydropicrotoxinin [11,12,86] or TBPS [82].

The clinically used anxiolytic, hypnotic and anticonvulsant BZDs, such as diazepam (4) (*Figure 2.2*) and flunitrazepam (11) bind very tightly to the particular site at the GABA receptor complex, and these compounds and the BZD antagonists, such as Ro 15-1788 (12), have been shown to affect the GABA-mediated neurotransmission *in vivo* and under a variety of *in vitro* conditions [12,79,87,88].

The interaction of GABA agonists with the GABA receptor complex *in vitro* has been extensively studied [49,53,89–91]. These studies have disclosed striking differences between the effects on BZD binding of different structural classes of GABA agonists and appreciable effects of temperature, and, in particular, chloride ions on the degree of GABA agonist-induced stimulation of BZD binding [53,92]. Whilst there generally is a positive correlation between the potencies of these GABA analogues *in vivo* and as inhibitors of GABA receptor binding [49,53,93,94], there is no simple correlation between the potencies of GABA agonists and their ability to stimulate BZD binding *in vitro*. For the latter effect of GABA agonists, three structural parameters appear to be of major importance [49,53,89–91]:

(1) the structure of the acid moiety of the agonist;
(2) the degree of substitution at the basic nitrogen atom; and
(3) the conformational mobility of the entire molecule.

The pharmacological importance of these dissimilar effects of GABA agonists on BZD binding *in vitro* is unclear. Whilst GABA agonists with a certain degree of conformational mobility and with primary amino groups, such as (5), (7) and (*RS*)-5-aminomethyl-2-isoxazolin-3-ol (dihydromuscimol, 6) (*Figure 2.2*), are capable of enhancing the binding of BZD to a level similar to that obtainable by GABA, GABA agonists like THIP, (29) (*Figure 2.4*) and, in particular, the aminosulphonic acid (47) are less effective in this *in vitro* system [89–92,95,96]. These effects actually are reminiscent of partial GABA agonist activities, but, so far, electrophysiological studies have not disclosed partial agonist profiles of these very potent GABA agonists. Furthermore, it has recently been demonstrated [97] that the depressant action of GABA, THIP and (47) on cultured neurones is equally enhanced by BZD. Still, these different effects of GABA agonists *in vitro* may reflect dissimilar mechanisms of interaction with the postsynaptic GABA receptors, which, in turn, may

suggest that it is possible to develop specific BMC-sensitive GABA agonists with different behavioural pharmacological profiles.

The binding to postsynaptic GABA receptor sites of the radiolabelled BMC-sensitive GABA agonists (5) [98–100], THIP [59,101], (29) [102–105] and (47) [59,101,106] has been characterized and compared with that of GABA. The receptor binding of radioactive (29) has been studied under slightly different experimental conditions, and these binding data [104,105] are not directly comparable with those obtained for the other GABA agonists. Whilst the binding characteristics for GABA, (5) and (47) are similar, though not identical [59,98–100,106], striking differences were observed between those of THIP and GABA [59] (*Figure 2.3*). The K_D values for GABA and THIP are comparable, whereas considerable differences between the B_M values were measured. Due to the very low affinity of both ligands for the low-affinity receptor sites, these sites eluded satisfactory characterization [59]. Similar differences between the binding characteristics of GABA and THIP have recently been observed by others [107]. Thus, THIP interacts selectively with medium-to-low-affinity GABA receptor sites, and since these sites appear to mediate the GABA-BZD coupling [11,103,108], the low 'efficacy' of THIP in activating BZD binding (cf. above) is difficult to rationalize. On the other hand, since THIP has pronounced pharmacological effects in animals and man (cf. later section), the medium-to-low-affinity sites of the postsynaptic GABA

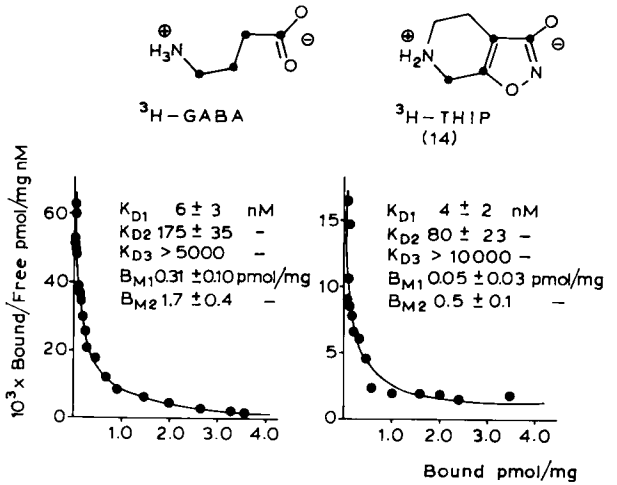


Figure 2.3. Scatchard plots of the specific binding of radioactive GABA and THIP (14) to rat brain synaptic membranes. The binding parameters are derived from computer-fitted non-linear regression analysis of the data [59].

receptors seem to reflect the receptor sites of major pharmacological and, perhaps, physiological relevance.

MULTIPLICITY OF GABA RECEPTORS

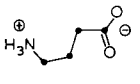
In the present review we have so far mentioned only one type of GABA receptor, namely, the postsynaptic BMC-sensitive GABA receptors (*Figures 2.1* and *2.2*). Accumulating evidence derived from studies of GABA analogues does, however, indicate the existence of different types of GABA receptor, but in the absence of specific agonists and antagonists for most of these proposed subpopulations of receptors, the exact number of physiologically relevant GABA receptors is unknown. Based on electrophysiological and receptor-binding studies, the GABA receptors can apparently be divided into three groups, and each of these groups may actually be heterogeneous [52,54,109,110]:

(1) GABA receptors sensitive to the competitive antagonists BIC (2) or BMC (*Figures 2.1* and *2.2*), at which THIP, (29) and (47) are potent and specific agonists (*GABA-A receptors*).

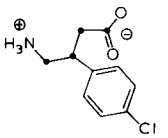
(2) BMC-insensitive GABA receptors, at which baclofen (15), notably the (−)-isomer of (15), is an agonist. So far, specific antagonists for these receptors (*GABA-B receptors*) have not been developed.

(3) BMC-insensitive GABA receptors, at which neither (29) nor (−)-(15) is an agonist, but which seem to bind selectively *cis*-4-aminobut-2-enoic acid. These GABA receptor sites have tentatively been named *GABA-C sites*.

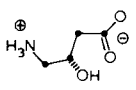
The GABA-A receptors include presynaptic (axo-axonic) and postsynaptic (axo-somatic or axo-dendritic) receptors, which have very similar pharmacological characteristics [6,111]. Extrasynaptic GABA receptors, which are



GABA



Baclofen (15)



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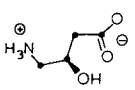

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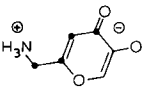

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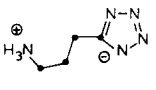


(19)

also sensitive to BMC or (2), apparently have agonist specificities different from those of synaptic BMC-sensitive GABA receptors [6,112,113].

There is some evidence supporting the view that GABA-A receptors can be further subdivided. The agonist selectivities of GABA receptors in the cat cerebral cortex and spinal cord are dissimilar [65,114], and whilst 5,6,7,8-tetrahydro-4*H*-isoxazolo[3,4-*d*]azepin-3-ol (iso-THAZ, 135) has a GABA antagonist profile after injection into the rat substantia nigra [71], (135) does not interact significantly with GABA receptors in the cat spinal cord [115]. These differences between spinal and supraspinal GABA receptors have recently been further elucidated. Compounds (5), (29) and GABA are agonists with decreasing potency at GABA-A receptors in the cat spinal cord (see *Table 2.3*, p. 106) [58,116], but seem to activate GABA receptors that regulate the release of GLU in rat brains, with a different order of potency [117]. At these receptors, which also are sensitive to the antagonist BIC (2), (5) is only a moderately potent agonist, weaker than GABA, whereas (29) and 3-aminopropanesulphonic acid (3APS, 24) are the most potent agonists so far tested.

Relatively little is known about the pharmacology of the GABA-B receptors, which have been detected in the periphery as well as in the CNS [110]. The GABA analogue (15) is a BMC-insensitive depressant of neuronal firing [119,120]. The (−)-isomer of (15) [110,118], the only potent and selective GABA-B agonist so far described, interacts with a population of receptor sites which bind GABA but not BMC, BIC or the GABA-A agonists (14), (29), and (47) [121]. These GABA-B receptors seem to modulate the release of monoamines in the CNS [122], but the relevance of this receptor interaction of (15) to the therapeutic effects of (15) in spastic patients [123] is unclear. This latter compound potently reduces monosynaptic excitation in the spinal cord, an effect probably not mediated by GABA receptors [124].

A number of GABA analogues, such as kojic amine (18) and 4-amino-3-hydroxybutanoic acid, interact with the GABA-B receptor sites, but these compounds are far from being specific GABA-B agonists. Kojic amine also is a moderately potent GABA-A agonist [125,126], and whilst the (*S*)-(+)-isomer (16) of 4-amino-3-hydroxybutanoic acid interacts slightly more effectively with the GABA-A receptors than the (*R*)-(−)-isomer (17) [94], (16) and (17) are equally effective at GABA-B receptor sites [127].

The heterocyclic GABA analogue 5-(3-aminopropyl)tetrazole (19) has recently been shown to interact weakly with GABA-A as well as GABA-B receptor sites *in vitro* [128]. 5-Aminopentanoic acid (DAVA) has a very weak antagonist profile at peripheral GABA-B receptors [129,130], but DAVA does not affect significantly central GABA-B receptors [110], and it interacts more or less effectively with all recognition sites at synapses containing GABA-A

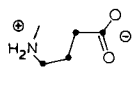
receptors [8,51]. A detailed analysis of the physiological and pharmacological importance of the GABA-B receptors must await the development of specific GABA-B agonists and, in particular, potent and specific antagonists at these receptors.

Other groups of BMC-insensitive GABA receptors may exist in the mammalian CNS, although the evidence is still indirect and very incomplete [52]. *cis*-4-Aminobut-2-enoic acid, *cis*-2-aminomethylcyclopropanecarboxylic acid, 5-(2-aminobutyl)-3-isoxazolol, and 5-(2-aminopropyl)-3-isoxazolol (82) are moderately potent neuronal depressants insensitive to BMC as well as to strychnine [131–133]. The detection of GABA-binding sites, different from GABA-A and GABA-B receptor sites, to which *cis*-4-aminobut-2-enoic acid appears to bind with some selectivity has recently been reported [134]. These binding sites [134,135], which have tentatively been named GABA-C sites, may be the sites of action of some of the BMC-insensitive GABA-like neuronal depressants mentioned above.

ALIPHATIC BIO-ISOSTERES OF GABA

Systematic alterations of the structural parameters characterizing the molecule of GABA have led to a wide variety of GABA analogues. The effects of these analogues on GABA synaptic mechanisms have been studied using different *in vivo* and *in vitro* techniques. These structure-activity studies include *N*-methyl-GABA (20), *N,N*-dimethyl-GABA (21) [136,137], and GABA analogues containing more bulky substituents on the nitrogen atom [138,139]. Furthermore, GABA analogues having methyl groups in the 2-, 3- and/or 4-positions [47,51,137–139], some GABA homologues [136,140,141], and a number of GABA analogues containing a variety of substituents in different positions [47,51,137,141] have been synthesized and tested. These simple and conformationally unrestricted GABA analogues interact more or less effectively with all GABA synaptic mechanisms [47,51]. Some analogues, such as (21) are inactive [136,137], and (20) shows a certain degree of selectivity, having some affinity for GABA-T and GABA uptake but not for the GABA receptors [47,136,137]. The unsaturated GABA analogue *trans*-4-aminobut-2-enoic acid (22), on the other hand, is a potent GABA-A agonist, and an effective substrate for GABA-T and the neuronal GABA uptake system [47,131,142].

A number of carboxylic analogues, in which the GABA backbone has been incorporated into 3- [132], 4- [143], 5- [144–147] and 6-membered rings [148–150], have been synthesized and tested. Structure-activity studies on



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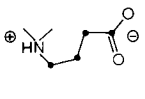

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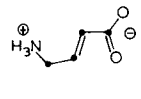

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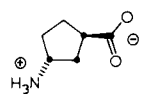

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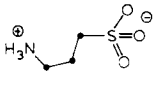

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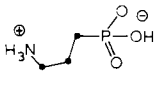

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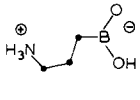

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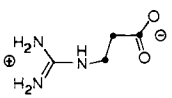
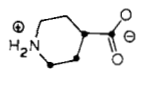


(27)

these analogues, and in particular on the stereoisomers of some of these compounds, such as *trans*-(23) and *cis*-3-aminocyclopentanecarboxylic acid, have shed some light on the structural constraints imposed on GABA-A agonists and GABA uptake inhibitors [47,51,151]. However, since none of these aliphatic amino acids is capable of penetrating the BBB to any significant extent, their applicability as GABA-ergic tools is restricted to *in vitro* experiments and to pharmacological studies on single cells.

The amino group and, in particular, the carboxyl group of GABA have been subjected to bio-isosteric modifications. Whilst the phosphonic (25) and boronic (26) acid analogues of GABA are totally devoid of affinity for GABA synaptic mechanisms [51,52,136,137,141], the sulphonic acid analogue 3APS (24) is a very active GABA-A agonist [53,60,106,136]. It does not show any significant affinity for the GABA uptake systems [89,137], and this lack of effect on GABA uptake is shared by various other aminosulphonic acid analogues of GABA, including (47) [53,89,93]. The selective GABA agonist effect of (24) actually prompted the development of (47) [93] and various other specific and potent cyclic aminosulphonic acid GABA-A agonists [53,89]. In light of the flattened structures of the carboxylate group and the anionic groups of the very active GABA-A agonists (5), (6) and (7) (*Figure 2.2*), the observation that GABA analogues containing the tetrahedrally arranged sulphonate group interact very effectively with the GABA-A receptors is quite surprising. There is, however, some evidence suggesting that the mechanism of receptor activation of (24), (47), and other GABA agonists containing sulphonate groups is somehow different from that of for example GABA and (5) [53,89,152,153].

Within certain limits, the primary amino group of GABA can be replaced by other basic functionalities without substantial loss of GABA-ergic activities. As mentioned above, (21) is totally inactive and (20) interacts only with GABA-T and GABA uptake, but 3-guanidinopropanoic acid (27) is moderately potent as a GABA-A agonist, as an uptake inhibitor and as a substrate for GABA-T [51,141,154,155].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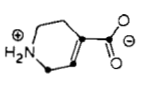
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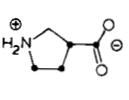
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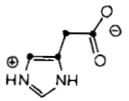
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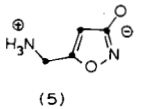
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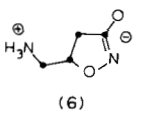
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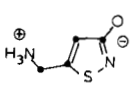
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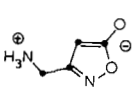
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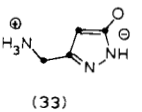
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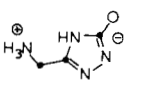
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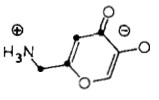
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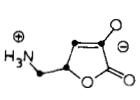
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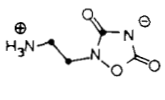
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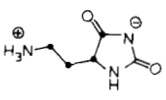
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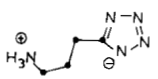

(19)

HETEROCYCLIC BIO-ISOSTERES OF GABA

As mentioned earlier, there is a pharmacological and therapeutic interest in agonists at the GABA-A receptors, and such compounds have proved to be essential as tools for studies of the molecular mechanisms of the postsynaptic GABA receptor complex (*Figure 2.2*). This scientific challenge has prompted medicinal chemists to design and synthesize a variety of analogues of GABA as potential GABA agonists, and during the past decade this interest has been concentrated on heterocyclic bio-isosteres of GABA.

By incorporation of the basic or acid moieties of GABA analogues into ring structures, it has been possible to immobilize different parts of the molecules and, furthermore, to develop zwitterionic GABA analogues with protolytic properties making penetration of the BBB possible. This approach has led to the development of conformationally restricted GABA analogues with specific actions on the GABA-A receptors and the GABA uptake systems, and structure-activity studies on these compounds have shed light on the 'active conformations' of GABA at the synaptic mechanisms concerned. Furthermore,

a number of GABA-A agonists suitable for animal behavioural studies have been developed. Among these compounds, the GABA-A agonists (5), imidazole-4-acetic acid (31) and, in particular, (14) have been the subject of clinical studies in different groups of patients.

The GABA analogues isonipecotic acid (28) and its 1,2,3,6-tetrahydropyridine analogue isoguvacine (29) interact specifically with GABA-A receptors [48–50,58,116]. Structure-activity studies on (28) and (29) and a number of related compounds have shown that the 'GABA-A receptor-active conformations' of GABA and its unsaturated analogue (22) are partially folded and relatively flattened. The 5-membered ring analogue of (28), β-proline (30), is a much weaker GABA-A agonist [49], and (30) also interacts with GABA uptake, showing some selectivity for the glial uptake system [8,156]. Replacement of the aminoethyl group of GABA by an imidazole ring gives (31), a potent and relatively specific GABA-A agonist [154,155,157,158].

Structure-activity studies on (28)–(31) indicate that GABA analogues with secondary amino groups are capable of activating effectively the GABA-A receptors, provided that they can adopt or are locked in conformations recognizable by these receptors. Thus, the lack of GABA-A agonist activity of (20) appears not to be caused by its secondary amino group *per se*, but the *N*-methyl group may make it difficult for (20) to adopt a conformation capable of binding to the GABA-A receptors. Furthermore, these structure-activity studies indicate that a certain degree of delocalization of the positive charges of GABA analogues is accepted by the GABA-A receptors, (31) and (27) being potent and moderately potent agonists, respectively [52]. The compounds (28)–(31) have been used as leads for the development of series of related GABA analogues (see subsequent sections).

The observations that (5) is a very powerful neuronal depressant [159], which acts through activation of the GABA-A receptors [58,65,133], prompted the syntheses and structure-activity studies of a variety of aminoalkyl-substituted acidic heterocyclic compounds. These studies have shown that the effects of these GABA analogues are strictly dependent on the structure of the heterocyclic ring. Thus, (6) and (7) are approximately equipotent with (5) as GABA-A agonists (see *Table 2.1* and *Figure 2.5*), whereas 3-aminomethyl-5-isoxazolol (isomuscimol, 32) and 5-aminomethyl-3-pyrazolol (azamuscimol, 33) are only weak GABA-A agonists [48,49,58], and the 1,2,4-triazole analogue (34) is totally devoid of receptor affinity [160]. Although the structural parameters of importance for the effects, or lack of effects, on the GABA-A receptors of (5)–(7) and (32)–(34) have not yet been mapped out in detail, the degree of delocalization of the negative charges of these compounds seems to be a factor of major importance [58,160]. Thus, the negative charge of (32) (and

probably also those of (33) and (34)) is delocalized to a much higher extent than that of (5) [161] and probably also those of (6) and (7) [49,58].

Whilst kojic amine (18) is a moderately potent GABA-A agonist [125,126] with some affinity for the GABA-B receptors [109], the 5-membered ring analogue 5-aminomethyl-3-hydroxyfuran-2(5*H*)-one (35) has, quite surprisingly, been shown to be completely devoid of affinity for the GABA-A receptors [162]. Similarly, 5-(2-aminoethyl)hydantoin (37) does not interact significantly with any GABA synaptic recognition site [47], and although 2-(2-aminoethyl)-1,2,4-oxadiazolidine-3,5-dione (quisqualamine, 36) shows some neuronal depressant activities, no effects of (36) on GABA synaptic mechanisms have been demonstrated with certainty [163]. As mentioned earlier, (19) interacts weakly with GABA-A as well as GABA-B receptor sites *in vitro* [128]. The compounds (5), (7), (18), (19), (32) and (33) have been used as leads for the development of series of related GABA analogues (see subsequent sections).

MUSCIMOL AS A LEAD COMPOUND

Muscimol (5) (*Figures 2.2* and *2.4*), which is a constituent of the fly agaric mushroom *Amanita muscaria* [50,164], is a powerful agonist at GABA-A receptors [58,65,133], and (5) is capable of displacing GABA and other radioactive GABA agonist ligands from the GABA receptor sites at concentrations in the low nanomolar range [47,49,58–60,106]. It does, however, also show some affinity for the GABA-B receptor sites [110,121], and interacts with the neuronal [58,62,165] as well as with the glial [62] GABA uptake mechanisms. Finally, it is a substrate for GABA-T [166], and this GABA-metabolizing enzyme is likely to be responsible for the degradation of (5) in animals [167,168]. Metabolites of (5) may contribute to its toxicity after systemic administration to animals and man [55]. It is, however, capable of penetrating the BBB [77] (see earlier section), a property which has been of particular importance for the choice of this natural product as the primary lead compound for the development of specific GABA-ergic agents.

The use of (5) as a lead for the development of specific GABA-A agonists and GABA uptake inhibitors is exemplified in *Figure 2.4*. Incorporation of the side-chain of (5) into a ring to give THIP (14) and the synthesis of the isomeric compound 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol (THPO, 38) resulted in a separation of the GABA receptor and uptake affinities of (5). Whilst (14) is a potent and specific GABA-A agonist [58,115,116], (38) is a highly

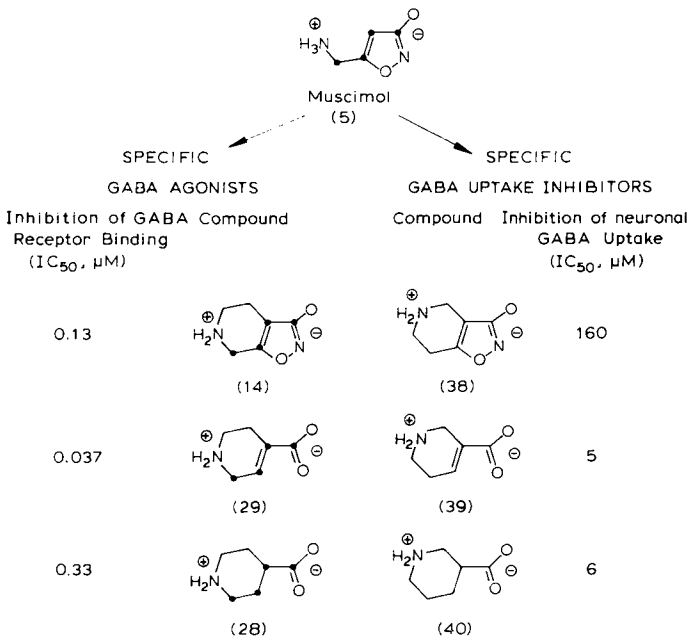


Figure 2.4. The development of a series of specific GABA-A receptor agonists and a series of specific GABA uptake inhibitors using muscimol (5) as a lead.

selective but relatively weak inhibitor of GABA uptake [8,169], interacting selectively with glial GABA uptake [170,171].

Replacement of the 3-isoxozolol unit of THIP (14) by a carboxyl group led to (28) and (29), of which the latter amino acid is even more potent than THIP as a specific GABA-A agonist [58,115,116]. The fact that THIP is more potent than (28) as a GABA-A agonist *in vivo* and *in vitro* [49,116] may indicate that a rather flattened conformation of GABA is recognized by the GABA-A receptors. Similarly, the high potency of (29) may reflect that a relatively flattened conformation of the entire molecule of (29) is quite stable due to the presence of a conjugated carboxylate group.

Replacement of the 3-isoxazolol unit of (38) by carboxyl groups to give guvacine (39) and nipecotic acid (40) results in substantial increases in affinity for the GABA uptake systems (*Figure 2.4*). Both of these cyclic amino acids are very potent inhibitors of GABA uptake [169,172], being approximately equipotent as inhibitors of the neuronal and glial uptake systems [8,62,171]. It has been shown that (40) is actually a substrate for the neuronal [8,173,174] as well as for the glial [175] GABA transport carriers, and, based on kinetic

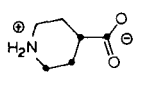
studies, (39) appears to be transported by the neuronal transport carrier [172]. These findings are surprising in light of the fact that neither (39) nor (40) is an analogue of GABA in the strict sense of the word (*Figure 2.4*). Recent studies on radiolabelled THPO (38) have shown that this selective glial GABA uptake inhibitor is not a substrate for the transport carrier concerned [176].

In spite of the structural similarities of the GABA-A agonists and the GABA uptake inhibitors listed in *Figure 2.4*, structure-activity studies on these compounds indicate distinctly different structural requirements for activation of the GABA-A receptors and for binding to the GABA transport carriers and, thus, probably different 'active conformations' of GABA with respect to these synaptic mechanisms. All of the compounds shown in *Figure 2.4* have been used as leads for the development of related heterocyclic GABA analogues (see subsequent sections).

ISONIPECOTIC ACID AND RELATED GABA AGONISTS

A number of analogues of isonipecotic acid (28) have been synthesized and tested as potential GABA agonists. In most cases, introduction of polar substituents into the ring of (28) results in substantial reduction of the affinity for the GABA-A receptors, and none of these cyclic GABA analogues has shown any significant affinity for GABA-B receptor sites [121,127]. *cis*-3-Hydroxypiperidine-4-carboxylic acid (41) and, in particular, its *trans*-isomer (42) are much weaker than (28) as GABA-A agonists, and (43) is inactive [49,177]. Similarly, (44) and (45) are completely devoid of affinity for or intrinsic activity at the GABA-A receptors [178]. Since (41), (42) and (44) still contain an unsubstituted 'GABA structure element', the present structure-activity studies seem to indicate that the GABA receptors recognize not just one side of the molecules of (28) and related compounds, but rather the entire molecules. It is possible that the hydroxy or primary amino groups of (41), (42) or (44) force these molecules to adopt conformations substantially different from that preferred by (28). However, X-ray crystallographic and ^{1}H-NMR spectroscopic investigations have demonstrated that (41), (42) and (44) preferentially adopt chair conformations with the carboxylate groups in equatorial positions similar to low-energy conformations of (28) [177–179].

The inactivity of (43) and (45) can hardly be related to the fully substituted 4-position of these compounds, which corresponds to the 2-position of GABA, since the epoxide (46) is a specific GABA-A agonist slightly more potent than (28) [76,180,181]. The epoxide actually was designed as an agent capable of reacting irreversibly with the GABA-A receptors [180], but neither *in vivo* nor



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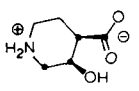

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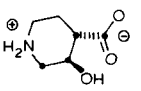

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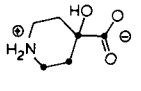

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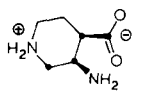

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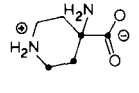

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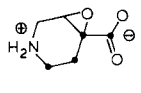

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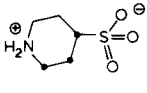


(47)

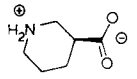
in vitro studies revealed any detectable irreversible receptor interaction of (46) [181]. As mentioned earlier, P4S (47) is a specific and very powerful GABA-A agonist [93,106], indicating that (47) represents the 'receptor-active conformation' of 3APS (24). In light of the relative potencies of (28) and (29) (*Figure 2.4*), it is surprising that (47) is more potent than its unsaturated analogue, 1,2,3,6-tetrahydropyridine-4-sulphonic acid [53,89], suggesting that the double bond in the compound forces the sulphonate group to adopt conformations which are not optimal for receptor activation. Two stereoisomeric *cis*-decahydroquinoline-5-carboxylic acids which are bicyclic analogues of GABA have been synthesized and shown to be virtually devoid of affinity for the GABA synaptic recognition sites [182].

NIPECOTIC ACID AND RELATED GABA UPTAKE INHIBITORS

STRUCTURE-ACTIVITY STUDIES

Nipecotic acid (40) is a specific inhibitor of GABA uptake, which is a substrate for the neuronal [173,174] as well as the glial [175] GABA transport carriers, making (40) a useful tool and lead structure for studies of the substrate specificity of the GABA transport carriers.

Like guvacine (39), (40) is almost equally effective in inhibiting neuronal and glial GABA uptake *in vitro* [8,62], and in an attempt to develop inhibitors for each carrier a number of analogues have been synthesized and tested. The (*R*)-(−)-isomer (49) of (40) is more potent than the (*S*)-(+)-isomer (48). The latter shows some selectivity for the neuronal system, whereas (49) is proportionally more potent as a glial GABA uptake inhibitor [62,183]. This selectivity for the glial system is more pronounced for *cis*-4-hydroxypiperidine-3-carboxylic acid (51), which is the most potent glial GABA uptake inhibitor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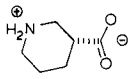
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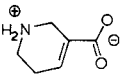
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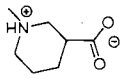
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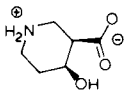
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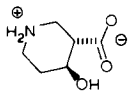
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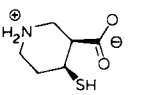
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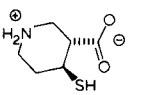
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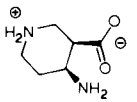
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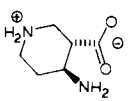
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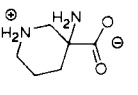
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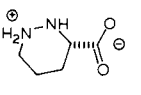

(58)

so far known [62], although THPO (38) is the most selective one [8,170,171]. Attempts to develop other more potent and selective glial GABA uptake inhibitors *via* structure modifications of (51) have, so far, been unsuccessful. The *trans*-isomer (52), which, like (51), is a racemic mixture, is almost two orders of magnitude weaker than (51) [177]. Similarly, *cis*-4-mercaptopiperidine-3-carboxylic acid (53) is much weaker than (51) [180,183]. The corresponding *trans*-form (54) and *cis*-(55) and *trans*-4-aminopiperidine-3-carboxylic acid (56) are inactive [178,183]. Furthermore, (57) is inactive [178], whereas (*S*)-(−)-hexahydropyridazine-3-carboxylic acid (58), which has the same absolute stereochemistry as (*R*)-(−)-nipecotic acid (49), is an inhibitor of neuronal GABA uptake 4-times weaker than (49) [183,184]. The (*R*)-(+)-form of (58) is inactive [184]. Kinetic data indicate that (58), like (49), is a substrate for the neuronal GABA transport carrier [184]. The oxa analogue of (58), perhydro-1,2-oxazine-6-carboxylic acid, is much weaker than (49) and (58) [169,184].

N-Methylnipecotic acid (50) is weaker than (40) as an inhibitor of GABA uptake [172,185], and the *N*,*N*-dimethyl derivative is even weaker [185]. Similarly, incorporation of a methylene group between the carboxylate group and the piperidine ring to give piperidine-3-acetic acid, which is an analogue of GABA, results in a substantial loss of effect on GABA uptake [186]. Surprisingly, however, analogues of (39), (40) and (51) with the very bulky 4,4-diphenyl-3-butenyl substituent on the nitrogen atom are more than an order of magnitude more potent than the parent amino acids [187].

PHARMACOLOGICAL STUDIES

The effects on single cells *in vivo* of (39), (40) and (51) have been studied using microelectrophoretic techniques. The depressant action of GABA on spinal [133,188], cerebellar [188] and cerebral cortical neurones [188,189] was reversibly enhanced by simultaneous microelectrophoretic administration of (40). Enantiomer (49) was more potent than (48) in enhancing the depressant action of GABA on spinal neurones [188], in agreement with the relative potency of these optical isomers as uptake inhibitors *in vitro* [62,174]. Using the same technique, (39) [190] and (51) [191] have been shown to enhance the depressant action of GABA on single cat spinal neurones. On the basis of an analysis of the effects of GABA uptake inhibitors on the depressant effects of different GABA agonists, it can be concluded that the enhancing effects of the uptake inhibitors are primarily the results of inhibition of the GABA transport processes rather than of heteroexchange phenomena [191]. Electrophoretically administered (38) also enhanced the depressant action of similarly administered GABA on cat spinal neurones [75,133], indicating that the glial uptake system plays an important part in the termination of the GABA-mediated neurotransmission [7,171].

In contrast to this enhancement by uptake inhibitors of GABA administered microelectrophoretically on single cells, application of (49) near Purkinje cells in cat cerebellum using this technique failed to prolong the GABA-mediated basket cell inhibition of these cells [188,190]. More recently, electrophoretic administration of (39) on rat hippocampus neurones was, however, shown to enhance substantially the duration of GABA-mediated inhibition [192].

In contrast to THIP (14) (*I/U* 1,500 or 500) (see earlier section), THPO (38) (*I/U* 2,500) [191] penetrates the BBB with difficulty, and (38) is pharmacologically active only in animals in which the BBB is not fully developed. Thus, (38) has anticonvulsant effects in young chicks but not in adult mice [171,193]. High doses of (38), administered intraperitoneally (i.p.) in mice, do, however, protect mice against audiogenic seizures [194], and there seems to be a positive correlation between the ability of (38) to increase the concentration of GABA in nerve endings and its anticonvulsant effects [171,195].

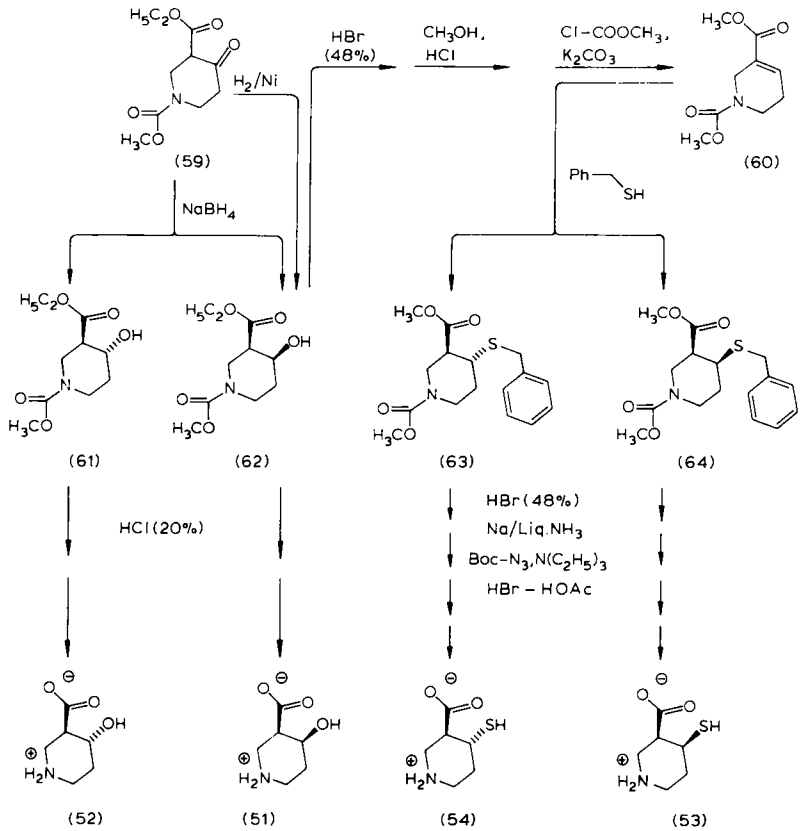
The compounds (40) (*I/U* 250,000) and (51) (*I/U* 300,000) do not pass the BBB [191], but both of these GABA uptake inhibitors have anticonvulsant properties after intracerebroventricular (i.c.v.) administration in animals [194]. The pivaloyloxymethyl ester of (40), the ethyl ester of (49), and the methyl ester of (51) have been used as prodrugs for the respective amino acids. All of these esters have anticonvulsant effects in different animal models of epilepsy [171,191,194–198]. On the basis of an analysis of the pharmacological effects

of GABA uptake inhibitors, it has been tentatively concluded that inhibitors of the glial rather than the neuronal GABA uptake system are potential anti-epileptic agents [171,191,194,197].

CHEMISTRY

The syntheses and structure determinations of (38) [199], (39) [200], (40) [201], (48) and (49) [202], (51) [200,203], (52) [177], (53) and (54) [180], (55)–(57) [178] and (58) [204] have been reported.

The syntheses of (51)–(54) are outlined in *Scheme 2.1.* Whilst catalytic reduction of the cyclic β-oxoester (59) gave (62), borohydride reduction of (59)

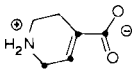


Scheme 2.1. The syntheses of the nipecotic acid (40) analogues (51)–(54). For details see Refs. 177,180,200.

gave a separable mixture of (61) and (62). Thiols (53) and (54) were prepared *via* nucleophilic addition of phenylmethanethiol to (60). The respective intermediates (64) and (63) were separated chromatographically and deprotected by acid hydrolysis and subsequent debenzylation. Attempts to isolate the desired mercaptoamino acids directly from the reaction mixtures were unsuccessful, apparently owing to rapid formation of disulphides. The hydrobromides of (53) and (54) were isolated after introduction of Boc groups on the amino and mercapto groups followed by acid deprotection.

ISOGUVACINE AND RELATED COMPOUNDS

The synthesis of isoguvacine (29) [205,206] and the demonstration of its potency and specificity as a GABA-A agonist [58,116] prompted the development of a number of structurally related amino acids. The affinity of the lower ring homologue of (29), 3-pyrroline-3-carboxylic acid (65) [207] for the GABA-A receptor sites is some two orders of magnitude lower than that of (29), and, in contrast to (29), (65) interacts with the neuronal as well as the glial GABA



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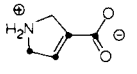

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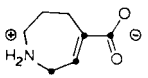

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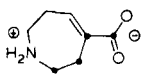

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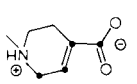
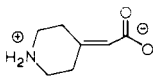


(68)



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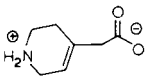
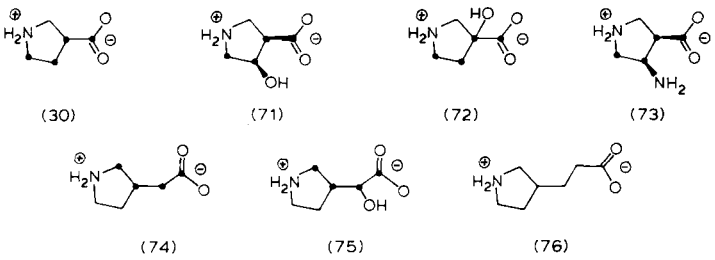


(70)

uptake systems [62]. The seven-membered ring analogues (66) and (67) do not affect GABA uptake *in vitro*, but these compounds are even weaker than (65) as inhibitors of the binding of GABA to the GABA-A receptor sites [206]. Quite surprisingly, (66) and (67) have been shown to antagonize the depressant action of glycine on cat spinal neurones [115]. *N*-Methylisoguvacine (68) [205], 1,2,3,6-tetrahydropyridine-4-acetic acid (70) and the isomeric compound (69) [208] also are very weak inhibitors of the binding of GABA to GABA-A receptor sites. Isomer (70) does not affect GABA uptake, whereas (69) is a relatively potent inhibitor of synaptosomal GABA uptake [208].

The structure-activity analysis of these analogues of (29) and a variety of structurally related compounds [206,208] emphasizes the pronounced structural specificity of the GABA-A receptors.



(30) (71) (72) (73)

(74) (75) (76)

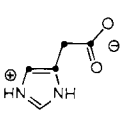
β-PROLINE, HOMO-β-PROLINE AND RELATED COMPOUNDS

In agreement with the findings for the five-membered ring analogue (65) of (29), β-proline (30) interacts much less effectively with GABA-A receptor sites than isonipecotic acid (28) [62], and, in contrast to (28), (30) is a moderately potent inhibitor of GABA uptake [62], and it quite selectively inhibits glial GABA uptake [156]. In light of the pharmacological interest in selective inhibitors of the glial GABA uptake system (see earlier section) and of the observation that *cis*-4-hydroxynipecotic acid (51) is more potent and selective than nipecotic acid (40) in inhibiting this system [8,62], *cis*-4-hydroxypyrrolidine-3-carboxylic acid (71) was synthesized [207] and tested *in vitro* [62]. It proved to be weaker than (30) as a GABA uptake inhibitor, and, furthermore, it showed approximately the same affinity for the neuronal and the glial systems [62]. The β-proline analogues (72) and (73) were totally inactive [63]. Pyrrolidine-3-acetic acid (homo-β-proline, 74) [207], on the other hand, is a potent GABA uptake inhibitor [63], and is equipotent with (49), and like (49), (74) interacts effectively with the glial as well as the neuronal GABA uptake systems and seems to be a substrate for both systems [63]. In contrast to (49), which does not interact with the GABA receptors [8,62], (74) activates the GABA-A receptors with a potency similar to that of (28) [52,63]. Thus, the effects of (74) on the GABA-A synaptic mechanisms actually are the 'sum' of the effects of (28) and (40) (see *Figure 2.4*). Neither the hydroxylated analogue (75) nor the homologue (76) of (74) interacts significantly with the GABA receptors or the GABA uptake systems *in vitro* [209].

IMIDAZOLE-4-ACETIC ACID AND RELATED COMPOUNDS

The heterocyclic GABA analogue imidazole-4-acetic acid (31) is a relatively potent depressant of the firing of cat cortical neurones [158]. This effect has

been shown to be the result of activation of GABA-A receptors [1,157], and accordingly, (31) quite effectively inhibits the binding of GABA to GABA-A receptor sites *in vitro* [47,62]. Although (31) at high concentrations reduced ganglionic accumulation of radioactive GABA [157], it does not significantly affect the uptake of GABA in rat brain slices or in cultured astrocytes [62].



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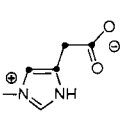

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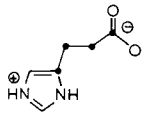
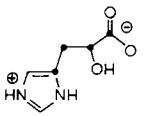


(78)



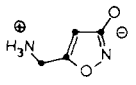
(79)

The imidazole (31) is present in the mammalian CNS, being a metabolite of histamine [210], and it may play a rôle in regulating the central GABA system. It does affect the level of GLU, the precursor for GABA, in the brain, but, so far, it has not been shown to alter the steady-state concentrations of GABA [211]. In animal models, (31) is absorbed from the gut and it readily penetrates the BBB [212,213]. The pharmacological profile of (31) includes hypnotic and analgesic effects [212,213], but, even at very high doses, (31) or prodrugs of (31) failed to produce behavioural or motor changes in patients with Huntington's chorea [214]. In these studies in animals and man, the correlation between blood and CNS levels of (31) was not studied in detail, and since (31) is a naturally occurring metabolite in the brain, it may be rapidly excreted from the CNS. In any case, (31) is an interesting GABA-ergic compound, the pharmacological and clinical potential of which has not yet been exhaustively studied. Some analogues of (31) have been synthesized and tested biologically. Whilst the homologue (78) of (31) is equipotent with (31) as a depressant of the firing of cat cortical neurones, the hydroxylated analogue (79) of (78) is considerably less active, and 1-methylimidazole-4-acetic acid (77) is totally inactive [158].

MUSCIMOL AND MONOCYCLIC MUSCIMOL ANALOGUES

STRUCTURE-ACTIVITY STUDIES

The identification of muscimol (5) as a naturally occurring heterocyclic isoster of GABA, which acts as a very powerful, though not specific, GABA-A agonist [65,133,159], and the confirmation of the unique structure of (5) by X-ray crystallography [215] prompted the synthesis of numerous analogues.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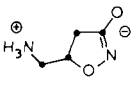
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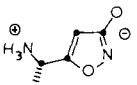
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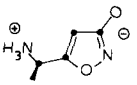
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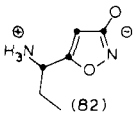
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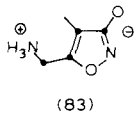
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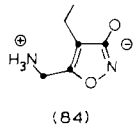
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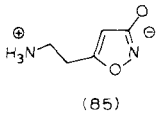
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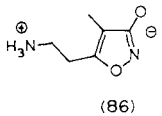
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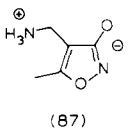
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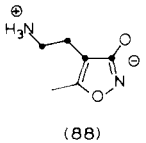
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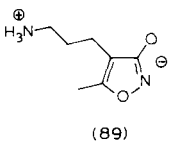
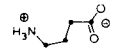
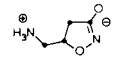
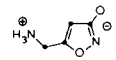
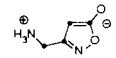
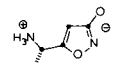
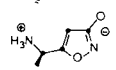
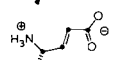
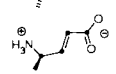

(89)

With the exception of (6), which is equipotent with (5) as a GABA-A agonist, and which has a GABA-ergic profile similar to that of (5) [49,50,58] (*Table 2.1*), none of the simple muscimol analogues so far tested has GABA-A agonist activities comparable in potency with that of (5) [48,49,133,205]. Among these aminoalkyl-3-isoxazolols, (*S*)-(−)-5-(1-aminoethyl)-3-isoxazolol (80) shows the highest affinity for the GABA-A receptor sites *in vitro*, (80) being some 30-times more potent than the (*R*)-(+)-isomer (81). *In vivo* studies did, however, fail to detect significant differences in potency as GABA-A agonists of (80) and (81) [94] (*Table 2.1*). It is interesting to compare these *in vivo* and *in vitro* effects of (80) and (81) with those of (*S*)-(−)-(90) and (*R*)-(+)-*trans*-4-aminopent-2-enoic acid (91) (*Table 2.1*). Like (80) and (81), (90) does not interact with the GABA uptake systems *in vitro*, whereas (91) is a relatively potent inhibitor of these systems, showing virtually no affinity for the GABA-A receptor sites [43,49,52,53]. This stereoselectivity of the GABA-A receptors and the GABA uptake systems is quite remarkable, and this phenomenon has recently been used to develop new specific GABA-ergic agents [216]. The different relative potencies of (80) and (81) on the GABA-A receptor sites *in vivo* and *in vitro* emphasize that the GABA-A receptor sites detectable on synaptic membranes are not identical with the postsynaptic GABA receptors on living cells.

5-(2-Aminoethyl)-3-isoxazolol (homomuscimol, 85) and 4-methyl-5-aminomethyl-3-isoxazolol (83) are relatively potent and weak GABA-A agonists, respectively [133], whereas none of the compounds (84) and (86)–(89) shows any detectable GABA-A agonist effects [133]. With the exception of (5)

Table 2.1. STRUCTURE, BIOLOGICAL AND *IN VITRO* ACTIVITY OF GABA, MUSCIMOL (5) AND RELATED COMPOUNDS

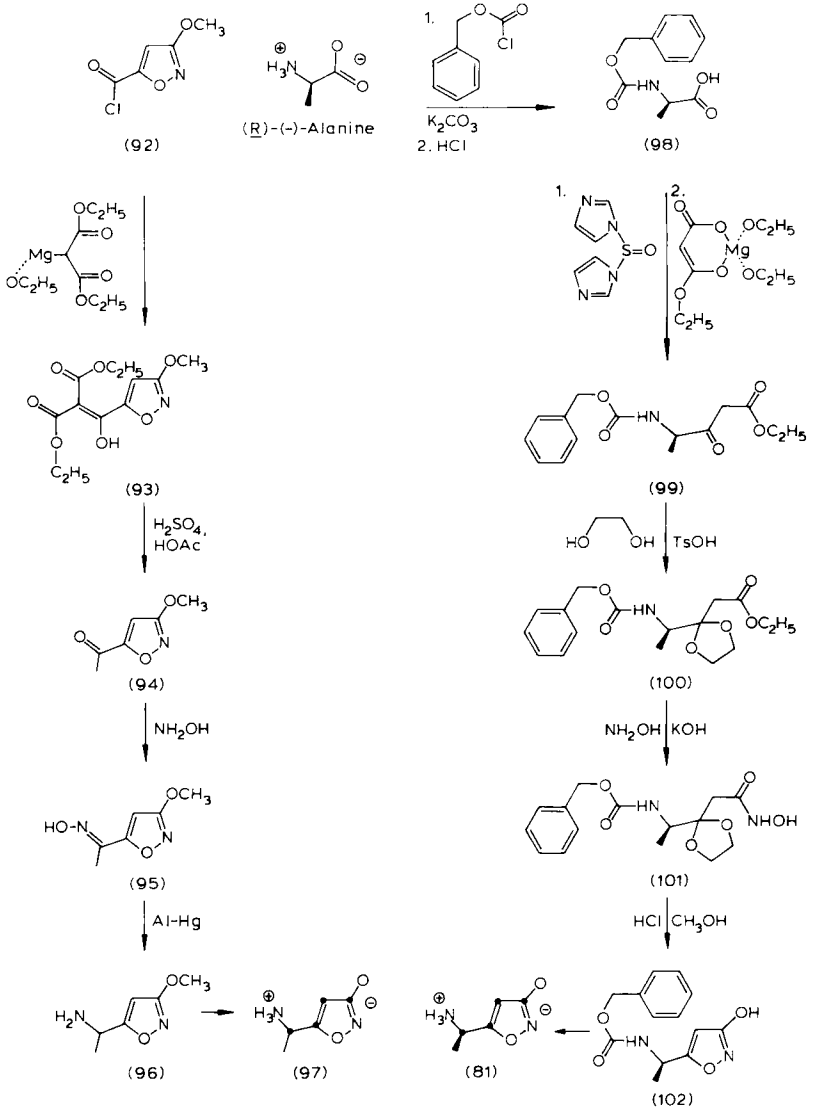
For details see Refs. 58,62,94,133. n.t., not tested.

Cmpd.	*Formula*	*GABA agonist activity in vivo (rel. potency)*	*Inhibition in vitro (IC_{50}, μM) of GABA:*		
			receptor binding	*neuronal uptake*	*glial uptake*
GABA		— — —	0.033	15	35
(6)		— — — —	0.009	1500	4000
(5)		— — — —	0.006	2500	2000
(32)		—	29	N.t.	N.t.
(80)		— — (—)	0.64	>5000	>5000
(81)		— — (—)	19	>5000	>5000
(90)		— — — (—)	4.1	>5000	>5000
(91)		N.t.	148	160	500

[110,121], none of the 3-isoxazolol zwitterions shown interacts significantly with GABA-B receptor sites *in vitro* [217]. Thus, the relatively weak BMC-insensitive depressant action of (82) on cat spinal neurones [133] does not appear to be mediated by GABA-B receptors.

Muscimol (5) has been extensively used as a pharmacological tool in different animal models (for reviews see Refs. 4,5,10,15,57,67,68), and has been administered to patients with Huntington's chorea [218] and schizophrenia [219] without any significant improvements of the psychiatric or motor symptoms of these patients. Although the majority of the pharmacological effects of (5) in animals and man are undoubtedly the results of activation of the central GABA-A receptors, some caution must be exercised in the interpretation of such observations. In light of the metabolic instability of (5) and the fact that it interacts more-or-less effectively with all GABA synaptic mechanisms in

addition to its potent effect on the GABA-A receptors (see earlier sections), (5) is of limited value as a tool for molecular and behavioural pharmacological studies.



Scheme 2.2. The syntheses of (RS)-(97) and (R)-(+)-5-(1-aminoethyl)-3-isoxazolol (81). For details see Refs. 220,221.

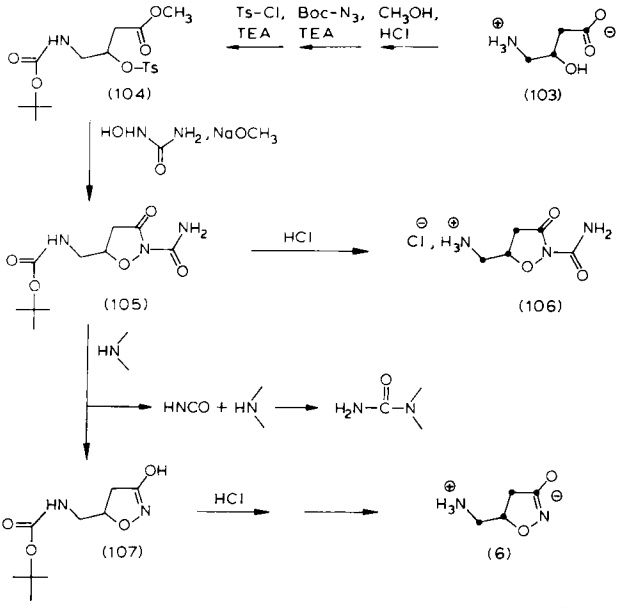
CHEMISTRY

The syntheses of some analogues of (5), including (6), (80) and (81) [220], (82) [221], (83) and (84) [220,222], (85)–(87) [220,222,223], (88) [224] and (89) [225] have been reported. A number of methods for the preparation of (5) have been published [50].

In *Scheme 2.2* the syntheses of (*R*)-(+)-(81) and (*RS*)-5-(1-aminoethyl)-3-isoxazolol (97) [220,221] are outlined. The starting material for the synthesis of (81) was (*R*)-(−)-alanine, the protected form of which (98) was converted into the imidazolide, and treatment of this reactive intermediate with the magnesium salt of malonic acid monoethyl ester gave (99). The ketalized hydroxamic acid (101) was deprotected and cyclized to give (102), which in turn was deprotected to give (81). The corresponding (*S*)-(−)-isomer (80) was synthesized analogously using (*S*)-(+)-alanine as starting material [220].

The isoxazole (97) was synthesized *via* the enolized β-oxodiester (93), which was prepared by treatment of (92) with the magnesium salt of malonic acid diethyl ester. The reduction of the oxime (95), which actually was obtained as a separable mixture of the *Z*- and *E*-forms, was accomplished without any significant cleavage of the N-O bond using aluminium amalgam.

The synthesis of (6) is illustrated in *Scheme 2.3* [220]. The fully protected



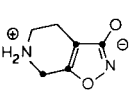
Scheme 2.3. The synthesis of dihydromuscimol (6). For details see Refs. 220,221.

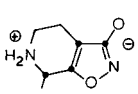
form (104) of (*RS*)-4-amino-3-hydroxybutanoic acid (103) was treated with the sodium salt of hydroxyurea to give (105) *via* an elimination-nucleophilic addition-cyclization reaction sequence [226]. Whilst treatment of (105) with hydrogen chloride in ethyl acetate gave (106), which can be used as a prodrug for (6) [74], the carboxamide group of (105) could be removed by treatment with dimethylamine. Acid deprotection of (107) under anhydrous conditions gave (6), which was most conveniently isolated as the hydrochloride.

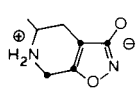
THIP AND RELATED BICYCLIC ANALOGUES OF MUSCIMOL

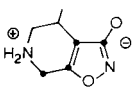
STRUCTURE-ACTIVITY STUDIES

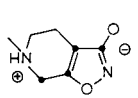
A number of analogues of the specific GABA-A agonist THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol, 14) (see earlier section) have been synthesized and tested. These structure-activity studies include analogues of THIP, in which the 3-isoxazolol unit has been replaced by other heterocyclic systems (see subsequent sections), and bicyclic 3-isoxazolol zwitterions such as (108)–(114). The *C*-methylated analogues, (108)–(110), are virtually devoid of affinity for the GABA-A receptors [227,228] suggesting that the GABA receptors do not recognize and bind a limited part of the molecule of THIP, but rather the entire molecule. Similarly, the *N*-methylated analogue (111) is completely inactive as a GABA agonist [115], and none of the seven-membered ring analogues THIA (112), THAZ (113) or THAO (114) interacts significantly with the GABA-A receptors (*Table 2.2*) [133,205]. However, in agreement with the findings for the seven-membered ring homologues (66) and (67) of isoguvacine (29), the corresponding 3-isoxazolol zwitterions (112) and (113) have been shown to be glycine antagonists [115]. Compound (114) is a ring homologue of the selective glial GABA uptake inhibitor, THPO (38) (*Figure 2.4*), and, accordingly, (114) interacts with this uptake mechanism with a potency only slightly lower than that of (38) [8,170].

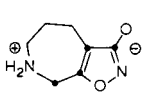

(14)

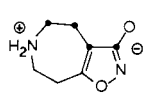

(108)

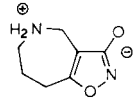

(109)


(110)


(111)


(112)


(113)


(114)

PHARMACOKINETICS OF THIP

As mentioned earlier,THIP does not affect GABA-T activities *in vitro* [58]. In animals, it penetrates the BBB very easily [76,77], and it distributes unevenly into the brain [77]. The pharmacokinetics of THIP in man have recently been studied [57,229–232]; it is rapidly absorbed after oral (or) [232] or intramuscular (i.m.) [230] administration, with peak concentrations in serum 0.5 h after administration of clinically relevant doses (10–15 mg) of ^{14}C-labelled THIP [57,232]. THIP is excreted mainly unchanged in the urine, although two or three metabolites are detectable in the urine from man and mouse, respectively. In man, the main metabolite, which corresponds to approx. 30% of the total dose of THIP, appears to be an *N*-glucuronide, glucuronic acid being coupled to the isoxazole ring [57]. The structure of this metabolite has not yet been unequivocally established, but its chromatographic and chemical properties are identical with those of the synthetic *N*-glucuronide of THIP [232,233].

In animals, peak concentrations of THIP in the organs, including the brain, coincide approximately with that in serum [232]. Assuming that the same relationship exists between serum and brain concentrations of THIP in man, there is a need for a sustained release preparation of this compound. Attempts to develop such a preparation have encountered difficulties.

PHARMACOLOGICAL AND CLINICAL STUDIES ON THIP

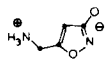
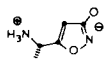
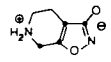
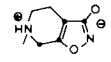
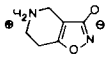
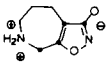
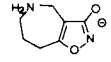
There is an overwhelming amount of indirect evidence, derived from experimental models of epilepsy, supporting the view that pharmacological stimulation of the GABA neurotransmission may have therapeutic interest in epilepsy [5,10]. The anticonvulsant effects of THIP (14) and muscimol (5) have been compared in a variety of animal models. THIP typically is 2–5-times weaker than (5) in suppressing seizure activities (*Table 2.2*). In mice [234] and in gerbils with genetically determined epilepsy [235], systemically administered THIP has proved very effective in suppressing seizure activity, and is capable of reducing audiogenic seizures in DBA/2 mice [236]. However, THIP failed to protect baboons with photosensitive epilepsy against photically-induced myoclonic responses [236]. In acute as well as chronic studies, THIP is capable of reducing seizures in mice induced by various agents [237]. An analysis of these effects [237] and the effects of THIP on pento-geniculo-occipital (PGO) activity in cats [238] seems to indicate an as yet unclarified involvement of the serotonin system. THIP has been subjected to a single-blind controlled trial in patients with epilepsy, in which it was added to the

concomitant antiepileptic treatment [239]. Under these conditions no significant effects of THIP were detected, although a trend was observed for lower seizure frequency during a period of submaximal doses of THIP.

The effects of THIP on ethanol withdrawal symptoms in rats have recently been studied [240]. Whilst intracisternally administered THIP proved effective in reducing audiogenic clonic-tonic seizures, no effects on forelimb tremor were observed. These selective effects may have clinical interest.

In recent years the involvement of the central GABA system in pain mechanisms has been the subject of intensive studies [22,24]. The demonstration of potent analgesic effects of THIP in different animal models (*Table 2.2*) [23,72] has made studies of the clinical prospects of GABA-mediated analgesia possible. THIP-induced analgesia is insensitive to naloxone [23–25,72], indicating that the effect is not mediated by the opiate receptors. Quite surprisingly, THIP analgesia can not be reversed by bicuculline [23–25,241], which may indicate the involvement of a distinct class of GABA receptor. On the other

Table 2.2. STRUCTURE AND BIOLOGICAL ACTIVITY OF MUSCIMOL (5), THIP (14) AND RELATED COMPOUNDS
For details see Refs. 58,72,94,116,133.

Compound		*Biological actions*		
		GABA agonism on cat spinal neurones (Rel. potency)	*Antagonism of isoniazide convulsions (ED_{50}, μmol/kg, i.p.)*	*Analgesia grid shock test (ED_{50}, μmol/kg, i.p.)*
(5)		– – – –	2	3
(80)		– – (–)	23	77
(14)		– – – (–)	9	16
(111)		0	> 200	> 200
(38)		0	> 200	> 200
(112)		0	> 200	> 200
(114)		0	N.t.	> 200

hand, THIP-induced analgesia can be reduced by atropine [25,241] and potentiated by cholinergics such as physostigmine, reflecting as yet unclarified functional interactions between GABA and acetylcholine neurones and the central opiate systems rather than a direct action of THIP on muscarinic receptors [25,241].

THIP and morphine are approximately equipotent as analgesics, although their relative potencies are dependent on the animal species and experimental models used [23,25]. Acute injection of THIP potentiates morphine-induced analgesia, and chronic administration of THIP produces a certain degree of functional tolerance to its analgesic effects [241]. In contrast to earlier findings [72], the results of recent studies have been interpreted in terms of some cross-tolerance between THIP and morphine [241]. In contrast to morphine, it does not cause respiratory depression [242]. Clinical studies on post-operation patients and patients with chronic pain of malignant origin [243] have disclosed analgesic effects of THIP, in the latter group of patients at doses of 5–30 mg (i.m.) [243]. In these cancer patients and also in patients with chronic anxiety [27] the desired effects of THIP were accompanied by side-effects, notably sedation, nausea and, in a few cases, euphoria. The side-effects of THIP have been described as mild and similar in quality to those of other GABA-mimetics [27]. These undesirable effects of THIP may to a certain extent be ascribed to its non-optimal pharmacokinetics [27], emphasizing the need for a sustained release preparation of it.

It is assumed that the postsynaptic GABA receptor complex is mediating the anxiolytic effects of the BZD [244], and, consequently, it is of interest to see whether GABA agonists have anxiolytic effects. Muscimol (5) has proved effective in conflict tests, though with a pharmacological profile different from that of diazepam [245], and in humans, (5) in low doses was found to sedate and calm some schizophrenic patients [246]. In a recent study on a number of patients with chronic anxiety, the effects of THIP were assessed on several measures of anxiety [27]. Although these effects were accompanied by side-effects [27], the combination of analgesic and anxiolytic effects of THIP would seem to have therapeutic prospects.

There is very strong evidence that GABA is involved in the regulation of cardiovascular mechanisms [19,22,247]. Whilst i.c.v.-administered THIP reduced blood pressure as well as heart rate, systemically administered THIP did not affect these functions significantly [248,249]. On the other hand, systemically administered GABA and (29) had significant cardiovascular effects, which can be blocked by BMC [249]. Since THIP, but not GABA and (29), is capable of penetrating the BBB [74–76,232], these results seem to indicate that peripheral GABA receptors are involved in the regulation of cardiovascular

functions [249]. It is interesting to note that GABA and a number of GABA agonists produce a dose-dependent dilation of isolated cat and dog cerebral artery segments apparently by activation of GABA receptors with pharmacological characteristics similar to those of central postsynaptic GABA receptors [250].

The results of pharmacological studies on the spastic mouse are consistent with a rôle of GABA in spasticity [251]. Systemic administration of the GABA agonists (5), THIP and (29) to the cat affected spinal cord activities [252]. Since (29) does not readily penetrate the BBB [74–76,232], its pharmacological effects in this animal model [252] suggest that some parts of the spinal cord are not effectively protected by a BBB. THIP has recently been studied in spastic patients [253]. At oral doses of 15–25 mg, THIP clearly reduced the monosynaptic T-reflexes without affecting the flexor threshold significantly [253].

Studies in recent years have disclosed very complex interactions between different neurotransmitter systems in the basal ganglia, notably the DA, GABA and cholinergic systems [5,10,55,254]. These interactions have been extensively studied with the intention of gaining a better insight into the mechanisms underlying schizophrenia, Parkinson's disease and different dyskinetic syndromes and, furthermore, of developing new strategies for the treatment of these severe diseases. Much interest has been focused on the nigrostriatal DA neurones, which form part of the nigrostriatal 'feedback' pathway, of which the striatonigral GABA neurones terminate within the substantia nigra (SN) pars reticulata, possibly on cholinergic neurones [255]. The DA neurones of this system originating in SN pars compacta as well as the mesolimbic DA neurones involved in an analogous 'feedback' loop are assumed to be under inhibitory control [5,10,55,254,255]. These aspects have opened up the prospects of using GABA-stimulating therapies in the treatment of schizophrenic patients and, consequently, THIP has been quite extensively studied in different animal models.

Whilst activation of GABA receptors in SN pars reticulata of rats has dramatic behavioural consequences [70], the DA neurones in SN pars compacta and the mesolimbic DA neurones are much less sensitive. Direct application of THIP in the respective brain areas actually have weak inhibitory effects on both types of DA neurone, whereas systemically administered THIP weakly stimulates these DA neurones [256,257]. No simple explanation of these apparently self-contradictory observations has, so far, been forwarded. The behavioural effects of acute and chronic administration of THIP have been studied [55,258]. DA agonist-induced locomotor activity and stereotypies are altered by simultaneous treatment with GABA agonists, the former activity

being depressed and the latter intensified [259]. From a clinical point of view, the interactions between (14) and neuroleptics may be particularly interesting. Most neuroleptic drugs inhibit DA-induced stereotypy and induce catalepsy in animals, the former effect being related to clinical antipsychotic effects and the latter to extrapyramidal side-effects of neuroleptics [260]. Since THIP and also scopolamine antagonize the antistereotypic effects of some neuroleptics, it has been tentatively concluded that GABA agonists such as THIP would probably not potentiate the antipsychotic effect of neuroleptics, but rather antagonize it [260].

The interactions between THIP and the central DA systems have also been studied in monkeys [261]. Analyses of the complex pharmacological profile of THIP in this animal, which to some extent was similar to that of diazepam (4), led to the conclusion that THIP would probably have limited therapeutic effect in different kinds of dyskinesia, and THIP did actually prove ineffective in reducing the symptoms of dyskinetic patients [262].

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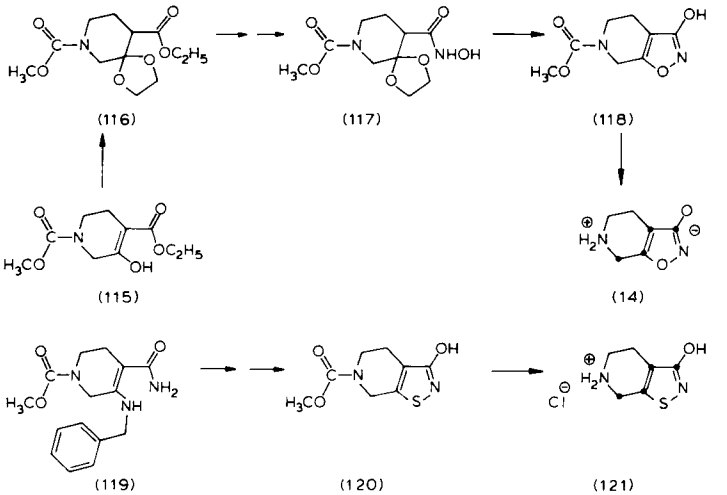
The syntheses of THIP (14) [263], (108)–(110) [227,228], (112) and (114) [263] and (113) [199] have been reported. In *Scheme 2.4* the last steps of the synthesis of THIP are outlined. The enolized β-oxoester (115) was ketalized to give (116), which was converted into the hydroxamic acid (117). This step

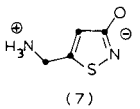


Scheme 2.4. The syntheses of THIP (14) and thio-THIP · HCl (121). For details see Refs. 75,263.

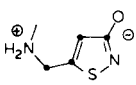
and the conversion of (117) into the protected form, (118) of THIP are the key steps in the synthesis, which is a ten-step reaction sequence [57,263]. The deprotection and subsequent cyclization of (117) are most conveniently accomplished using concentrated sulphuric acid as a reagent.

THIOMUSCIMOL AND RELATED COMPOUNDS

Besides dihydromuscimol (6) [58] (*Table 2.1*), thiomuscimol (7) [58], is the only analogue of muscimol (5), which is approximately equipotent with the parent compound as a GABA-A agonist [58]. Thus, the IC_{50} value of (7) (19 nM) as an inhibitor of the binding of GABA to GABA-A receptor sites *in vitro* is comparable with that of (5) (6 nM) [58,59] and (5) and (7) are equally effective in depressing the firing of cat spinal neurones in a BMC-sensitive



(7)



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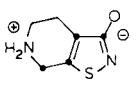
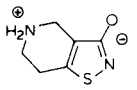
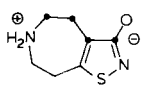


(123)



(124)



(125)

manner [58] (*Figure 2.5*). Like (5) [166], (7) is a substrate for GABA-T [265], but in contrast to (5), which is a substrate for the neuronal GABA transport carrier [58,62,264], (7) has no significant effect on GABA uptake [58]. From a pharmacological point of view, this difference between (5) and (7) is interesting, since the toxic effects of (5) to some extent may be attributable to metabolites formed intracellularly by the GABA-T catalyzed degradation of (5) after the uptake into nerve terminals. Thus, like (5) [167,168], (7) may be subjected to a partial degradation by GABA-T after peripheral administration, but (7) is likely to be less toxic than (5). These aspects are at present under investigation.

Like *N*-methylmuscimol [115], *N*-methylthiomuscimol (122) is only a weak GABA-A agonist [54], but whereas conversion of (5) into THIP gave a specific GABA-A agonist only slightly weaker than the parent compound (*Figure 2.4* and *Table 2.3*), an analogous transformation of (7) to give 4,5,6,7-tetrahydroisothiazolo[5,4-*c*]pyridin-3-ol (thio-THIP, 123) resulted in a substantial decrease in activity. The latter is only a weak GABA-A agonist [75], and, in

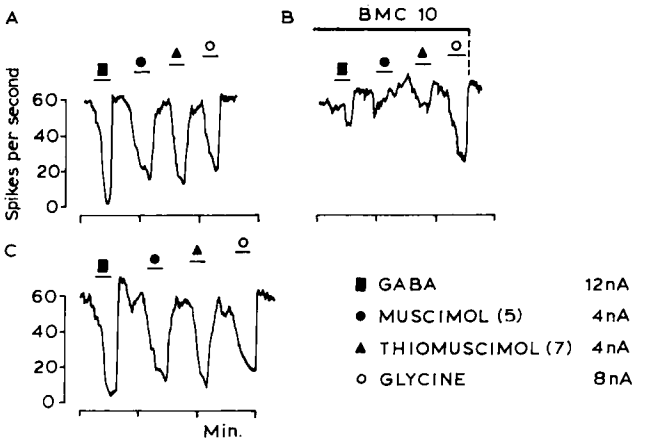


Figure 2.5. Comparison of the effects of GABA (12 nA), muscimol (5) (4 nA), thiomuscimol (7) (4 nA) and glycine (8 nA) on single cat spinal neurones using microelectrophoretic techniques, before (A), during (B) and after (C) simultaneous administration of the GABA agonist BMC. For details see Ref. 58.

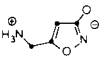
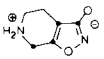
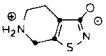
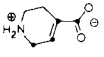
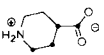
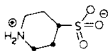
contrast to THIP and to the parent compound (7) [58], (123) does affect GABA uptake (*Table 2.3*) [75].

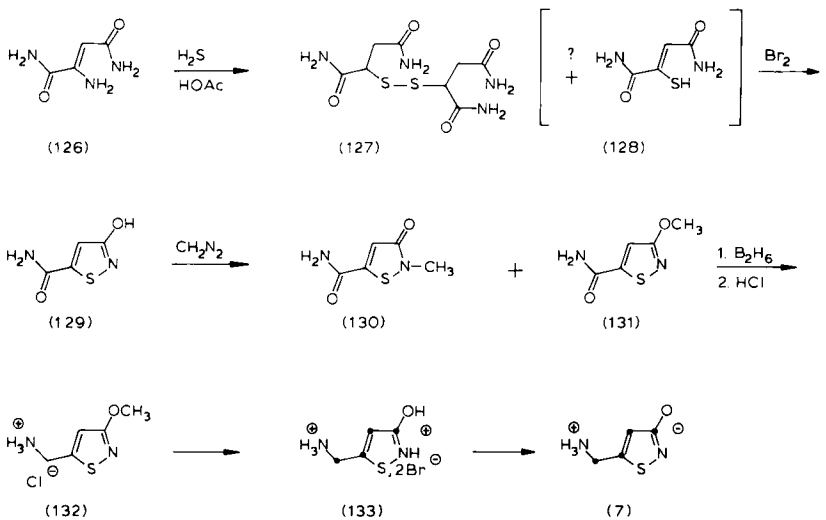
Like THPO (38) [8,169–171], thio-THPO (124) is an inhibitor of GABA uptake weaker than (38) [75], and it seems to lack the glial-selectivity which is characteristic for (38). 5,6,7,8-Tetrahydro-4*H*-isothiazolo[4,5-*d*]azepin-3-ol (thio-THAZ, 125) has only marginal effects on GABA synaptic mechanisms, but it is capable of blocking the depressant effect of glycine on cat spinal neurones [75]. This pharmacological profile of (125) is similar to that of its oxa-analogue, THAZ (113) [115,133,205].

The syntheses of (7) [266], (122) [267] and (123)–(125) [75] are published or under publication. The last steps of the reaction sequence for the preparation of the hydrochloride (121) of (123) are outlined in *Scheme 2.4*. The enamine amide (119), which is a derivative of (115), was treated with hydrogen sulphide, and the complex reaction product, assumed to contain as the major product the enethiol analogue of (119), was oxidized with bromine to give (120) (*cf.* the conversion of (126) into (129) in *Scheme 2.5*). Analogous reaction sequences were used for the preparation of (124) and (125), although the reaction conditions for the preparation of these bicyclic analogues of (7) were quite different [75].

Scheme 2.5 illustrates the synthesis of (7) [266]. From the reaction mixture obtained after treatment of aminofumaramide (126) with hydrogen sulphide and assumed to contain (128), only (127) could be isolated in a pure state.

Table 2.3. STRUCTURE, BIOLOGICAL AND *IN VITRO* ACTIVITY OF GABA AND SOME GABA ANALOGUES
For details see Refs. 53,58,75,93,116.

Cmpd.	*Structure*	*GABA agonism Rel. potency*	*GABA binding IC_{50} (μM)*	*GABA uptake IC_{50} (μM)*
GABA		– – –	0.033	2
(5)		– – – –	0.006	240
(14)		– – – (–)	0.130	> 300
(123)		–	42	220
(29)		– – – –	0.037	> 300
(28)		– – –	0.33	> 300
(47)		– – – –	0.034	> 300

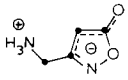


Scheme 2.5. The synthesis of thiomuscimol (7) For details see Ref. 266.

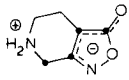
Oxidation of the crude reaction product with bromine gave 3-hydroxyisothiazole-5-carboxamide (129) in moderate yields. Treatment of (129) with diazomethane gave a separable mixture of (130) and (131), of which the latter was converted into (132). Deprotection of (132) by hydrogen bromide in glacial acetic acid gave (133), which could be converted into the monohydrobromide of (7).

ISOMUSCIMOL, AZAMUSCIMOL AND RELATED COMPOUNDS

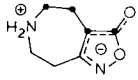
As discussed earlier, the very low GABA-A agonist activity of isomuscimol, (32), as compared with those of the structurally related compounds (5) and (6) (*Table 2.1*) probably is a consequence of the marked delocalization of the negative charge of (32) [49,58]. Interchange of the isoxazole oxygen and



(32)



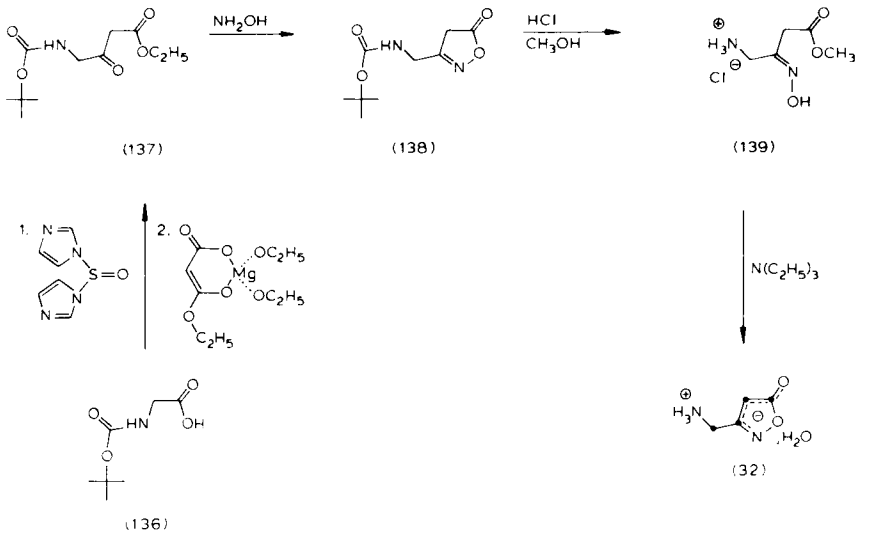
(134)



(135)

nitrogen atoms of THIP to give 4,5,6,7-tetrahydroisoxazolo[3,4-*c*]pyridin-3-ol (iso-THIP,134) has even more dramatic consequences, as this compound binds weakly to the GABA-A receptor sites *in vitro* [206] but has weak antagonistic rather than agonistic properties [71]. Similarly, iso-THAZ (135), has GABA antagonist properties after local injection into the substantia nigra pars reticulata of rats, and in this model (135) is more potent than (134) [71]. Microelectrophoretic application of (135) on cat spinal neurones does, however, not affect the depressant action of simultaneously administered GABA, whereas (135) antagonizes the depressant action of glycine on these neurones [115]. This effect on glycine receptors of (135) is similar to but slightly more potent than those of a number of structurally related compounds such as (112), (113) and (125) [75,115]. This rather complex pharmacological profile of (135) seems to indicate that the GABA receptors in the rat substantia nigra and in the cat spinal cord are different [54].

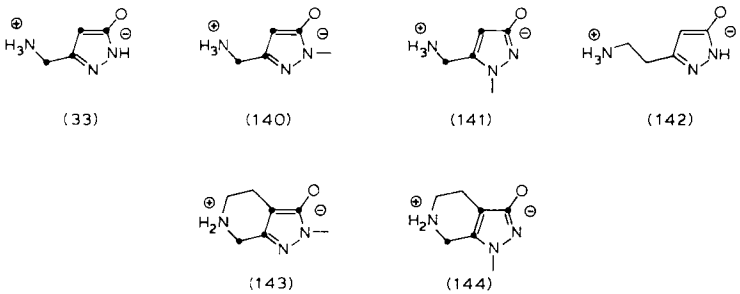
The syntheses of (32) [268], (134) [206] and (135) [269] have been published. In *Scheme 2.6* the synthesis of (32) is outlined. Treatment of the imidazolide of Boc-protected glycine (136) with the magnesium salt of malonic acid monoethyl ester gave (137), which reacted regiospecifically with hydroxylamine to give (138). Removal of the Boc group by methanolic hydrogen chloride was accompanied by opening of the 2-isoxazolin-5-one ring to give



Scheme 2.6. The synthesis of isomuscimol (32). For details see Ref. 268.

(139), which cyclized to (32) by treatment with triethylamine. The structure of (32) monohydrate was confirmed by X-ray crystallography [161].

Like (32), azamuscimol (33) is a very weak inhibitor of the binding of GABA to GABA-A receptor sites [58]; it is, however, more potent than (32) as a depressant of neuronal firing, but this *in vivo* effect of (33) was not readily antagonized by BMC [58], suggesting that at least part of this effect is not mediated by the GABA-A receptors. The 2-methyl analogue (140) of (33) also shows very low affinity for the GABA-A receptor sites *in vitro* [58,270]. These weak effects may reflect that the distribution of the negative charges of (33) and (140) is similar to that of (32) [268]. 1-Methyl-5-aminomethyl-3-pyrazolol, (141), is, however, only marginally more potent than (33) and (140), and it is



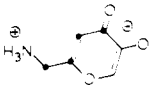
unlikely that the negative charge of this compound is delocalized to the same extent as those of (33) and (140) [270]. The homologue (142), as well as the bicyclic 3-pyrazolol zwitterions (143) and (144) [206], are completely inactive. The structure of (143) was confirmed by X-ray crystallography [271].

In contrast to (141), (33) and (140) do inhibit the activity of GAD *in vitro* [58] in a dose-dependent manner. Being completely devoid of affinity for the GABA receptors, the GABA uptake systems, and for GABA-T, (140) actually appears to be a specific, though relatively weak, inhibitor of GAD [58].

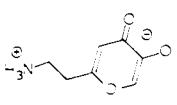
KOJIC AMINE AND RELATED COMPOUNDS

Kojic amine (18) was designed as a GABA-A agonist capable of penetrating the BBB [126]. It is behaviourally active, although it was found to possess only low affinity for the GABA-A receptor sites [126,272]. It was found that (18) resembled baclofen (15) on the spinal cord, although it had a GABA-A agonist profile in the cerebral cortex [125]. Recent studies have shown that (18) interacts quite potently with GABA-B receptor sites *in vitro* [109,273]. In agreement with the low affinity of (18) for the GABA-A receptors, (18) was found to be a relatively weak stimulator of BZD binding, an effect which is mediated by the GABA-A receptors (see earlier section). Thus, the behavioural effects of (18), notably anticonvulsant and skeletal muscle relaxant activities [126], apparently are mediated primarily by the GABA-B receptors [109].

A number of analogues of (18) have been synthesized and tested biologically [126], and structure-activity studies of these analogues have shown that even minor structural modifications of (18) result in biological inactivity. Thus, 2-aminomethyl-3-hydroxy-4*H*-pyran-4-one (146), the aza-analogue (147), and the homologue (145), failed to produce any significant pharmacological effects in the animal models used for the pharmacological evaluation of (18) [126].



(18)



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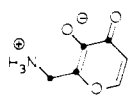

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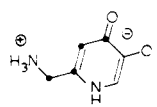

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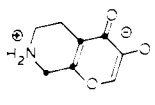


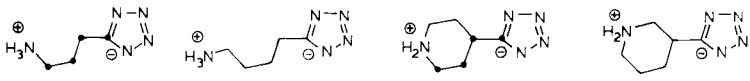
(148)

Compound (148), which has been obtained by isosteric replacement of the 3-isoxazolol ring of THIP (14) by the 3-hydroxy-4*H*-pyran-4-one ring, also proved to be inactive [227].

Pyranopyridine (148) and a number of analogues of (5) were synthesized and tested in an attempt to develop analgesic GABA agonists devoid of the undesired sedative effects of THIP [227]. Some of the analogues of (5) showed analgesic effects, but in no case was the analgesic effect separated from sedation [227]. On the basis of extensive structure-activity analyses of these analogues, it was proposed that compounds with non-zwitterionic structures can bind to GABA-A receptor sites [227]. This proposal may open up the possibility of designing new types of GABA agonist of pharmacological interest.

5-(3-AMINOPROPYL)TETRAZOLE AND RELATED COMPOUNDS

In some areas of drug design, isosteric replacement of carboxyl groups by tetrazole rings has been accomplished with success [274]. Accordingly, some mono- and bicyclic zwitterionic tetrazole compounds have recently been synthesized as potential GABA-ergic agents [128]. As mentioned earlier, the tetrazole GABA analogue (19) showed some affinity for the GABA-A as well



(19) (149) (150) (151)

as the GABA-B receptor sites *in vitro* [128]. In agreement with isoguvacine (29) being a potent and specific GABA-A agonist [49,58,110,116,121], the tetrazole analogue of (29) showed some selectivity for the GABA-A receptor sites, but with an affinity some three orders of magnitude lower than that of (29) [128]. The tetrazole analogue (150) of isonipecotic acid (28) and the homologue (149) of (19) were virtually devoid of any affinity for GABA receptor sites *in vitro*, and none of these tetrazole zwitterions, including (151), an analogue of nipecotic acid (40), had any effect on GABA uptake *in vitro* [128]. In agreement with the results of numerous other studies on GABA analogues (see earlier sections) the structure-activity relationships of the tetrazole analogues (19) and (149)–(151) emphasize that strict structural constraints are imposed on GABA agonists and GABA uptake inhibitors.

CONCLUSION

Bio-isosteric replacements of the amino and carboxyl groups of GABA by certain heterocyclic ring systems have led to many GABA-ergic agents which have been subjected to pharmacological and, in some cases, clinical studies. By incorporation of the GABA backbone into these ring systems, a variety of conformationally restrained GABA analogues have been developed which interact specifically with different GABA synaptic mechanisms. Structure-activity studies on such compounds have demonstrated that GABA adopts different conformations during its interaction with the GABA receptors and transport carriers. The active conformation of GABA with respect to the GABA-A receptors is relatively planar and partially folded as reflected by THIP and isoguvacine (29), whereas nipecotic acid (40) and guvacine (39) somehow reflect the conformation in which GABA is transported into the nerve terminals and glia cells. By proper choice of acid and basic heterocyclic rings, it has been possible to design GABA-ergic agents with protolytic properties making penetration of the BBB possible. Thus, the GABA-A agonists muscimol (5), dihydromuscimol (6), thiomuscimol (7), THIP (14), imidazole-4-acetic acid (31) and the mixed GABA-A–GABA-B agonist kojic amine (18) are pharmacologically active after systemic administration. Similarly, the glial-selective GABA uptake inhibitor THPO (38) does, to some extent, penetrate the BBB. Clinical studies on muscimol (5) and, in particular, THIP have disclosed interesting effects, including analgesic, anxiolytic, antispastic, and muscle relaxant activities. Thus, THIP is a potent non-opioid analgesic without effects on respiration and blood pressure in man, and it has anxiolytic and antispastic effects in man.

Based on the effects of GABA uptake inhibitors, notably THPO (38) and different analogues and derivatives of nipecotic acid (40), such compounds, particularly glial-selective uptake inhibitors, are considered to be potential antiepileptic agents.

The clinical effects of GABA-ergic agents quite often are accompanied by undesired effects, and a major aim in the GABA field must be to design specific drugs in which the desired actions have been separated from the undesired effects. In this field, medicinal chemists are faced with biological mechanisms characterized in pronounced 'structural specificities'. These aspects, supported by the apparent therapeutic prospects of GABA-stimulating drugs, represent a great challenge to intuitive and imaginative medicinal chemists.

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3 Recent Advances in β-Adrenergic Blocking Agents

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INTRODUCTION

β-Adrenergic receptor antagonists, commonly referred to as β-blockers, have been one of the most important groups of drugs introduced into clinical practice during the last 20 years. Although introduced initially for the treatment of angina pectoris and arrhythmias, they are now used widely in the treatment of hypertension, glaucoma, anxiety and numerous lesser indications. The vast amount of literature dealing with all aspects of β-blockers attests to the intense research activity in this area. In this review we have concentrated on what we consider to be the most significant advances since 1977.

TEST SYSTEMS FOR EVALUATING β-BLOCKERS

Before beginning a discussion of the structure-activity relationships of β-blockers, it is important to outline the biological parameters which characterize a β-blocker and describe how they are measured. Traditionally the parameters measured were potency, selectivity for β_1- or β_2-receptors, and degree of partial agonist activity (P.A.A.). Another property, lipophilicity, has assumed a greater importance in more recent times.

Potency is a measure of the ability of a test compound to inhibit competitively the actions of a β-agonist on a test system. In *in vitro* systems, the β_1-response is commonly the heart rate increase caused by isoprenaline in spontaneously beating guinea-pig or rat atria, while the β_2-response is the relaxation of tracheal or uterine smooth muscle by the same agonist in these species. Data are usually analyzed by standard pharmacological methods such as Schild plots [1] and potency is expressed as a pA_2 value, selectivity being simply the relative potency in the two systems. It has been suggested that for more accurate estimates of potency and selectivity, a β_1-selective agonist (e.g., noradrenaline) should be used in β_1 tissue and a β_2-agonist (e.g., fenoterol) in a β_2 tissue [2].

The same principles apply to the *in vitro* situation, in which the β_1-response is measured as a change in heart rate and the β_2-response as a change in blood pressure. The *in vivo* situation is, however, more complex because of cardiovascular reflex mechanisms which would obscure the results [3]. To a large extent these reflex mechanisms are removed by vagotomy. The vagotomized rat and cat models have provided a simple, high-throughput method for estimating β_1- and β_2-potency [4,5], and are probably the most widely used. These models can be further refined by removing the influence of endogenous catecholamines by pretreatment of the animal with a reserpine derivative [6]. By employing

vagotomy, reserpinization and section of the sympathetic nerves in the dog, and by measuring blood pressure in an artery perfused at constant flow, a virtually isolated β_1- and β_2-system *in vivo* has been obtained [7]. All these models provide a measure of selectivity of a compound in blocking the β_1- or β_2-receptor, in which the β_2-response is based on blood pressure effects. Bronchial effects can be considered as the *in vivo* equivalent of the isolated trachea and have been used to measure β_2-effects in guinea-pig [8] and dog [9]. A crude measure of β_1-selectivity was obtained using a guinea-pig bronchospasm model in which the protective action of isoprenaline against histamine-induced bronchospasm is unaffected by β_1-selective blockers [10]. The large variety of test systems and species used in the evaluation of β-blockers makes a direct comparison of potencies extremely difficult. Potency can also be measured by biochemical techniques in which the ability of a test compound to displace a radioactive ligand from a β-receptor preparation is measured [11], but this technique does not distinguish between agonists and antagonists. The accuracy will be critically dependent on the purity of the β-receptor preparation. The inhibition of agonist-promoted production of cyclic AMP in isolated membrane preparations has also been used [12].

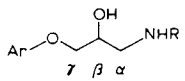
In the context of β-blockers, the partial agonist activity (P.A.A.) is understood to be the maximum increase in heart rate observed on administration of a high dose of the compound to a catecholamine-depleted animal [13]. The animal used is usually the rat, and the P.A.A. is expressed in beats per min. Other workers have used cat [14] and dog [7] models, and have expressed P.A.A. as a percentage of the maximum value obtained with isoprenaline.

Lipophilicity is the relative affinity of a compound for a non-aqueous phase compared with water. The non-aqueous phase normally used is *n*-octanol, and lipophilicity is expressed as the logarithm of the partition coefficient, *P*. The lipophilicity of β-blockers has implications for the degree of metabolism, the distribution of the drug throughout the body, and the local anaesthetic activity or, as it is also known, membrane-stabilizing activity (m.s.a.). A direct relationship between lipophilicity and m.s.a. has been reported [15]. M.s.a. is not relevant clinically, since it is observed only at doses far in excess of those needed for β-blockade.

STRUCTURE-ACTIVITY RELATIONSHIPS (S.A.R.)

INTRODUCTION

With a few notable exceptions, β-blockers which have reached clinical evaluation belong to the 'aryloxypropanolamine' series depicted in structure (1). A



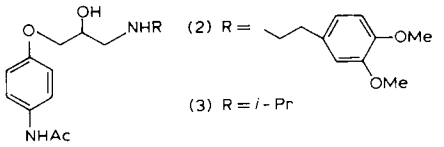
(1)

definitive review of the chemical features required for β-blocking activity in this series has appeared [16]. The general requirements for activity are as follows:
(i) The oxypropanolamine side-chain. Substitution in the α, β or γ positions or of the hydroxy or secondary amino groups causes a marked reduction in activity. Where β-blockers have been resolved, the β-blocking activity has resided with the *S*-enantiomer. This has the same absolute configuration as the naturally occurring agonists, noradrenaline and adrenaline, which as a consequence of the priorities of the Cahn-Ingold-Prelog rules have the *R*-configuration.
(ii) An aromatic ring which may be benzenoid, bicyclic aromatic, heterocyclic or benzoheterocyclic. The most detailed S.A.R. has been studied in the benzenoid series, where the nature and position of the substituent has a dramatic effect on the pharmacological profile. The *ortho* position has a considerable degree of steric freedom. There are greater constraints on the size and nature of groups which can be accommodated in the *para* position; many substituents markedly reduce activity, but as a rule amidic functions (in the broadest sense) and groups capable of accepting hydrogen bonds confer cardioselectivity. *Ortho*-substituted β-blockers are usually more potent than their *meta*- or *para*-substituted analogues.
(iii) An amine substituent which is a branched chain alkyl group. The isopropyl and *tertiary*-butyl groups have been found to confer optimum potency.

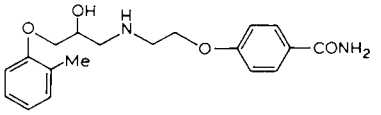
CARDIOSELECTIVE β-BLOCKERS

One of the more interesting aspects of the medicinal chemistry of β-blockers during the period under review has been the emergence of alternative amine substituents to the branched chain alkylamines. The 3,4-dimethoxyphenylethylamine group was shown [17] to confer cardioselectivity on a series of β-blockers, even improving selectivity of known, cardioselective compounds such as practolol. For example, in an *in vitro* test system, (2) was 22-times more β_1-selective than practolol (3).

Using a competitive binding assay in rat lung and ventricle membranes with (−)-[^{3}H]dihydroalprenolol as ligand, (2) was only twice as cardioselective as practolol [11], which highlights some of the inherent problems of making comparisons using two different test systems. Furthermore, these authors concluded that the amine component played only a minor rôle in determining

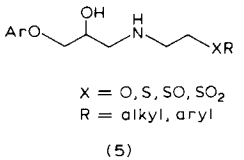


cardioselectivity. More recent findings with β-blockers containing the 3,4-dimethoxyphenylethylamino group and other amine substituents do not support this. A number of β-blockers incorporating the 3,4-dimethoxyphenylethylamino substituent are currently undergoing clinical trials, e.g., crinolol, bevantolol and bometolol (see *Table 3.2*, p. 139). In 1973, the cardioselectivity of a class of 4-amide-substituted phenoxyethylamino-substituted β-blockers was described [18], one of which, tolamolol (4), was studied extensively but withdrawn because of the development of mammary tumours in animals.



(4)

ICI workers discovered later that the 4-amidic function of the tolamolol series was not essential for cardioselectivity, and aryloxyethylamino- and even alkoxyethylamino-substituted β-blockers were potent and cardioselective [19], as were the corresponding aryl- and alkylthioethylamine analogues [20] (5). It was hypothesized that the cardioselectivity was the result of an interaction between the oxygen or sulphur atom and a complementary site on the β-receptor.



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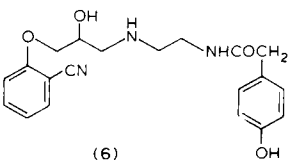
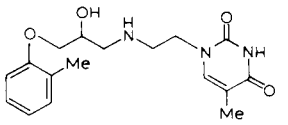


(6)

Replacement of the heteroatom, X, in (5) by a series of amidic substituents, e.g., -NHCO-, -$NHSO_2$-, -NHCONH-, furnished potent cardioselective β-blockers [21,22]. A feature of this series of compounds is that, although potency varies widely according to the nature of the R group, most of the compounds are cardioselective. The optimum chain length between the amino and the amidic nitrogen atoms was two carbon units; branching of the chain α to the amine nitrogen atom improved potency but reduced the degree of cardioselectivity, whereas substitution α to the amidic nitrogen atom reduced

potency. In general, an *ortho* substituent in the aryloxy ring had little effect on potency but caused a small reduction in cardioselectivity. A member of this series, epanolol (ICI 141292) (6), a cardioselective β-blocker with partial agonist activity, is undergoing clinical evaluation.

Primidolol (7) and imidolol (8) are two structurally related compounds in which the acylamino portion is contained in a heterocyclic ring. Primidolol is a cardioselective β-blocker with no partial agonist activity, but it has some α-blocking activity. It was withdrawn from the clinic because of the discovery of a higher-than-normal incidence of tumours in mice during toxicological evaluation [23,24]. Imidolol is reported to be a β-blocker with α-blocking activity [25].



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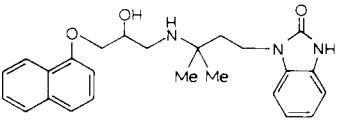
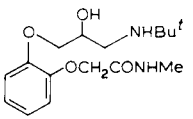


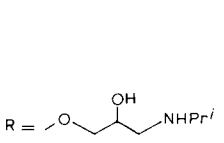
(8)

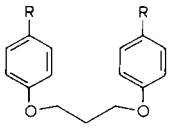
Many of the cardioselective β-blockers which have appeared in the past few years bear a close structural resemblance to established cardioselective agents. Thus metoprolol is the progenitor of H 87/07, betaxolol, bisoprolol and ciclo-prolol, while pafenolol and pamatolol are related to atenolol. Although there are many examples where *para*-amidic functions confer cardioselectivity on β-blockers, cetamolol (9) [26] is virtually the only example of a cardioselective compound bearing an *ortho*-amidic group.



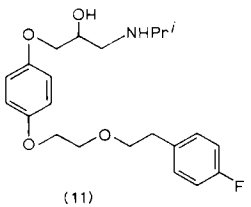
(9)

Modification of the aromatic ring substituents in β-blockers which retain the traditional isopropyl- or *t*-butylamino substituent has furnished selective β-blockers. Roche workers discovered that joining two β-blocker units together via oxyalkylene links through the 4,4′ positions resulted in β_1-selective compounds on the basis of *in vitro* results. The best of these, (10), had a short duration of action on intravenous dosing in the conscious cat, but was non-selective on oral dosing [4]. Taking this as a lead, it was discovered that replacement of one of the oxypropanolamine side-chains with simple non-interactive substituents, e.g., hydrogen, halogen or alkyl, resulted in even more potent β_1-selective blockers such as (11) (flusoxalol), with a cardioselectivity



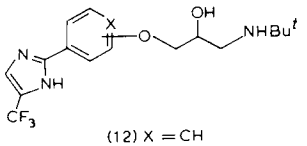


(10)

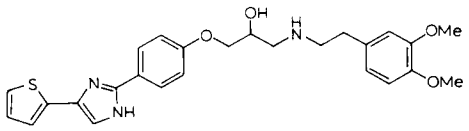


(11)

ratio of greater than 1000:1 being recorded *in vivo* in the rat [27]. These compounds had an 'acceptable' level of partial agonist activity but, surprisingly, no duration of action data were reported. Formally, these compounds fall within the metoprolol structure-activity patterns. In a more recent publication, the successful replacement of the pendant aryloxy group with heterocyclic moieties has been described [28]. In a search for a vasodilator-β-blocker, Merck workers prepared (12) and (13) (see later), which were found to be cardioselective. Substituting the 3,4-dimethoxyphenylethylamino group for *t*-butylamino, they prepared (14), which they describe as being 'virtually absolutely β_1-specific' on the basis of *in vitro* data [29]. The cardioselectivity ratio for (14) was over 10,000:1 and, for comparison, atenolol had a cardioselectivity ratio of 49:1 in the same test.

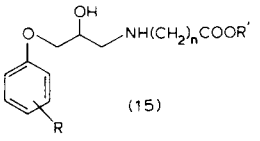


(12) X = CH
(13) X = N

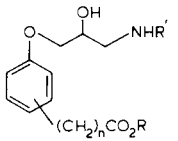


(14)

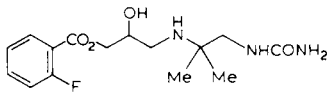
The use of an ultra-short-acting β-blocker in patients with ischaemic heart disease, where prolonged exposure to β-blockade could remove essential sympathetic support, leading to heart failure, is a novel concept. Introduction into a β-blocker molecule of substituents which would be readily metabolized to functional groups known to give inactive β-blockers was seen as a means of providing controlled and titratable therapy for post-myocardial-infarction patients.



(15)



(16) n = O
(17) n = 2, R = Me, R' = Pr^i, (para subst.)



(18)

The ester group was chosen as the rapidly metabolized moiety, and incorporation into the nitrogen substituent of a β-blocker via a spacer arm produced a short-acting β-blocker (15). However, following extended periods of infusion, the duration of action of this compound increased [30]. Compounds of structure (16), in which the ester function is attached directly to the aryloxy ring, also showed extended duration of action and poor cardioselectivity. Moderately potent cardioselective short-acting β-blockers were obtained by separating the ester from the aromatic ring by a two-carbon chain. Esmolol (17) was chosen for development [31] and, following a 3 h infusion, isoprenaline responses were restored to normal (i.e., no β-blockade) within 20 min; propranolol, however, was still fully effective 60 min after cessation of the infusion.

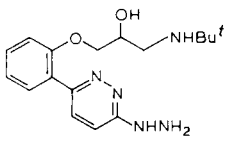
In a more recent approach, the ester group has been incorporated into the β-blocker as a linking group between the propanolamine side-chain and the aromatic nucleus. Many of the analogues prepared exhibited good potency and an acceptable duration of action. The *N*-ureidoalkyl analogue, ACC-9089 (18), was as potent as propranolol, had a duration of action of 21 min in the dog, and is undergoing clinical study [32]. It is interesting that a compound of this structure retains β-blockade.

β-BLOCKERS WITH DUAL ACTIVITIES

One of the major indications for β-blockers is in the treatment of hypertension. The effects of a β-blocker are additive or complementary to those of other common antihypertensive drug therapies such as diuretics and vasodilators where, for example, the reflex tachycardia associated with vasodilator therapy can be abolished by concomitant administration of a β-blocker. Most commercially available β-blockers are now marketed in combination with diuretics,

vasodilators or calcium-channel blockers. There has been a trend to produce single chemical entities incorporating β-blocking activity with another known antihypertensive activity. A perceived advantage of this dual-activity molecule is that absorption, metabolism and excretion would be at a single rate, thus optimizing the chances of a balanced profile during the course of action of the drug. Patient compliance, too, might be improved.

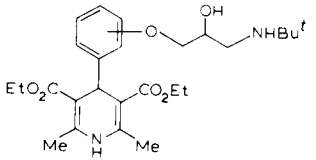
Two types of combination molecule can be distinguished: (i) those which clearly arise from the insertion of an 'oxypropanolamine' chain into an active pharmacophore; and (ii) β-blockers which have an additional pharmacological activity as an inherent property of the molecule. Introduction of a known vasodilator moiety into the *ortho* position of a β-blocker, which is known to be sterically undemanding, has given the β-blocker-vasodilator prizidolol, (19) [33]. Prizidolol, which is as potent as propranolol as a β-blocker, has



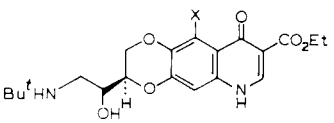
(19)

partial agonist activity, is not cardioselective, has arteriolar vasodilating activity, and lowers blood pressure in hypertensive patients. It was withdrawn from phase II clinical trials because of side-effects which included pigmentation and disorders of bone development [34]. Similarly, an 'oxypropanolamine' chain was introduced into the calcium slow-channel blocker-vasodilator, nifedipine. The 2- and 4-substituted analogues (20) and (21) were not β-blockers (measured in the dog) and exhibited only little antihypertensive activity in the spontaneously hypertensive rat. Insertion of a 2-methyl substituent in (21) greatly improved the antihypertensive activity, but the compound was not a vasodilator or a β-blocker and the mechanism of its antihypertensive activity is unknown [35]. This illustrates that introduction of an 'oxypropanolamine' side-chain into an active molecule does not necessarily guarantee β-blocking activity or retention of the original biological activity. This is highlighted, too, in the unsuccessful attempt to obtain β-blocker-diuretic combination molecules using quincarbate as the diuretic entity. In this case, the target molecules (22) and (23) exhibited neither diuretic nor β-blocking activity [36].

Perhaps the best known example of the successful combination of another pharmacophore with a β-blocker is the α,β-blocker, labetalol (24). Labetalol is marketed for the treatment of hypertension and is the subject of many reviews [37,38]. In man, labetalol is more potent at inhibiting β-receptors than α-recep-

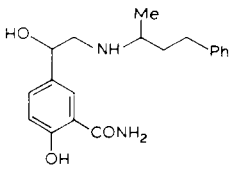


(20) 2-subst
(21) 4-subst

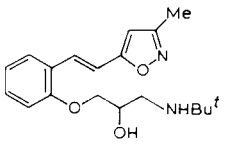


(22) X = H
(23) X = Cl

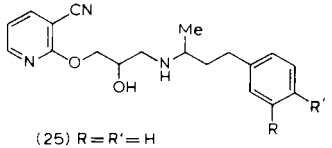
tors, the relative ratio being approx. 3 : 1. Labetalol, an α,β-blocker of the ethanolamine series, possesses two asymmetric centres and is a 1 : 1 mixture of two racemates. The four enantiomers of labetalol have been prepared independently by two groups [39,40], who found that the *R,R* enantiomer possessed most of the β-blocking activity, while the *S,R* enantiomer had most of the α-blocking activity. The *R,R* enantiomer (Sch 19927) is currently the subject of clinical trials and its antihypertensive activity is ascribed to its β-blocking activity and a β-adrenergic-mediated vasodilation (β_2-stimulant activity). Closely related ethanolamines for which α,β-blocking activity is described include medroxalol [41] and sulphonamide analogues of labetalol [42].



(24)



(27)

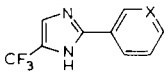


(25) R = R' = H
(26) R,R' = OCH_2O

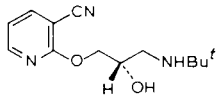
In an attempt to introduce α-blockade into an established, acutely active hypotensive β-blocker, the *t*-butylamino substituent of MK-761 was replaced by those arylalkylamino groups which had proved so effective in the labetalol series. Compounds (25) and (26) were equiactive with labetalol as α-blockers but were considerably more potent as β-blockers – which is to be expected for aryloxypropanolamines [43].

Isoxaprolol (27) was 16-times more potent than labetalol as a β-blocker and

4-times more potent as an α-blocker, and acutely reduced blood pressure in normotensive and spontaneously hypertensive rats [44]. Other β-blockers which have α-blockade as an ancillary property include primidolol, arotinolol and YM 09538.



(28) X = CH
(29) X = N

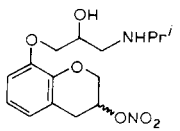


(30)

Merck workers sought to prepare β-blocker-vasodilators by introducing oxypropanolamine side-chains into their known vasodilators (28) and (29). When the side-chain was introduced *ortho* to the imidazole ring (2-position for the pyridine), the compounds exhibited modest β-blockade but the vasodilator activity was much reduced. The corresponding *para*-substituted compounds (6-position for the pyridine) were cardioselective β-blockers with vasodilator activity, but this was predominantly attributable to undesirable β_2-agonist activity [45]. The acidic imidazole proton was considered to be responsible for this β_2-agonist activity and a series of structural modifications were tried out in an attempt to eliminate this property [46].

This work culminated in the replacement of the imidazole moiety of 6-oxypropanolamine-substituted pyridines by electron-withdrawing groups such as -CN or CF_3 [47]. The best compound of the series, MK-761 (30), was equipotent with timolol as a β-blocker and 3.8-times more potent than hydralazine as a vasodilator, and was not a β_2-agonist. Unlike the other combination-molecules discussed, MK-761 is not clearly identifiable as a pharmacophore plus a β-blocker side-chain; the vasodilation is an intrinsic property of the whole molecule. Acute studies in man with MK-761 were terminated following the observation of teratogenicity in rabbits on chronic administration at high doses [47].

Of the other heterocyclic aromatic substituents investigated, only the 4-cyanothiazole and 4-cyanoisothiazole derivatives exhibited activity comparable with that of MK-761 [48].

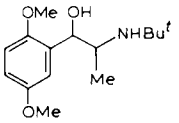


(31)

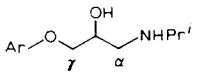
Although it is not formally described as a combination β-blocker-nitrate molecule, K 351 (31) clearly owes its vasodilator activity to the presence of the nitrate ester. K 351 is a non-selective β-blocker with no P.A.A.; it has some α-blocking activity and vasodilator activity which resembles that of nitroglycerin [49]. It is orally effective and has a long-lasting antihypertensive activity in the spontaneously hypertensive rat with no reflex tachycardia. Removal of the nitrate group results in a compound devoid of α-blocking and vasodilator activity, with much reduced β-blocking activity, and is ineffective in reducing blood pressure in the spontaneously hypertensive rat or dog.

β_2-SELECTIVE BLOCKERS

Prior to the proposal in 1967 [50] that the β-receptor could be subdivided into β_1- and β_2-subtypes, there was evidence that certain α-methyl-substituted analogues of dichloroisoprenaline [51] and butoxamine (32) [52] were capable of blocking vascular β-receptors at doses which had no effect on the positive inotropic or chronotropic actions of isoprenaline. Butoxamine, the best of these, was not particularly potent or specific in its pharmacological actions. It was a generally accepted dictum in the ethanolamine series of β-agonists and antagonists that α-methyl substitution conferred a degree of β_2-selectivity. In the aryloxypropanolamine series (33), however, this situation was not as clear-cut. There have been conflicting reports on the β_2-selectivity of the *threo* isomer of α-methylpropranolol [53,54]. It is now accepted that this is as β_2-selective as propranolol itself but is less potent; it is also less potent and selective than the corresponding *erythro* isomer [55].



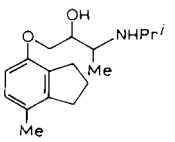
(32)



(33)

In a series of α-methyl-substituted aryloxypropanolamines (33) (mainly *threo* isomers), introduction of the α-methyl group alone did not confer β_2-selectivity and, in fact, among the analogues studied were to be found examples of cardioselective, non-selective and β_2-selective blockers, all of which were less potent than the parent unsubstituted compounds [56]. A recent publication on the pharmacology of α- and γ-methyl-substituted aryloxypropanolamines concluded that the γ-methyl analogues may show a shift towards β_2-selectivity, whereas none of the α-methyl analogues was active [57]. This work is at variance with results previously reported for α- and γ-methyl-substituted

aryloxypropanolamines [56,58,59]. An extension of the work [56] led to the potent β_2-selective blocker, ICI 118551 (34). A β_2-selectivity ratio of 123 : 1 has been reported from *in vitro* studies and greater than 250 : 1 *in vivo* [60]. ICI 118551 has no partial agonist activity; it has the same degree of membrane-stabilizing activity as propranolol and is currently undergoing clinical evaluation for potential use in migraine and essential tremor. The β_2-selectivity has been confirmed in man [61]. ICI 118551 is more β_2-selective than its *threo* isomer and a general stereoselective synthesis of both isomer types has been reported [62].



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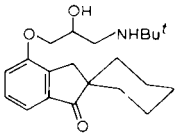

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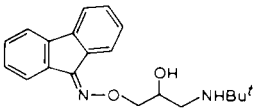

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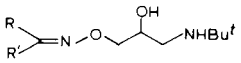
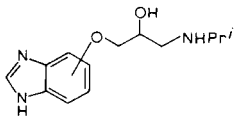
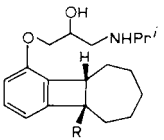


(37) R = Me, R' = Me
(38) R = Me, R' = CH_2CH_2Ph



(39) 4 – subst.
(40) 2 – subst.



(41) R = H
(42) R = OH

Spirendolol (LI 32468) (35) is another β_2-selective blocker in clinical trial. It is effective in controlling essential tremor at doses which have no effect on heart rate [63]. No pharmacological studies or S.A.R. on this compound have appeared to date.

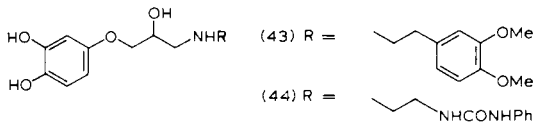
A novel series of oxime ethers also exhibit β_2-blocking activity, and the best of these, IPS 339 (36), had a β_2-selectivity ratio of 155 : 1 *in vitro* and 23 : 1 *in vivo* [64]. Other workers have been unable to confirm this result, finding only modest β_2-selectivity [65,66]. The enantiomers of IPS 339 have been synthesized using chiral 3-carbon precursors derived from D-mannitol [67]. Unlike the findings with other pairs of enantiomers of β-blockers, both *R*- and

S-enantiomers of IPS 339 were equiactive. An explanation proposed for this observation was that, as a consequence of the pseudosymmetry introduced by the oxime function, the aromatic ring, hydroxyl group and amine nitrogen atom of both enantiomers could occupy the same positions in space [66]. The dominant rôle of the aryl ring in these oxime ethers has been called into question with the finding that the alkyl oxime ether (37) has modest β_2-selectivity, while the analogue (38) tends towards β_1-selectivity [68].

Preliminary studies on the two benzimidazole analogues (39) and (40) indicate that (39) has a β_2-selectivity ratio of 17 : 1, whereas (40) is essentially non-selective [69]. Good β_2-selectivity has recently been reported for the tricyclic compounds (41) and (42) [70].

PARTIAL AGONISTS

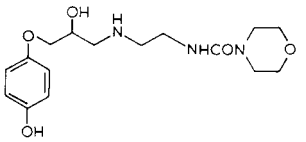
The retention of the catechol moiety in the arylethanolamine and aryloxypropanolamine series gives powerful agonists with an efficacy as high as that of isoprenaline. The main difference between the two series is a tendency towards β_1-selectivity with aryloxypropanolamines [71]. Introduction of amino substituents such as homoveratryl (43) or acylaminoalkyl (44), which are known to confer β_1-selectivity to β-blockers, has given β_1-selective full agonists [72,73].



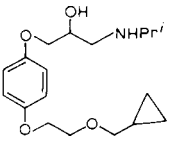
Removal of one of the hydroxyl groups in the ring gives potent partial agonists. Prenalterol, the *p*-hydroxy analogue of isoprenaline, has been studied widely in the clinic as a cardiotonic both in volunteers [74] and in patients with heart failure [75]. The drug is effective in the treatment of chronic heart failure [76], but tachyphylaxis occurs during prolonged treatment and for this reason prenalterol has been withdrawn from the clinic. The pharmacology, pharmacokinetics and clinical studies of prenalterol are summarized in a comprehensive review [77].

A series of compounds based on acylaminoalkyl-substituted aryloxypropanolamines has been described by ICI workers [73]. The acylamino group was shown to control both the degree of agonism and the cardioselectivity in these compounds, with small changes in structure producing profound biological effects, as shown in *Table 3.1*. The effect of changing from di- to trisubstituted ureas is particularly noteworthy; in the latter case, no β_2-stimulant

activity was detected. From this group of compounds, xamoterol (ICI 118587) (45) was selected and is undergoing clinical trials in heart failure patients [7,78]. Although some patients have received xamoterol for more than 1 year there is no evidence of tachyphylaxis, possibly because the greater β_1-selectivity of xamoterol prevents the reflex release of catecholamines found with prenalterol. As a consequence of their partial agonist properties, prenalterol and xamoterol act as β-stimulants in patients when sympathetic tone is low (at rest, asleep), and as β-blockers when the patient is under mental or physical stress. Patients are therefore stabilized at a sympathetic stimulation level controlled by the degree of agonism of the drug used [7].



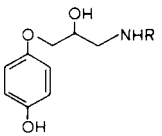
(45)

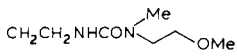
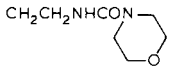


(46)

Recently, cicloprolol (SL 75.177.10) (46) has been examined as a cardioselective partial agonist. It has been shown to be qualitatively similar to xamoterol, but quantitatively it exhibits a lesser degree of agonism [79].

Table 3.1. AGONISM AND SELECTIVITY OF A SERIES OF ACYLAMINOALKYLAMINES



R	*Agonism (isopren = 100%)*	*β_1/β_2 selectivity*
i-Pr(prenalterol)	56	0.9
$CH_2CH_2NHCONH_2$	92	1.7
$CH_2CH_2NHSO_2NHPh$	92	3.0
$CH_2CH_2NHCONHCH_2Ph$	80	4.1
$CH_2CH_2NHCONHPh$	52	2.8
$CH_2CH_2NHCOCH_2Ph$	40	3.3
$CH_2CH_2NHCONMe_2$	29	> 40
	27	> 250
	43	> 250

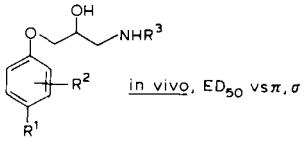
QUANTITATIVE ASPECTS OF S.A.R.

A number of different methods have been used to describe and define the relationships between chemical structure and various descriptors of biological activity. In the case of β-blockers, the biological parameters used may be potency (*in vivo*, *in vitro*), selectivity, partial agonism and nonspecific or membrane activity. The Hansch approach of multivariable regression analysis has been the most widely used, and this employs an extension of the Hammett equation as shown:

$$\log[\text{activity}] = \alpha + \beta\pi + \gamma\pi^2 + \rho\sigma$$

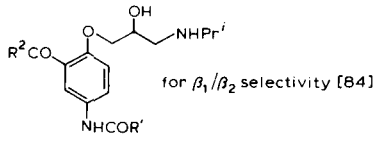
where α, β, γ, ρ are constants, π is an additive parameter representing the difference between the (log) partition coefficients of a substituted vs. unsubstituted molecule [80] and σ is the Hammett value representing the electronic effect of the substituent [81]. These last two parameters have some importance regarding drug distribution and binding, and detailed lists of published values are available [82].

Using the Hansch method, many successful analyses have been carried out *within closely defined series*. For example, the three series below (47–49) exhibited good correlations between experimental and calculated results within a series, but data from the different, though closely related, series did *not* conform to the same regression equation [83].

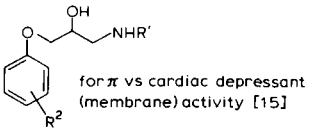


(47) R = $NHCOR^4$
(48) R = $NHCONHR^4$
(49) R = $CONHR^4$

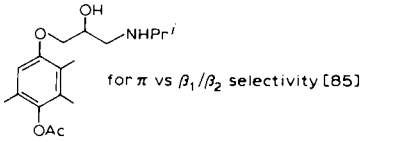
Other successful examples of the Hansch approach are (50–52), while this method has proved unsuccessful for the following series (53,54):



(50)

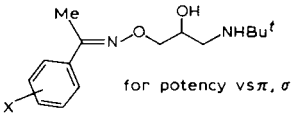


(51)



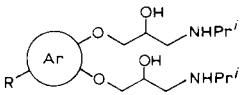
for π vs β_1/β_2 selectivity [85]

(52)



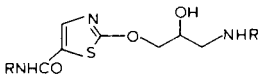
for potency vs π, σ, F, Es, parachor [64]

(53)

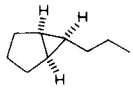


(54) for potency vs π, Es, R', σ [86]

Free-Wilson [87] analysis of the ureido series previously described (48) agrees with the results from the multi-regression approach [83].



(55)

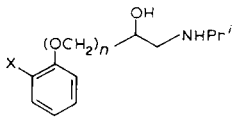


(56)

In a series of thiazole β-blockers (55), where π was shown to be unimportant, attempts were made to quantify the effects of structural changes in the side-chain R [88]. Assignment of numerical values to the presence or absence of various structural features finally yielded an optimum compound with R = (56). It has been suggested that the equations describe the 'molecular fit' of the side-chain to the receptor.

It is well known that '*ortho*' substitution in aryloxypropanolamine β-blockers (57) tends to reduce the degree of partial agonism. Two groups have attempted to quantify this effect. In the first approach [89], agonism was related to the amount of a (minor) *gauche* isomer of the side-chain, and this correlated well in the series studied, but did not extend to other (e.g. arylethanolamine) series. In the second approach [90], the degree of agonism was related to the bulk of the *ortho* substituent as expressed by Taft's steric factor [91]. This method was extended to several other series, including arylethanolamines (58) [90].

The field of QSAR in β-blockers has been reviewed [92].

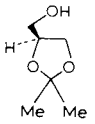


(57) $n = 1$
(58) $n = 0$

Table 3.2 contains an alphabetical list of β-blockers for which clinical or pharmacological data have been published. Compounds which are described only in patents are not included. The origin of the compound, its chemical formula and, where possible, its potency in relation to propranolol (= 1) are given; selectivity and partial agonism are indicated qualitatively and lipophilcity data are included where possible, data being taken either from Ref. 93 or ICI internal data using the same methods as in this reference. Aromatic ring structures are shown in the table with the nature of the side-chain abbreviated to a single letter. The side-chains are drawn in full at the end of the table. Formulae which appear elsewhere in the text are shown by their numbers, e.g., (9), which appears on p. 126.

CHEMISTRY

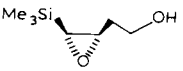
The basic method of synthesis of β-blockers of the aryloxypropanolamine series has changed little from that described in 1969 for the synthesis of propranolol analogues [154]. The major advance in recent years has been the development of a synthetic route to optically active β-blockers using (*S*)- and (*R*)-epichlorohydrin (60,61) obtained from a common, chiral three-carbon unit, (*S*)-glycerol acetonide (59) [155]. The *R*-enantiomers of the aryloxypropanolamines can be prepared from (*S*)-epichlorohydrin by base-promoted reaction with a phenol, followed by reaction of the resulting (*R*)-epoxide with the appropriate amine. However, it was not necessary to prepare (*R*)-epichlorohydrin, since the triflate of the (*R*)-epoxyalcohol (62) reacted with the sodium salt of phenols by direct S_N2 displacement of the triflate group to yield the (*S*)-epoxides (63) [156].



(59)

(60) R = Cl (S)
(62) R = OH (R)
(63) R = OAr(S)

(61)



(64)

In another elegant approach, (*E*)-3-trimethylsilylallyl alcohol was oxidized under Sharpless's conditions to the (2*S*,3*S*)-epoxyalcohol (64), the tosylate of which was reacted with 1-naphthol. The resulting trimethylsilyl epoxide was selectively desilylated using tetra-*n*-butylammonium fluoride to the (*S*)-epoxide, which was converted to (*S*)-propranolol in 79% yield by standard methods [157].

Table 3.2. STRUCTURE AND PROPERTIES OF β-BLOCKERS

The structures of the side-chains bearing the letters (a) to (s) are to be found at the end of the table.

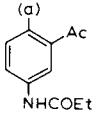
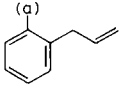
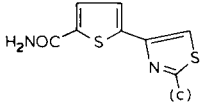
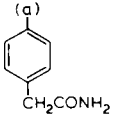
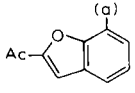
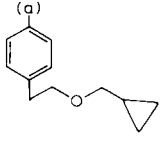
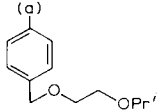
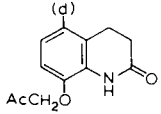
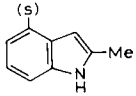
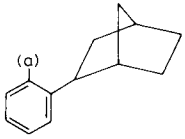
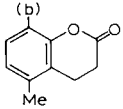
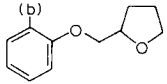
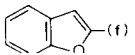
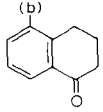
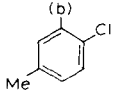
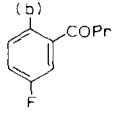
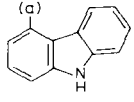
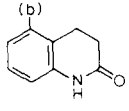
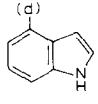
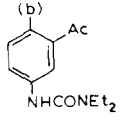
Name	*Structure*	*Potency*	*Selectivity*	*P.A.A.*	*log* P	*Comment*	*Ref.*
acebutolol (May and Baker)		0.3	β_1	+	1.87		94
alprenolol (Hassle)		1	–	+	2.61		95
arotinolol (S 596) (Sumitomo)		5	–	–		α/β-blocker	96
atenolol (ICI/Stuart)		1	β_1	–	0.23		97
befunolol (BFE-60) (Kakenyaku-Boots)		4	–	–			98
betaxolol (SL 75212) (Synthelabo-Searle)		1	β_1	–			99
bevantolol (CI 775) (Warner-Lambert)		2	β_1	–			100

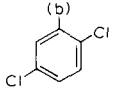
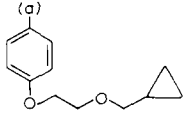
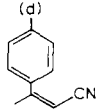
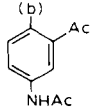
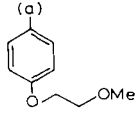
Table 3.2. continued

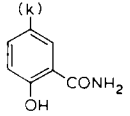
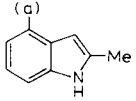
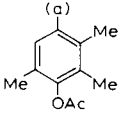
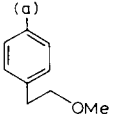
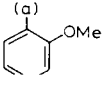
Name	*Structure*	*Potency*	*Selectivity*	*P.A.A.*	*log* P	*Comment*	*Ref.*
bisoprolol (EM-33512) (E. Merck)		10	β_1	–			101
bometolol (Otsuka)		1	β_1	–			102
bopindolol (Sandoz)		50	–	+		prodrug of mepindolol	103
bornaprolol (FM 24) (Pharmuka)		0.05	–	–			104
bucindolol (MJ 13105) (Mead Johnson-Bristol Meyers)		3	–	+		antihypertensive	105
bucumolol (CS 359) (Sankyo)		3	–	–			106
bufetolol (Y-6124) (Yoshitomi)		1	–	–			107
bufuralol (Roche)		1	–	+			108

bunitrolol (Kö 1366) (Boehringer-Ingelheim)		3	–	+	2.25	109
bunolol (Merrell-Warner Lambert)		40	–	–		110
bupranolol (KL 255) (Sanol-Kakenyaku)		0.5	–	–		111
butofilolol (Labaz-Clin. Midy)		1	–	–		112
carazolol (Boehringer-Mannheim)		100	–	–		111
carteolol (Abbott/Otsuka/Reckitt/Berk)		10	–	+		113
carvedilol (BM 14.190) (Boehringer-Mannheim)		4	–	–	vasodilator-β-blocker	114
celiprolol (Chemie Linz-Revlon)		0.5	β_1	+		113

(continued)

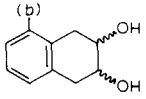
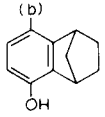
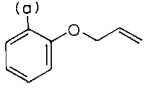
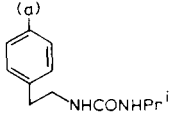
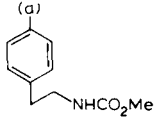
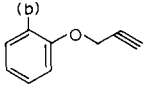
Table 3.2. continued

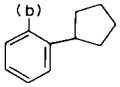
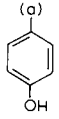
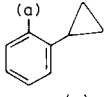
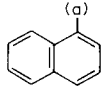
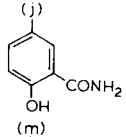
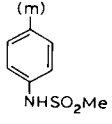
Name	*Structure*	*Potency*	*Selectivity*	*P.A.A.*	*log* P	*Comment*	*Ref.*
cetamolol (Ayerst)	(9)	4	β_1	+	1.61		115
chlorpranolol (GYK 1.41099) (Richter)		3–8	–	–			116
cicloprolol (Synthelabo)		–	–	–		part. agonist	79
crinolol (Hoe 224) (Hoechst)		?	β_1	–			117
diacetolol (EU 4891) (May and Baker)		0.3	β_1	+			118
epanolol (ICI 141292)	(6)	4	β_1	+	0.87		119
esmolol (ASL 8052) (Am. Crit. Care)	(17)	0.025	β_1	+		short-acting	31
flusoxalol (Ro 31-1115) (Roche)	(11)	30	β_1	+		withdrawn	27
H 87/07 (Hassle)		0.3	β_1	+	1.23		120

ICI 118551	(34)	–	β_2	–	3.56	β_2-blocker	60
indenolol (YB-2) (Yamanouchi-Schering Plough)		1	–	+			121
IPS 339 (Synthelabo)	(36)	5	β_2	–			122
isoxaprolol (BASF)	(27)	5	–	–		α/β-blocker	44
labetalol (Glaxo-Allenbury)	(24)	0.3	–	–		α/β-blocker	37
medroxalol (Richardson-Merrell)		0.1	–	–		α/β-blocker	123
mepindolol (LF 17895) (Schering/Sandoz)		100	–	+			124
metipranolol (Boehringer/Spofa) (Mannheim)		2	–	–			125
metroprolol (H 93/26) (Hassle)		1	β_1	–	2.15		120
MK-761 (Merck)	(30)	30	–	+			126
moprolol (Simes)		2	–	–			127

(continued)

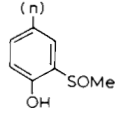
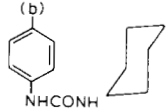
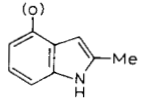
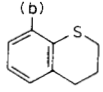
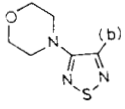
Table 3.2. continued

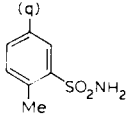
Name	*Structure*	*Potency*	*Selectivity*	*P.A.A.*	*log* P	*Comment*	*Ref.*
nadolol (Squibb)		1	–	–	0.71		128
nafetolol (K 5407) (Carbo Erba)		4	–	–			129
nipradolol (K 351) (Kowa)	(31)	2	–	–		α_2/β-blocker nitrate comb.	49
oxprenolol (Ciba-Geigy)		1	–	+	2.18		130
pafenolol (Hassle)		1	+	–			131
pamatolol (H 104/08) (Astra-Hassle)		1	β_1	–		withdrawn	132
pargolol (Kö 1400) (RU 21824) (Boehringer-Ingelheim-Thomae)		3	–	+			133

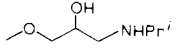
penbutolol (Hoe 893d) (Hoechst)		4	–	–			134
pindolol (Sandoz)		6	–	+	1.75		135
practolol (ICI)	(3)	0.3	+	+	0.79	withdrawn	136
prenalterol (Hassle)		–	–	+	1.1	partial agonist: (–)-isomer, withdrawn	137
primidolol (UK 11443) (Pfizer)	(7)	?	+	–		vasodilator β-blocker, withdrawn	138
prizidolol (S.K.F.)	(19)	1	–	–		withdrawn	33
procinolol (S.I.F.A./Diamant)		~10	–	–			139
propranolol (ICI)		1	–	–	3.65		140
SCH 19927 (Schering)		1	–	–		α/β-blocker	141
sotalol (Mead Johnson)		0.05	–	–	–0.79		142

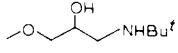
(continued)

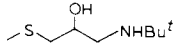
Table 3.2. continued

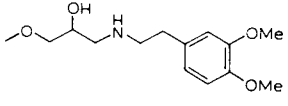
Name	*Structure*	*Potency*	*Selectivity*	*P.A.A.*	*log* P	*Comment*	*Ref.*
spirendolol (Sandoz)	(35)	?	β_2	?		β_2-blocker	63
Sulfinalol (WIN 40808-7) (Sterling-Winthrop)		?	+	+		vasodilator β-blocker, withdrawn	143
talinolol (V.E.B.)		0.15	β_1	+			144
tazolol (Syntex)		0.1	–	+		partial agonist	145
teoprolol (Chemiewerke Hamburg)		5	–	+		withdrawn	146
tertatolol (Servier)		0.1	β_2	–			147
timolol (Merck)		6	–	–	2.10	*S*-isomer	148
tiprenolol (DU 21445) (Philips Duphar)		3–6	–	+			149

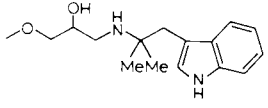
tolamolol (Pfizer)	(4)	1	+	–	2.21	withdrawn	150
toliprolol (Kö 592) (Boehringer)		0.5	–	–	2.82		151
YM 09538 (Yamanouchi/Lilly)		0.3	–	–		α/β-blocker	152
xamoterol (ICI 118587)	(45)	12	+	+	0.03	partial agonist	7
xibenolol (D-32) (Teikoku)		1	–	+			153

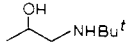
(a)= 

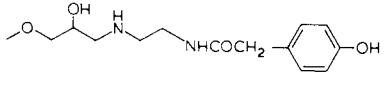
(b)= 

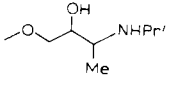
(c)= 

(d)= 

(e)= 

(f)= 

(g)= 

(h)= 

(continued)

(continuation, Table 3.2 structure codes)

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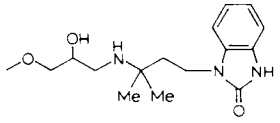

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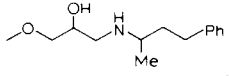

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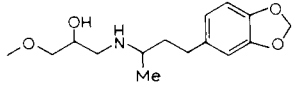

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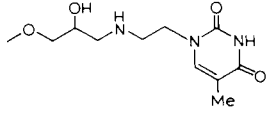

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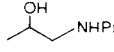

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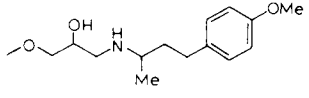

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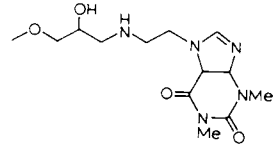

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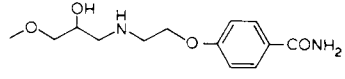

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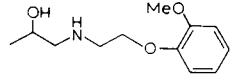

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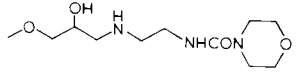

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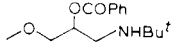


BIOCHEMISTRY OF THE β-RECEPTOR

β-Agonists exert their pharmacological actions by interacting with specific receptors located on the surface of the target cells. The agonist-receptor interaction leads to the activation of the enzyme adenylate cyclase which produces cyclic AMP (cAMP) from ATP located within the target cells. The increased levels of cAMP activate cAMP-dependent protein kinases that initiate the physiological response. Current evidence on the nature of the β-receptor and theories of the mechanisms by which β-agonist-receptor interactions activate adenylate cyclase are presented below. A number of detailed reviews have been addressed to this subject [158–162].

The basic components of the β-receptor-adenylate cyclase system are the β-receptor (R), a guanine nucleotide regulatory protein (N) and the catalytic unit of the enzyme adenylate cyclase (C). Each of these has been shown to reside on a separate macromolecule.

In recent years, much progress has been made in techniques for the purification and characterization of β-receptors, and these are the subject of detailed reviews [158,159,162]. The β-receptors from frog erythrocytes and from hamster and guinea-pig lung tissue have been purified to apparent homogeneity. These are of the β_2-subtype and the binding subunit resides on a single polypeptide of 58–64 kDa [163]. The β-receptor-binding subunit isolated from the turkey erythrocyte, which is of the β_1-subtype, is contained on two proteins, of 40 and 45 kDa, which are present in the ratio of 4 : 1 and each of which binds β-adrenergic ligands with identical β_1-specificity [164]. Evidence from photoaffinity-labelling studies indicates that for mammalian systems both β_1- and β_2-receptor binding sites reside on a single peptide of 60–65 kDa [162,163]. Each of the purified β_1- and β_2-receptor proteins binds β-adrenergic ligands with the appropriate specificity. To date, no amino acid sequencing studies of the binding sites of the individual β-receptors have been reported, neither have the structural differences between β_1- and β_2-receptors been ascertained.

The nucleotide regulatory protein (N) from a number of sources has been purified and shown to consist of two subunits, of 35 and 45 kDa. The 45 kDa protein contains the GTP-binding site [165,166]. The instability of the catalytic unit of adenylate cyclase (C) when separated from the nucleotide regulatory protein has precluded its purification.

Interaction of a β-agonist with its receptor results ultimately in the activation of membrane-bound adenylate cyclase. The β-antagonist presumably exerts its actions by denying the agonist access to the receptor. According to one theory, the agonist-receptor complex binds to the nucleotide regulatory protein to form

a ternary complex. On binding GTP, the ternary complex is destabilized and dissociates with the liberation of GDP to the agonist-receptor complex and an N · GTP unit which is presumed to be the active form of the N protein. The N · GTP unit binds to the catalytic site (C) of adenylate cyclase to form N · GTP · C, the active form of the enzyme. The activation is switched off by the cleavage of GTP to GDP by a GTPase possibly associated with the N protein. In this mechanism, the N protein acts as a shuttle which conveys information between the agonist-receptor complex and the enzyme [162].

$$H + R \rightleftharpoons HR$$
$$HR + N.GDP \rightleftharpoons HR.N.GDP$$
$$HR.N.GDP \longrightarrow HR + N.GTP$$
$$N.GTP + C \longrightarrow N.GTP.C$$
$$N.GTP.C \longrightarrow N.GDP + C + P_i$$

Scheme 3.1.

An alternative mechanism, based essentially on kinetics arguments, considers that the N-protein and catalytic unit of the adenylate cyclase C are permanently attached [158,161]. Activation of the enzyme requires the simultaneous binding of the agonist and GTP to their respective binding sites. The active form of the enzyme decays to the inactive form with concomitant hydrolysis of GTP to GDP at the N-protein unit. The function of the agonist is to catalyze the conversion of the enzyme from its inactive to its active form; it is not involved in catalyzing the release of GDP or the binding of GTP.

It has emerged from radioligand binding studies that there are high-affinity and low-affinity forms of the β-receptor [167]. β-Agonists bind to the high-affinity state only, while antagonists do not distinguish between high- and low-affinity states. β-Agonists can also stabilize the high-affinity form and the degree of stimulation of adenylate cyclase by an agonist correlates with the fraction of the total receptor population which is in the high-affinity state. Guanine nucleotides decrease the affinity of β-agonist binding to the β-receptor with no effect on antagonist binding. This effect is attributable to the apparent ability of guanine nucleotides to convert the high-affinity forms of the β-receptor into low-affinity forms.

Recently, β-receptor subunits isolated from amphibian and mammalian sources were purified to apparent homogeneity. These receptor proteins were incorporated in lipid vesicles and fused with receptor-deficient cells which contained the N and C units. The reconstituted cells exhibited β-adrenergic responsiveness of appropriate specificity. The results from these 'reconstitution' experiments indicated that the β-receptor polypeptides isolated contain both

the ligand-binding site and the site responsible for mediating stimulation of adenylate cyclase activity, presumably via interaction with the N protein [168].

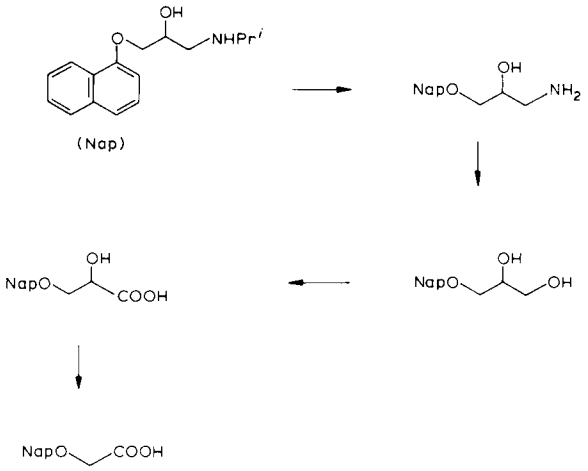
Desensitization describes the phenomenon whereby repeated exposure of β-receptors to β-agonists results in a decreased receptor responsiveness. It is thought that multiple mechanisms are involved in mediating catecholamine-induced desensitization of adenylate cyclase. Thus for the β-receptor system isolated from frog erythrocytes, desensitization occurs via agonist-induced internalization of the receptor, which may then be recycled or degraded [169]. A different mechanism was observed for the β-receptor isolated from the turkey erythrocyte [170]. In this case, the receptors remain on the cell surface, but on interaction with an agonist there is no activation of adenylate cyclase. Possible mechanisms accounting for this include defects in the activity of the N protein and structural alteration of the β-receptor, possibly involving phosphorylation by some as yet unexplained mechanism [162].

The classification of β-responsive tissues into β_1- and β_2-subtypes may be an oversimplification, since evidence has emerged in recent years that these tissues can contain mixed populations of β_1- and β_2-receptors. This evidence has come from both pharmacological [171] and biochemical studies [160,172,173]. Because different groups of workers have used different species, their conclusions differ quantitatively, but qualitatively their results agree. In atrial tissue, for example, the β_1-to-β_2 ratios are usually 3–4 : 1, while in ventricular tissue they are 10–20 : 1 and in trachea about 1 : 4. In intact animals, β_1-responses are usually mediated via noradrenaline, so the significance of β_2-receptors in predominantly β_1-tissues is not yet clear. The same applies to predominantly β_2-tissues, where adrenaline is the agonist.

METABOLISM AND PHARMACOKINETICS OF β-BLOCKERS

Two types of metabolism occur with beta-blockers; the first of these is common to all and involves oxidation of the oxypropanolamine side-chain, and the second is specific for each particular aromatic ring substitution pattern. Dealkylation of the side-chain may be followed by successive oxidations to give, as exemplified by propranolol, the corresponding aryloxyacetic acid (*Scheme 3.2*).

At the same time, metabolism of the aromatic ring may occur, and this is dependent on the exact nature of the substituent. Bourne [174] has reviewed the metabolism of a wide range of clinically available β-blockers and has also shown that the degree of metabolism is directly related to the lipophilicity of the compound. In general lipophilic compounds are metabolized extensively in



Scheme 3.2.

the liver, giving rise to the so-called 'first pass' effect, while hydrophilic compounds are excreted essentially unchanged.

Lipophilicity also affects absorption and distribution of these drugs; the pharmacodynamic aspects of this have been reviewed in some detail for the most widely used β-blockers [175].

CLINICAL APPLICATIONS OF β-BLOCKADE

β-Blocking drugs were developed initially for the treatment of ischaemic heart disease, particularly angina pectoris, and were soon shown to be effective. Other clinical applications became apparent over the next few years. Some of these were predictable, since the disease state was the direct consequence of overactivity of the sympathetic nervous system, for example, essential tremor and phaeochromocytoma. The effects of β-blockers on hypertension, glaucoma and migraine were surprising and cannot be explained adequately to this day. The clinical aspects of β-blockers have been reviewed extensively [176,177], and in this section progress in the most important areas is discussed.

ANGINA PECTORIS

All β-blockers provide effective treatment for angina pectoris. The relevance of subsidiary properties such as cardioselectivity, partial agonism and membrane-

stabilizing activity (m.s.a.) has caused much discussion [178,179], the arguments centring on both efficacy and safety. Cardioselectivity is seen to cause fewer problems due to blockade of β_2-mediated bronchodilation in asthmatic patients, though this added safety is by no means absolute.

The rôle of partial agonism is controversial. β-Blockers with P.A.A. have been claimed to be safer, the perceived advantages being that they might cause less myocardial depression and less likelihood of provoking bronchospasm than pure β-antagonists [180]. This is contested by other workers, who find no clinical difference between β-blockers with or without P.A.A. with regard to either safety or efficacy. These aspects have been reviewed [181] and the importance of P.A.A. in β-blockers is still the subject of active research.

Membrane-stabilizing activity, at one time implicated in myocardial depression, is now accepted as being clinically irrelevant at therapeutic doses.

HYPERTENSION

Since the initial unexpected finding that pronethanol reduced blood pressure in man [182], the treatment of hypertension has become the largest indication for β-blockers. All β-blockers reduce blood pressure in man to approximately the same extent, regardless of secondary properties such as β_1-selectivity or partial agonist activity, which indicates that β-blockade itself is the critical parameter. The secondary properties, however, can be important to the overall acceptability of the drug to the patient; as an example, β_1-selective compounds are less likely to precipitate bronchospasm, affect carbohydrate metabolism, renal function or cause CNS disturbances [93,183].

It is also reported that partial agonism plays no rôle unless it reaches levels higher than that of pindolol [184]. On the other hand, it was concluded from a retrospective study of 85 trials of different β-blockers in hypertension, that compounds with P.A.A. did not raise the total peripheral resistance on initial administration as much as β-blockers without P.A.A., and this could have implications for the long-term treatment of hypertension [185]. Overall, there is no clear clinical evidence for a rôle for P.A.A. in a β-blocker for the treatment of high blood pressure.

The mechanism by which β-blockers exert their antihypertensive actions is still the subject of much debate. The solution to this problem is hampered by the absence of any laboratory models which mirror the effects seen in man. Among the mechanisms which have been proposed are: (i) the reduction in cardiac output; (ii) the resetting of baroreceptors as a consequence of a reduction in the cardiac-mediated stress responses in everyday life; (iii) direct actions of β-blockers on central neurones responsible for the regulation of the

cardiovascular system; (iv) β-blocker-mediated suppression of plasma renin activity and aldosterone secretion; and (v) the presynaptic actions of β-blockers in antagonizing adrenaline-mediated transmitter release [186]. There is no overwhelming body of evidence supporting any of these mechanisms as the sole means by which β-blockers reduce blood pressure. It is probable that all of these mechanisms, and others as yet unidentified, contribute to the final hypotensive effect [187–190]. Typically, only 60% of hypertensive patients respond to β-blockers alone.

Current clinical practice uses β-blockers in combination with other antihypertensive therapies for the control of moderate to severe hypertension. Often the two agents act synergistically. In the case of β-blocker-diuretic combinations, the β-blocker ameliorates certain adverse effects associated with diuretic monotherapy such as having a potassium-sparing action and blocking the diuretic-induced renin release. Likewise β-blocker-vasodilator combinations have a more rapid onset of action and the β-blocker inhibits the reflex tachycardia produced by the vasodilator. Most commercial β-blockers are now available in fixed combinations with a diuretic, vasodilator or calcium channel blocker. A current research target is the combination of a β-blocker with another antihypertensive agent in a single molecule.

MYOCARDIAL INFARCTION

From middle age onwards, coronary heart disease is the single most common cause of death in the Western World. As early as 1965, there was a report [191] of a trend to lower mortality following myocardial infarction in patients treated with propranolol. Subsequent trials also demonstrated a trend towards decreased mortality, but conclusive proof was not forthcoming either because of flaws in the trial design or because of inadequate numbers of patients participating [192]. Retrospective analysis of the results from the practolol trial [193] showed a statistically significant reduction in non-fatal re-infarctions and a reduction in mortality in a subgroup of patients with anterior infarcts. However, the first definitive evidence of benefit came with the large-scale, long-term Norwegian Multicentre Study using timolol in over 3000 patients, starting treatment 1–4 weeks after myocardial infarction with a follow-up period of 17 months [194]. A highly significant reduction in total deaths and in sudden cardiac deaths was observed, regardless of the site of infarction. There was also a reduction in the rate of re-infarction.

The large-scale Beta Blocker Heart Attack Trial (BHAT) carried out in the U.S.A. using propranolol with a treatment regime similar to that of the Norwegian study showed similar results over a follow-up period of

2 years [195]. The results from these long-term trials have been reviewed [192,196–200] and also those from smaller trials carried out on alprenolol, oxprenolol, pindolol, practolol and sotalol [201]. The results from a number of trials in which β-blockers are administered acutely (i.e., within 72 h of the onset of infarction) are equivocal [201].

It would appear that among those β-blockers which are effective, the benefit is a consequence of β-blockade; however, at present there is no firm evidence that all β-blockers will be effective in the treatment of myocardial infarction. This has raised a problem for newer β-blockers, since it is felt that further studies comparing these drugs with placebo would be unethical [197].

The exact mechanisms by which β-blockers reduce cardiac death and the rate of re-infarction following myocardial infarction are not clear, but possible contributing factors are (a) a reduction in myocardial oxygen demand as a consequence of the prevention of tachycardia, elevation of blood pressure and increased contractility, (b) the anti-arrhythmic actions of β-blockers, and (c) possible beneficial effects on myocardial metabolism [196].

It is worth emphasizing that the patients who are treated with β-blockers are the survivors of myocardial infarction, since many of the deaths occur in the first few hours following infarction. Current views on the use of β-blockers in myocardial infarctions are that all those patients who can tolerate β-blockers should be treated with those β-blockers for which there is clear clinical evidence of utility. Treatment should start 5 days to 1 month after myocardial infarction and should continue for 2 years [192].

MIGRAINE

Chance clinical observations between 1966 and 1968 indicated that patients receiving propranolol for cardiovascular disease often obtained relief from concomitant migraine [202]. Since then, many controlled clinical trials have confirmed the efficacy of propranolol in the prophylaxis of migraine [203]. The variable clinical manifestations of this disease and the subjective end-point in defining therapeutic benefit have made the evaluation of drug-related effects difficult. However, it appears that atenolol, timolol, metoprolol and nadolol have a significant prophylactic effect in migraine, while acebutolol, alprenolol and oxprenolol are ineffective [203]. Equivocal findings are reported for pindolol [204,205]. Clearly, the fact that a compound is a cardiac β-blocker does not necessarily mean that it will have an antimigraine action and neither do the ancillary properties such as cardioselectivity or lipophilicity correlate with activity. However, those β-blockers with partial agonist activity seem to be less effective.

The therapeutic efficacy of β-blockers is in the prophylaxis of migraine; they do not alleviate the migraine attack on acute administration. Moreover, not all migraine sufferers respond to treatment with β-blockers, although recent findings indicate that the proportion of migraine patients benefiting from propranolol therapy increased as the duration of the therapy increased, reaching 84% after 1 year [206].

Neither the primary causes of migraine nor the mechanisms by which certain β-blockers exert their beneficial actions are understood. A number of mechanisms have been proposed and critically reviewed [207,208]: these include β-blockade, antagonism of 5-hydroxytryptamine, actions on platelet function, inhibition of thromboxane synthesis and increased β-receptor number and responsiveness following β-blockade. None of these mechanisms alone accounts for the efficacy of some β-blockers and the apparent ineffectiveness of others.

ANXIETY

The first observations of a possible therapeutic effect of β-blockers in anxiety states also arose by chance. Analysis of the results showed that it was not the psychological symptoms of anxiety which improved, but the somatic symptoms [209]. This can be understood to some extent, since the somatic symptoms, which include tachycardia, palpitations, tremor and sweating, arise through overactivity of the sympathetic nervous system. There is a vicious circle in some anxiety states where an awareness of the somatic symptoms serves to make the patient even more anxious. In this state, β-blockers are superior to placebo, although possibly inferior to benzodiazepines; however, in psychic anxiety states, benzodiazepines are superior to β-blockers. In one trial, a combination of propranolol with diazepam proved to be better than either agent alone [210]. In different forms of situational anxiety such as public speaking, car racing, examination stress and solo instrument playing, β-blockers decreased nervousness and did not impair performance [211]. This was found for non-selective β-blockers (propranolol and oxprenolol) and β_1-selective blockers (atenolol). It is not clear whether β-blockers exert their beneficial anxiolytic actions by blockade of central or peripheral β-receptors, but their efficacy in the treatment of somatic anxiety symptoms is well established.

Reports in 1970 [212] that high doses of propranolol were effective in psychotic disorders were followed by a number of largely uncontrolled trials in schizophrenic patients. The results were conflicting and current evidence does not support the use of β-blockers in this condition. A clear antipsychotic effect for β-blockers was found in patients who were also receiving neuroleptics [213]

and this has been confirmed in patients taking propranolol and chlorpromazine. The clinical improvement may have been due to the higher plasma levels of chlorpromazine and metabolites found on combined therapy [214].

TREMOR

Essential tremor is a condition of unknown aetiology. In many respects it resembles the physiological tremor produced by catecholamines. β-Blockers are effective in treating essential tremor, but it is not clear whether this is a consequence of blocking peripheral or central β_1- or β_2-receptors. The efficacy of non-selective β-blockers such as propranolol, oxprenolol, timolol and sotalol is undisputed. There is controversy regarding the β_1-selective blockers, with some workers reporting that atenolol and metoprolol are less effective than non-selective β-blockers, while other workers disagree. The evidence and arguments have been reviewed [215] and appear to favour β_2-receptors as the site of action. This is supported by the efficacy of two new β-blockers, ICI 118551 and spirendolol, in essential tremor at doses which do not elicit β_1-responses [63,216]. The relative importance of central or peripheral actions is unresolved.

GLAUCOMA

Since the initial discovery [217] that propranolol lowered intra-ocular pressure in glaucoma patients, many other β-blockers, both non-selective and cardio-selective, have been shown to share this property. The only β-blocker clinically available for topical application in glaucoma is timolol, and this is used widely. Timolol is well tolerated, but side-effects due to systemic β-blockade have been reported; these are due to absorption of the drug through the nasal mucosa. The subject of β-blockers in glaucoma has been reviewed [218].

SIDE-EFFECTS

The side-effects commonly observed with β-blockers have been reviewed [176,177,188].

The most serious of these are heart failure, usually occurring in patients whose hearts are maintained by a large degree of sympathetic drive, AV (atrio-ventricular) conduction defects and bronchospasm in susceptible individuals. Other commonly reported side-effects include sedation, depression, vivid dreams, cold extremities, reduced capacity to exercise, and bradycardia. There are reports of a β-blocker-withdrawal syndrome in which abrupt

withdrawal of therapy in patients with coronary heart disease may lead to a rebound exacerbation of symptoms, more severe than before therapy [188]. There have been some doubts about the existence of this syndrome, but it appears that it is a real phenomenon, although the incidence is low [219]. A suggested mechanism of action is an increased sensitivity to catecholamines consequent to a β-blocker-promoted increase in the number of β-receptors. The withdrawal syndrome has been reported for propranolol, alprenolol, atenolol and metoprolol and there are suggestions that β-blockers with P.A.A. do not exhibit this effect.

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4 Thalidomide and Congeners as Anti-inflammatory Agents

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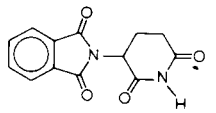
INTRODUCTION

For many people, the name thalidomide still evokes visions of crippled children born without limbs, eyes or ears after their mothers had taken the drug as a sleeping pill during their pregnancy. But now, more than two decades later, thalidomide is experiencing a renaissance as an experimental drug, with a surprising ability to cure a series of inflammatory skin and bowel diseases better than any other drug currently available.

The story of thalidomide is a tragic chapter in the generally progressive development of drug therapy in medicine. In its way, it was a turning point, an end of innocence. It marked the beginning of a new, greater sense of responsibility in clinical pharmacology, clinical investigation, and medicolegal relations [1]. Tragedy and triumph appear to be the two sides of the coin: in the brief period of 5 years, between 1956 and 1961, the apparently most innocent of drugs, as thalidomide was once regarded, became the drug that has been more thoroughly damned than any other in history, and now on the bright side of the coin a new brilliance has again emanated. It is the promise of success of thalidomide in the treatment of leprosy reaction and other lesions of the skin and mucous membranes.



$C_{13}H_{10}N_2O_4$ CAS[50-35-1]

Figure 4.1. Structure, molecular formula and Chemical Abstracts registry number of thalidomide.

Thalidomide (*Figure 4.1*) was synthesized in 1953 and designed into a drug by the researches from Chemie Grünenthal in West Germany and marketed there since 1956 under the name Contergan [2]. Subsequently, it was distri-

buted to other countries as a sedative or hypnotic drug, and it became quite popular because of its good activity, the absence of acute toxicity, and the lack of side-effects. It was known at this time under a variety of names, such as Distaval (United Kingdom), Softenon (Austria, Belgium, Portugal, Spain, Scandinavia), Talimol (Canada), Kevadon (Brazil, U.S.A.), Rosalon (Argentina), Isomin (Japan), and others [3–9].

Upon long-term medication, however, some unpleasant symptoms gradually became evident. The first reports of polyneuritis due to thalidomide intake were recorded in 1960, and they were subsequently confirmed many times by many authors in different parts of the world.

More serious in its consequence, however, was the confirmation of the drug's teratogenic action, after a number of unexplained foetal abnormalities had been observed through the late 1950's and early 1960's. In November 1961, Lenz and Knapp [3], at a meeting in Westphalia, for the first time raised the possibility of an association between those cases and the ingestion of thalidomide during the first trimester of pregnancy. The manufacturer instantly withdrew the drug from the market, but it has remained available after that date in several countries to clinicians for defined research purposes under strict control. This is because several new indications for the use of thalidomide have emerged after this incidence or have been mentioned in the literature later.

In 1965, Sheskin [10] first reported spectacular results in the treatment of leprosy reactions of the erythema nodosum type with thalidomide which have been confirmed by many others in the years thereafter. More recently, several authors have independently shown that thalidomide is a useful drug for treating chronic discoid lupus erythematosus. Other skin diseases, such as prurigo nodularis and actinic prurigo, were treated with thalidomide with considerable success, as is true for some less frequently found cutaneous lesions. Finally, favourable response has been achieved with thalidomide in some cases of ulcerative colitis, a fact that opens another rather important perspective for new therapeutic uses of the drug.

It must be emphasized at this point, however, that it is imperative to combine any future thalidomide therapy with adequate contraception in adult women of child-bearing age. This is certainly the gravest handicap of thalidomide and the reason why the search for less toxic, especially non-teratogenic, analogues is still going on.

The aim of this article is to present an overview of the findings on these new indications for the 'classical drug' thalidomide which are different from its original use as a sedative-hypnotic drug, as well as on those of a few 'novel analogues' which may be superior to thalidomide in certain respects. It is, however, not the aim of this review to rehabilitate thalidomide as a drug in its

former indications, i.e., as a tranquillizer or sleep inducer, but rather to review critically and objectively the pros and cons for its future use in those conditions which are believed to be (mainly) inflammatory in origin.

This review is devoted to those scientists who are interested or involved in drug research and might wish to participate in the search for new and non-teratogenic compounds related to thalidomide, capable of replacing it in those fields of medicinal use mentioned above and outlined in more detail in the following pages.

THE TERATOGENICITY OF THALIDOMIDE

The teratogenic action of thalidomide cannot be neglected in any considerations of this drug. Therefore, a brief review of the present 'state of the art' with respect of the elucidation of the drug's mechanism of (teratogenic) action, which after 25 years of continuing efforts by numerous researchers all over the world, are still far from complete, should precede the present considerations. This is only fair and correct, since it has been truly stated: "If the medical profession failed to learn anything from the thalidomide disaster and failed to investigate the mechanisms of its action, that would be the worst of the affair" [11].

The teratogenicity of thalidomide, and the same is true for any other teratogen, depends on several factors, the most important of which seem to be administration in the sensitive period (i.e., the organogenesis), the species, the genotype of the recipient, the dose size, the route of administration, and possibly other environmental conditions.

For instance, concerning with the last point, thalidomide cannot be dissolved in alkaline media prior to its administration because the compound is unstable at higher pH values and breaks down to inactive products. Limitation in absorption because of the low aqueous solubility of the drug or losses due to fast breakdown may be responsible for most of the controversial results from earlier animal experiments, and many failures of action reported in the literature may be due only to the improper use of the drug. This must be emphasized, since such factors may also influence thalidomide's activity upon inflammatory conditions [12–14].

The teratogenic effect of thalidomide has provided a considerable stimulus for research into the biochemical reactions and the possible mechanism of action of the drug. Investigations were performed either purely *in vitro* or *in vivo* on almost any kind of biological object such as intact animals, mostly mammals, but also in birds, reptiles, and invertebrates, in cultured cells and organs, in micro-organisms, and in lower and higher plants. There are several excellent

review articles on this special topic available to which the reader may be referred [7,16–18].

Many hypothetical explanations of thalidomide's mechanism of action on a molecular basis have been raised, but most of them have remained speculative or have gained no experimental support. The most noteworthy theories are based on one or the other of the following assumptions:

vitamin antagonism
amino acid antagonism
acylation of biogenic amines
interaction with various enzymes
interaction with nucleic acids
interference with energy metabolism
interference with hydroxyproline biosynthesis.

However, congenital malformations have not been restricted to the action only of thalidomide. Many other physical and chemical agents have been known for a long time to exert similar effects. Reviews on such events from the pre-thalidomide era can be found in the literature [16,19].

There is a striking divergence between the high teratogenic activity of thalidomide and the almost total absence of acute toxicity in whole animals.

Table 4.1. HIGHEST DOSES OF THALIDOMIDE WHICH HAVE CAUSED NO SIGNS OF ACUTE TOXICITY

From D.E. Hague [20].

Species	*Dose (g/kg)*	*Route*
Mouse	10	p.o
	5	s.c.
	4	i.p.
Rat	2	p.o.
	1	i.p.
Guinea-pig	0.65	p.o.
Hamster	2	p.o.
Dog	1.5	p.o.
	0.1	i.p.
Monkey	0.1	i.p.

This fact must also be mentioned in this context, since it throws light on the exceptional properties of the compound.

Thalidomide was found to be virtually non-toxic in a number of studies. Acute LD_{50} values could not be determined in animals because of the physical impossibility of giving enough of the drug by conventional routes. *Table 4.1* gives the highest doses of thalidomide which have been reported in the species studied and which have produced no acute lethalities [20].

A similar absence of acute toxicity of thalidomide in adult humans can be derived from several attempted suicides which have been reported in the literature [21–23].

The drug which was formerly used therapeutically and continues to be employed as an anti-inflammatory agent is the racemic compound. It is noteworthy that the optical isomers of thalidomide (*Figure 4.2*) differ considerably in their teratogenic potential (but not in sedative effect), and that they are also acutely more toxic than the racemate.

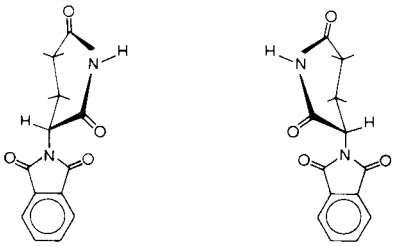


Figure 4.2. Optical isomers of thalidomide with absolute configuration and specific rotation as indicated.

Table 4.2 summarizes the acute toxicities observed with D- and L-thalidomide in three strains of mice [20]. Apart from the differences in the LD_{50} values of the three forms of the drug, there are also striking fluctuations in the susceptibility between sexes and between the strains. This reveals another important aspect of the conditions responsible for the drug's action: the biological effects of thalidomide must be highly dependent on the genetic background of the individual reacting to the drug.

No regard has been paid so far to these certainly important factors influencing thalidomide's biological activities with respect to the anti-inflammatory action of the drug. These experiments are still to be undertaken.

Table 4.2. LD_{50} VALUES OF DL-, D- AND L-THALIDOMIDE IN THREE STRAINS OF MICE

From D.E. Hague [20].

		LD_{50} (g/kg)			
		p.o.		i.p.	
Strain	*Sex*	*D*	*L*	*D*	*L*
ICI SAS	male	0.7	1.5	0.3	0.5
	female	1.5	3.0	0.5	1.5
TO ASL	male	0.75	1.0	0.2	0.2
	female	1.5	1.5	0.75	1.0
CF1	male	>3	>5	1.0	1.5
	female	>3	>5	1.5	1.5

OTHER ACTIONS OF THALIDOMIDE

There are other biological activities of thalidomide which have been detected during the extensive experimental investigation of the compound since its introduction as a therapeutic agent and, much more, after the recognition of its teratogenic potential. To a few of these activities, either desirable or undesirable, attention will be paid briefly in this section. These are the hypnosedative effect, neurotoxicity, immunosuppressive action, anticancer activity and anti-inflammatory action of thalidomide. Other activities, such as the proliferative or auxin-like effect in higher plants, for instance, cannot be more than mentioned here. Reviews on these and other 'thalidomide actions' have been previously presented by this author [15,17].

HYPNOSEDATIVE ACTION

Thalidomide had originally been used solely as a sedative and sleep-inducing drug. It is especially tragic that, by accident, in the thalidomide molecule this favourable property is associated with the teratogenic side-effect, since, as has been shown later, these two actions can easily be dissociated. This means that it is possible, by a slight structural modification of the original compound, to arrive at new entities which are still excellent sedative drugs but do not have the teratogenic effect. Taglutimide and supidimide (*Figure 4.3*), for instance,

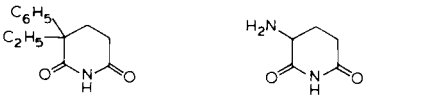
Taglutimide
(Biglumid, K-2004)
[CAS 14166-26-8]

Supidimide
(CG-3033, EM-87)
[CAS 49785-74-2]

Figure 4.3. Structure of taglutimide (INN) and supidimide (INN), together with previous denomination and code designation and Chemical Abstracts registry number, respectively.

retain the tranquillizing activity of the parent compound but are no longer teratogenic.

The sedative-hypnotic action of thalidomide and its congeners is most likely mediated by the glutarimide moiety substituted in the 3-position with a non-specific, space-filling group. This configuration exists in a number of sedative-hypnotic drugs, including glutethimide (*Figure 4.4*). The unsubstituted 3-aminoglutarimide, however, is inactive in this respect.



Glutethimide

3-aminoglutarimide

Figure 4.4. Structure of glutethimide (INN) and 3-aminoglutarimide.

It is worth mentioning in this context that the two optical isomers of thalidomide (*Figure 4.2*) are equally hypnosedative in the mouse, whereas they are quite distinct in their teratogenic potential: only the L- (or *S*-) isomer of thalidomide is teratogenically active.

Frederickson et al. [24] compared the sleep-inducing effects of thalidomide and pentobarbital. While thalidomide, over a dose range that did not produce ataxia, increased slow-wave sleep and rapid eye movement, pentobarbital had hypnotic activity and produced ataxia in over same dose range. In several simple screens for the central nervous activities of the drugs, it was shown that thalidomide probably acts by a mechanism of action different from that of the barbiturates, possibly involving the activation of a sleep centre in the forebrain, while the latter suppress the reticular arousal. It is concluded from this that thalidomide provides a much more physiological and a safer mechanism of

action than do the conventional hypnotics. Similar observations have been made by Kaitin [25]; he also states that sleep after thalidomide is physiologically and behaviourally similar to drug-free sleep.

This is not the place to review and discuss in detail the numerous pharmacological and clinical papers dealing with thalidomide's hypnosedative activity which have appeared throughout the years. A complete listing of these publications is available [26].

NEUROTOXICITY

Apart from the teratogenicity, the neurotoxic side-effects of thalidomide, which become manifest after chronic ingestion of the drug in relatively high doses, are the most striking negative features of this unique substance. It is because of the lepra reaction (which will be discussed in the next section, 'Thalidomide in leprosy') which is associated also with neuropathological symptoms that this aspect will be given attention in this section.

Florence [27] and Burley [28] were the first to draw attention to the neurotoxic effect of thalidomide. Previously, thalidomide had been considered as a practically atoxic and most effective hypnotic with no 'morning hangover'. It later became apparent, however, that patients who received the drug at doses of 100 mg a night for periods of 6 months to 2 years developed signs of peripheral neuritis, such as paraesthesia of hands and feet, pallor and coldness of fingers and toes, slight ataxia and muscular cramps in the extremities, and the like. The symptoms improved after cessation of the drug, but were still present after extended periods of time.

Then, early in 1961, Fullerton and Kremer [29] published a more extensive compilation of 13 cases of neuropathy after intake of thalidomide, and they wondered whether only these few patients had succumbed to the toxic effect when the population at risk was already relatively large. At this time, of course, no explanation for the mechanism of action was available. Soon after this, many more detailed reports on polyneuritic side-effects of thalidomide appeared in the literature, which confirmed all the earlier suspicions [30–37].

However, in the meantine, the disclosure of the teratogenicity of thalidomide superseded the further discussion of the neurotoxic effect, but in the mid-1960's several authors engaged themselves again in both the elucidation of pathogenesis and therapy of the so-called 'Contergan polyneuropathy'.

Amelung and Püntmann [38] published a comprehensive review on the etiology as well as the possible therapy of thalidomide-induced neural damage. Administration in high doses of vitamins of the B-group, of glutamic acid or antiallergic agents, and physical therapy were the only means available for

treatment of this polyneuropathy, but recovery of the patients proceeded, even under this treatment, slowly, if at all. Similar reviews were also published by other authors [39–42].

The therapy with B-vitamins might have its biochemical basis in an observation of Buckle [43], who found elevated blood pyruvic acid levels in patients with thalidomide neuropathy and concluded from this finding that it might be disturbance of some thiamine-dependent enzyme system and/or an abnormality that is responsible for the toxic effect.

Electroencephalographic and electronmicroscopic studies revealed that thalidomide affected not only peripheral nerves but also certain parts of the CNS [39,44,45]. Gibbels, especially, devoted tremendous effort to the investigation of the 'thalidomide polyneuropathy', and the interested reader should consider her special monograph on this subject [41].

In this context, it must be noted that several authors [46–48] reported similar neurological effects after administration of glutethimide, a sedative-hypnotic drug which shares with thalidomide the typical glutarimide moiety. It must thus be concluded that this part of the molecule is responsible for the neurological and cerebellar damage produced after prolonged exposure to these compounds.

Apart from its teratogenicity and neurotoxicity, thalidomide is not at all a harmless drug. In rare cases, it has induced allergic vasculitis and thrombocytopenic purpura, as was reported by Schulz and Jänner [49] and Kimmig [50]. The sensitizing component was found to be the glutarimide moiety in the thalidomide molecule, since tests with the imide of phthalic acid were negative, whereas signs of allergic cross-reactions to glutethimide were observed.

A further hazard of long-term use of thalidomide which has not become widely known is the appearance of myxoedema in some patients [51,52]. This complication could be related to a potential anti-thyroid activity of the drug [53–55], but further discussion of this phenomenon would certainly be beyond the scope of this review.

IMMUNOSUPPRESSIVE ACTION

An immunosuppressive effect of thalidomide has been discussed for a long time, but the experimental investigation of this hypothesis produced rather controversial results and even the theoretical mechanism of this action remains doubtful. However, since the assumed immunosuppression (if it were tenable) would explain the teratogenicity as well as some aspects of the anti-inflammatory action of the drug, attention must be paid to it in this context.

An early experimental approach was the attempt to demonstrate skin homograft survival in laboratory animals under thalidomide treatment.

Hellmann, Duke and Tucker [56], the strongest supporters of the immunodepressant theory, first found that thalidomide prolonged the survival of homografts transplanted across certain histocompatibility barriers in mice. The drug was also found to inhibit graft rejection in chicken embryos [57].

Later on, Turk, Hellmann and Duke [58] studied the mechanism by which the graft survival was prolonged. They administered thalidomide to mice both before and after the exchange of skin homografts and examined the lymph nodes that drained the area. Thalidomide was found to decrease the number of immunoblasts which developed as a result of the immunological stimulus, and the most marked effect occurred when both donor and recipient were treated with the drug.

Hellmann [59,60] then advanced the hypothesis that thalidomide might exert its teratogenic effect by an immunosuppressant mechanism in that it allows the continued development and eventual birth of a malformed foetus which would otherwise have been aborted, presumably via an immune mechanism.

This explanation of thalidomide's mechanism of action is no more than a curiosity, although it caused a big stir at the time of its advancement. Critical comments have been presented by several authors. [61,62], and summaries on this topic have been published [8,18,63].

The 'Hellmann hypothesis' has been tested in more-or-less direct fashion by several other researchers. Bore and Scothorne [64] treated pregnant rabbits with thalidomide (200 mg/kg per day i.p.) and found that the drug caused foetal malformations but did not prolong the survival of skin homografts in the mothers. Ellenrieder, Frankus and Krüpe [65] investigated the effects of thalidomide and of two derivatives on the survival of skin homografts in rats. The drugs were administered in the diet, beginning before grafting and continuing until the end of the experiment. Thalidomide at 2.5% in the diet was effective, while at the 1% level it was not.

Dukor and Dietrich [66] studied the immunological responses of mice treated with thalidomide and found some prolongation of skin homograft survival when both the donors and the recipients were treated with the drug. They also observed decreases in titres of circulating haemolysins and haemagglutinins after immunization with sheep erythrocytes when thalidomide was administered for several days after the immunization.

Contrary to this, Floersheim [67] reported experiments with thalidomide in mice to determine the drug's influence on the skin homograft reaction, but these results were unimpressive. Failure to affect the survival time of skin transplants in mice was also noticed [68].

Köhnlein, Lemperle and Kargas [69] tested the effect of thalidomide on skin homotransplants in inbred mice. When the transplants were incubated with thalidomide before transplantation, only slight retardation of rejection occurred, while following intraperitoneal injection of the drug at various doses significant prolongation of the transplant survival time was observed. The authors believe that this effect is probably due to the action of thalidomide as a folic acid antagonist.

Mouzas [70], in a preliminary communication, also reported a skin homograft rejection-prolonging effect of thalidomide in mice. Two years later, Mouzas and Gershon [71] confirmed these results. They administered the drug i.p. as a suspension in 0.5% carboxymethylcellulose in saline; the doses employed were 12.5 and 25 mg/mouse per day. Both donors and recipients were treated, commencing 10 days before grafting. Significant survival of skin transplants was noticed, and no toxicity was observed at these dose levels.

Jaffe [72] reported an experiment in chickens, a species which is particularly well suited for the testing of skin graft survival and graft versus host reaction. Adult hens received two grafts from a treated donor on one leg and from a control donor on the other one. Treatment consisted of 50 mg thalidomide per day for 4 days and 100 mg per day for 20 days, respectively. No difference was noted in the appearance or rejection pattern between treated and control grafts, and there was no difference in the graft versus host reaction, either.

Work with baboons and rhesus monkeys, on the basis of functional and pathological observations with *renal allografts* showed that thalidomide has some immunosuppressive properties but may be too toxic for human application [73].

Renal allotransplants in the baboon as an experimental model were used also by other workers [74], who found that thalidomide is an effective immunosuppressant agent, while conventional cytostatics such as azathioprin and cyclophosphamide were nephrotoxic and failed to prolong survival rate.

Erythropoietin release has been studied in renal allografted baboons with and without immunosuppression by various agents, including thalidomide (10 mg/kg per day i.m.), postoperatively [75]. Erythropoietin levels were progressively elevated after transplantation, and the animals died within a few days. Thalidomide lowered erythropoietin levels and increased survival by 21 days in the mean. Erythropoietin levels are considered to reflect the progress of renal rejection. Thalidomide was also found to be an effective immunosuppressive agent in the renal allograft survival rate in the dog [76]. The effect was most marked when donor and recipient were pretreated for 1 week prior to renal allotransplantation. No effect was noted on the second set response with thalidomide therapy. The immunosuppressive properties of thalidomide do not appear to be primarily mediated by suppression of the lymphoid system.

The clinical course of mongrel dogs after unilateral lung transplants was followed by periodic examinations including bronchography, angiocardiography, scintigraphy, spirometry and contralateral arterial clamping [77]. As immunosuppressants, methotrexate, azathioprine, chloramphenicol and thalidomide were administered in appropriate doses. Recipients not given immunosuppressive therapy rejected the transplants within 1 week. The animals treated with chloramphenicol and thalidomide did not survive significantly longer, and therefore it was concluded that these drugs do not have any immunosuppressive action. Azathioprine and methotrexate caused survival of several weeks, but the animals died finally from the toxic effect of these agents.

A review [78] summarizes the immunosuppressive methods employed in organ (lung) transplants and the results of antirejection therapy with synthetic drugs, among them thalidomide. Genovesi and Petrosillo [79], working with corneal transplants in rabbits, found some positive effects of thalidomide as a result of their experiments.

Monitoring of humoral antibody production is another approach to proving a potential immunosuppressive action. Gusdon and Cohen [80] investigated the effect of thalidomide on antibody synthesis in cultures of lymph nodes *in vitro*. They injected incompatible erythrocytes into rabbits and measured the haemagglutinin titres. Thalidomide was given from day 5 before until day 15 after immunization at doses from 50 to 200 mg per animal. The highest decrease was found at 100 mg, whereas 50 and 200 mg were less influential on antibody production. On the other hand, the authors found no effect upon antibody formation *in vitro*, but it must be remarked that they prepared solutions of thalidomide by heating and standing in solution for as long as 1 week, time enough for complete hydrolysis (and inactivation!) of the drug.

Investigation of the action of thalidomide upon the formation of antibodies in rabbits after stimulation with bacterial antigens (TAB vaccine) showed no effect [81]. The authors conclude that thalidomide is devoid of any immunosuppressive activity. Similar results have been reported by others [82].

One clinical observation, however, is in favour of an influence of thalidomide on antibody production. One single case of cold haemagglutinin disease, a haemolytic anaemia with a positive direct antiglobulin test and fairly high cold-agglutinin titres, was successfully treated with thalidomide after therapies with prednisone, penicillamine and azathioprine had failed. Three courses of thalidomide treatment of several weeks resulted in clinical improvement, significant fall in cold-agglutinin titre, a pronounced fall in the level of IgM antibodies and a rise in haemoglobin content [83].

In order to test the effect of thalidomide on the immune response in animals, Ogilvie and Kantor [84] used the following models: active delayed hypersensi-

tivity, passively induced delayed hypersensitivity, haemagglutinating antibody production, passive cutaneous anaphylaxis, and Arthus reaction in guinea-pigs with hapten-protein antigens. Drug dose levels varied up to 2200 mg/kg per day, administered either orally or intraperitoneally. In no instance, however, were these immunological reactions affected by the drug in any way.

Cerruti-Mainardi, Borrone and Tambussi [85,86], working with rats, could show that thalidomide produced a block of antibody formation in response to injections of human albumin.

The special rôle of lymphocytes in the function of the immune system and their participation in immunological responses, respectively, are presumably known. Thus, it may be reported without further explanation that several studies were undertaken in order to investigate the effect of thalidomide on these cells and on the drug's influence upon the blast transformation of human lymphocytes in the response to the nonspecific mitogen, phytohaemagglutinin (PHA).

Roath, Elves and Israels [87,88] have shown that the lymphoblastic transformation of human leucocytes in culture, stimulated by PHA, was inhibited by relatively high doses of thalidomide (25–50 μg/ml), as well as by some of its metabolites. D- and L-Thalidomide were equally active, as was the racemate. Aitken [89] observed a similar effect on lymphocytes, but as there was no effect upon the formation of antilymphocytic antibodies, he arrived at the conclusion that thalidomide has no immunosuppressive activity. The known immunosuppressors azathioprin and chlorambucil, on the other hand, strongly inhibited *in vitro* and *in vivo* both the lymphocytic transformation and the antibody synthesis.

Lindahl-Kiessling and Böök [90], working with human leukocyte cultures, obtained contradictory results insofar as thalidomide had no effect on the extent of blast transformation in response to PHA. Similarly, DL-, D- and L-thalidomide had no effect on the lymphoblastic transformation of cultured human lymphocytes, whereas some thalidomide derivatives showed considerable activity in this system [91].

A last approach in order to prove an immunosuppressant effect of thalidomide is to evaluate its usefulness in suppressing the manifestations of autoimmune diseases. For this purpose, two series of experiments, in guinea-pigs and in rabbits, were performed [92–94]. Experimental allergic encephalomyelitis, allergic neuritis, adjuvant disease and nonspecific granulomata were the models. The animals received the drug either intramuscularly (400 mg/kg daily) or intragastrically (800 mg/kg twice a week in oily suspension). Evaluation consisted of the observation of clinical features, survival time, histopathology, nerve conduction velocity, and of counting inflammatory lesions.

Thalidomide had mild sedative activity, if any, and it reduced the symptoms of adjuvant diseaese to a low extent, but it showed no effect in any of the other models. From these results it must be concluded that thalidomide does not exert any appreciable effect in autoimmune diseases.

The assumed immunosuppressive effect of thalidomide in guinea-pigs with experimental tuberculosis has been studied [95]. Two groups of animals were treated with thalidomide orally at doses of 500 mg/kg daily and infected with *Mycobacterium tuberculosis hominis*. In one group, thalidomide treatment was begun on day of infection, in the other group 14 days before infection. The response was similar in both groups and in the control group. Histological inspection of lungs and spleens showed less caseation in thalidomide-treated animals, which suggests some positive effect of the drug, but the assumed immunosuppression remains unclear. There is, however, no antimycobacterial effect of thalidomide *in vitro*.

Vladutiu [96] studied the possible immunosuppressive properties of thalidomide upon an experimental pattern of autoimmune disease after induced allergic encephalomyelitis in guinea-pigs. After inoculation, the animals received thalidomide treatment (500 mg/kg per day), but the results were of the kind which makes an immunosuppressive effect doubtful. Another attempt to prevent or to modify the symptoms of experimental allergic encephalomyelitis in the rat, a model reaction of human autoimmune disease, with thalidomide (300 mg/kg p.o.) was also unsuccessful [97].

Last but not least are immunological studies in leprosy patients who were under thalidomide therapy, in order to establish the assumed immunosuppressant action of the drug [98]. They involved 30 patients, 25 of whom received thalidomide, 5 being used as controls. Recorded were haematology, bacilloscopy, intradermal tests, T- and B-cell counts, immunoglobulins and complement. However, there was no important difference in any of the parameters between patients treated with thalidomide and those who were not.

Further immunological experiments have been performed in connexion with the aim of elucidating thalidomide's mechanism of action in the leprosy reaction. This aspect will be discussed later in more detail (section 'Mechanism of action...' pp. 213 ff). Hastings [18,99] is convinced that thalidomide works in this way, and he believes that the drug inhibits the antigen-antibody response both on the cellular and humoral level. There is ample evidence that thalidomide has anti-inflammatory properties in the leprosy reaction and in other skin diseases, but it remains open to question whether this is mainly, if at all, due to an immunosuppressant action or to any other mechanism whatsoever.

THALIDOMIDE AS AN ANTICANCER DRUG

During the early period of thalidomide's history, there was some interest in the compound as a chemotherapeutic agent for cancer treatment. This arose with the awareness of the teratogenic potential of the drug. The developing embryo may be considered as something like a 'physiological neoplasm' or a 'physiological transplant' [100], the growth of which is depressed by thalidomide's action in a hitherto unknown way: why should the drug not have a similar action on true neoplasms, too?

Some preliminary animal experiments seemed to be in favour of this theory. Sugiura and Wuest [101], for instance, observed a moderate inhibition of the growth of a Lewis bladder carcinoma at comparable high doses of thalidomide (1000 mg/kg per day), but there was no effect on 24 other transplantable tumours in mice, rats and hamsters. It is of interest that methylation of the nitrogen on the glutarimide ring abolishes the antitumour activity.

Mückter and More [102,103] performed a series of experiments to evaluate the potential anti-tumour activity of thalidomide and some of its congeners, e.g., the Mannich bases CG-601 and CG-603 (see section on Congeners, pp. 218 ff). Autonomously growing, virus-induced and hormone-independent tumours, with some exceptions, are not influenced by thalidomide; hormone-dependent tumours, however, e.g., tumours which have been induced with dimethylbenzanthracene, respond to prophylactic application of thalidomide as well as to curative treatment with it and the other substances. The effect is limited by the size of the tumour at the time of first administration of the drugs, and by the duration of the treatment. The mechanism of the antitumour action of thalidomide and its derivatives differs fundamentally from the cytostatic action of alkylating agents, e.g., cyclophosphamide, in that the drug seems to exert its effect mainly or perhaps exclusively through the endocrine system.

Chaudry and Schmutz [104] compared the effects of prednisolone and of thalidomide on dimethylbenzanthracene-induced submandibular gland tumours in the hamster. However, neither a suppressant nor a facilitating effect could be detected in any of the drugs. This is in obvious contrast to the findings of Mückter's group.

Pagnini and DiCarlo [105] studied the effect of thalidomide upon two experimental tumours, Ehrlich ascites carcinoma in the mouse and myeloma in the rat. Thalidomide was given at 500 mg daily p.o., starting from the day of inoculation. However, no significant change in the mortality rates of the treated animals and the controls could be found.

A model often employed for testing the cytostatic activity of chemical compounds is liver regeneration in partly hepatectomized rats. A reduction was

observed in the initial rate and degree of regeneration of liver in thalidomide-treated hepatectomized rats (1.5 or 6 mg/rat per day p.o. for 5–30 days) [106]. The livers reached almost prehepatectomy weight by day 15 in the controls, whereas it took 30 days for similar or lesser weight gain in the thalidomide-treated animals. In a different, non-tumour model, the regenerating tail of lizard, the same authors could not repeat the previous findings: thalidomide (30 to 360 μg) did not show any inhibitory effect on tail regeneration as measured by its linear growth; regeneration progressed in all groups without much variation [107]. Similarly, the tentacle regeneration in *Hydra littoralis* was influenced by thalidomide, but only a reduction in the number of tentacles and some malformations of the extremities were observed [108].

In striking contrast to the first report is the observation of Gershbein [109], who also investigated the effect of thalidomide on the regeneration of liver tissue in rats. At a dose of 0.2% in the diet (approximately 250 mg/kg per day), thalidomide significantly increased the regeneration rate of the liver cells as compared with the untreated control animals. In other paper, Gershbein [110] reported that tumour transplants and concomitant pregnancy tended to normalize the regenerative rate, while tumour growth was little affected in the presence of immunosuppressive agents like thalidomide.

These preliminary results, at least in part favourable, which suggest a potential antitumour activity of thalidomide, led to several studies in humans, too, but the outcome was meagre. Mauad [111] reports on some cancer patients who were treated with thalidomide and concomitantly with small doses of hormones (glucocorticoids, testosterone, thyroid hormones, anabolics); he claims to have achieved good results with this 'thalidomide micro-scheme', as he calls it.

Other workers treated 21 patients with fourteen types of advanced cancer, already resistant to all other therapies, for periods of 1–34 weeks. They used extremely high doses (600–1400 mg daily) but, except for one remission of a developing liposarcoma, no objective result could be obtained. Some subjective improvement, however, was due to the tranquillizing action of the drug.

Grabstald and Golbey [113] used thalidomide in high doses (800–1200 mg daily) in 71 cancer patients refractive to all other treatment. They, too, could not observe any antimitotic effect, although some temporary regression of pulmonal metastases in one case of renal cancer was seen. Allegri [114] treated 12 patients with various malignant tumours with thalidomide at doses from 50 mg to 1.05 g per day for periods of between 6 and 37 days, but there was no visible effect, except for some somnolence.

Woodyatt [115] had one case of a malignant mixed mesodermal tumour of endometrium, resistant already to deep X-ray therapy, which was treated with 400 mg thalidomide three times daily, but no regression occurred.

The inefficacy of thalidomide as a cytostatic agent has been confirmed subsequently in various animal experiments and in cell cultures. The possible anticarcinogenic effect of thalidomide could not be confirmed upon two implanted tumours in mice [116,117]. Juret and Aubert [118] employed an Ehrlich ascites tumour in the mouse and a solid epithelioma in the rat as models, but could not find any suppressive activity of thalidomide.

An extensive study of the problem in various experiments in connection with the teratogenicity of thalidomide yielded no objective verification of any antitumour activity of the drug [119–121]. Thalidomide did not inhibit dehydrogenase activity or growth of Ehrlich ascites tumour cells in agar culture. However, when mixed with the cells *in vitro*, it increased the mitotic activity; the effect of thalidomide was not altered by vitamins of the B-group, nor was the oxygen uptake by the tumour affected. A possible relationship between teratogenesis and carcinogenesis was considered as existing also in the case of thalidomide.

In a variety of eight transplantable tumour systems of different origin, DiPaolo [122] found again no evidence of effectivity of thalidomide as an anticancer agent. The most that can be said is that thalidomide did produce a slight delay in the growth of some transplantable tumours such as choriocarcinoma of human testicular origin and a testicular carcinoma of murine origin.

A different approach to confirm the potential cytostatic activity of thalidomide is the use of cell cultures of different origin. Mohri and Kitagawa [123] found that thalidomide and several of its metabolites had essentially no effect on the growth of HeLa cells in tissue culture, even when the medium was depleted of glutamine. Thus, it does not seem that thalidomide might work as a glutamine antagonist.

One observation seems to be in favour of the assumed cytostatic activity of thalidomide [124]. By prolonged cultivation (over 100 days) in media containing thalidomide (20 μg/ml), HeLa cells underwent marked genetic modifications, such as high polyploidy, multiple chromosomal aberrations, translocations, dicentric chromosomes, double-ring chromosomes, etc. This phenomenom seemed to exhibit a tendency towards normalization when the cells were transferred to thalidomide-free media again. Similar cytological effects were also observed [125–127] in chicken embryo blood cells which, in the presence of thalidomide, showed many mitotic figures with gross alterations or cessation of cell division.

Miller [128] studied the effect of a water-soluble derivative of thalidomide, 3-hydroxythalidomide, which is also one of the products of biotransformation of the parent compound, on a primary mouse embryo monolayer tissue culture. She found that even 300 μg/ml of this metabolite produces complete inhibition

of RNA synthesis and almost total inhibition of protein synthesis in these cultures.

Moiraghi-Ruggenini and Errigo [129], working with various experimental tumours (Ehrlich adenocarcinoma, sarcoma 180, lymphoid leukaemia, Yoshida tumour), could not find any antimitotic activity of thalidomide, although they admit that, at first, the substance decreased tumour growth to some extent. Gaetani [130] administered thalidomide (500 mg/kg per day) to tumour-inoculated mice and found no influence of the drug upon normal development of various tumours such as Ehrlich ascites, myeloma, sarcoma and transplantable teratoma. This proved to be the case for both racemic thalidomide and for the pure L-(−)-isomer. Body weight and mortality of the mice were not affected by either form of thalidomide.

An immunosuppressant action of thalidomide was found in rats with experimentally induced neoplasia (Yoshida sarcoma), but not in normal rats [131]. The complement activity elicited by reaction of a serum factor with RNA from a mutant of *Saccharomyces cerevisiae* was tested. Thalidomide and prednisolone, for comparison, were given i.p. at daily doses of 100 mg and 10 mg, respectively, on 10 consecutive days. The tumour factor showed progressive deficiency (20% on the first day up to 100% on the 9–10th day) in tumour-bearing rats with no treatment, as compared with the non-tumour-bearing controls; in thalidomide- and prednisolone-treated tumour-bearing animals there was an additional immunosuppressant action, as evidenced by a 100% deficiency of the tumour factor by the 5–7th day.

More recently, in an *in vitro* system, Braun and Dailey [132] showed that thalidomide and some of its metabolites inhibit tumour cell attachment to plastic surfaces coated with concanavalin A. Especially metabolites produced by incubation of thalidomide with murine liver microsomes were inhibitory. This finding suggests that an active metabolite of thalidomide alters cell-surface function leading to interference with normal morphogenic cell-to-cell interactions. Szydlowska and Dluzniewski [133] believe that thalidomide acts by blocking functional groups of protein, e.g., SH groups, and may exert its biological effect in this manner.

Pfordte [134] found that thalidomide is able to stimulate the serum properdin system, which is considered as part of the nonspecific defence system of the body against infectious agents of any kind. Following the first dose, the properdin level was increased to 119% of the starting value. After 10 successive doses, the level remained at about 125% of the basic value, and it continued at the same concentration even 5 days after cessation.

Several studies have also been conducted with various kinds of blood cell (lymphocytes, leucocytes, etc.) in culture in order to prove either an antimitotic

or an immunosuppressive activity of thalidomide (see subsection 'Immunosuppressant action...', pp. 216 ff).

While the antitumour activity of thalidomide remains doubtful, there are, on the other hand, reports of a cancer-promoting or even a carcinogenic action of the drug. Thalidomide has a potentiating effect on methylcholanthrene oncogenesis in mice [135].

Roe and Mitchley [136], in an attempt to test thalidomide for potential carcinogenicity by injecting oily suspensions of the drug (7.5 and 15 mg/0.2 ml) subcutaneously into the flanks of mice over prolonged periods of time, found well-differentiated spindle cell sarcomas at the injection site in some of the animals. In another experiment, the authors checked the tumour incidence in the progeny of thalidomide-treated mice, but even at high doses (total dose 165–195 mg) there was no carcinogenic effect observable [137]. The case history of a 15-year-old patient with thalidomide-induced malformations who, later on, developed a lymphoma of high malignancy has been reported [138].

On the contrary, Neubert [139], reflecting upon the relationship between teratogenicity (which is certain in thalidomide) and carcinogenicity (which is still doubtful), came to the conclusion that "the teratogen most widely known to affect humans and other primates is *not* considered to be carcinogenic".

A favourable effect, although not of the anticancer type, may conclude this section: thalidomide was given to patients with malignant neoplastic diseases (gastric cancer, lymphoblastoma) who underwent antimitotic treatment with mechlorethamine and who concomitantly suffered from severe nausea and vomiting. Although thalidomide had no influence on the neoplasm itself, it exerted a significant antiemetic activity in all of these patients [140].

ANTI-INFLAMMATORY ACTION

Among the favourable pharmacological activities of thalidomide, there is a remarkable anti-inflammatory component which has been known from the very beginning but has not attracted as much attention as the tranquillizing effect that finally led to the introduction of thalidomide as a sedative and sleep-inducing drug.

When surgical patients were treated with 100 mg thalidomide three times daily for 4–7 days, it was effective in preventing post-operative oedema of surgical wounds, with excellent results obtained in haemorrhoidectomies. It was felt, at this time, that thalidomide had limited effectiveness in alleviating the manifestations of existing oedema and inflammation. The authors [141] refer to a personal communication of G.J. Martin, who stated that thalidomide had been found to have an anti-inflammatory effect that was superior to that of the salicylates in rats.

There is also a certain antipyretic activity present in thalidomide, as has been reported in an earlier paper on the pharmacological properties of the drug. Somers [142] gave thalidomide to animals which had been pretreated with a suspension of heat-inactivated *E. coli* bacteria to induce fever; 200 mg/kg thalidomide significantly lowered and shortened the febrile response to the bacterial injection. An analgesia-enhancing effect of thalidomide has been reported by Harris and Allgood [143].

The most comprehensive investigation into thalidomide's anti-inflammatory properties, however, was performed much later by Hastings and Morales [18,144] who were predominantly interested in the application of the drug in the lepra reaction. They studied thalidomide in five systems in an effort to clarify its mechanism of action in humans with erythema nodosum leprosum (ENL). These are, in brief, as follows:

(1) Thalidomide is active in inhibiting the inflammation in the rat paw induced by carrageenan. This points to a specific step in the sequence of events in the post-immunological inflammatory mechanism. The effect is highly unusual and is non-linearly dose-related; it appears to be biphasic, with inhibition of the oedema occurring at 3–10 mg/kg and in the range of 100–300 mg/kg, and no

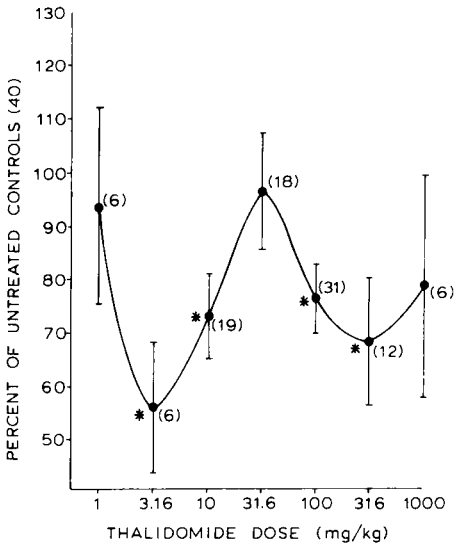


Figure 4.5. Effect of thalidomide on rat paw oedema 1 h after injection of carrageenan. Bars represent standard errors. The numbers is parentheses are the numbers of animals. Asterisks indicate significant difference from controls ($P \leq 0.05$) (from Hastings [18]).

significant net effect was seen at doses of 1, 30 and 1000 mg/kg (*Figure 4.5*). The authors explain this phenomenon as due to a biphasic mode of action of carrageenan in producing the oedema, and thalidomide acting on both phases.
(2) The interaction with various nucleotides was studied. The rate of hydrolysis of thalidomide is clearly related to the hydroxyl ion concentration, increasing directly as this increases. The nucleotides AMP, GMP, CMP and UMP retard the hydrolysis rate of thalidomide, and thus this effect reflects the strength of binding between the drug and the nucleotide.

Thalidomide was found to bind to CMP, somewhat less with GMP, and considerably less with AMP and UMP. The binding of thalidomide to nucleotides *in vitro* points to an interaction with nucleic acids, inhibiting cellular growth processes.
(3) The binding of thalidomide to bovine serum albumin (BSA) was tested. Intact, partly hydrolyzed and completely hydrolyzed thalidomide was incubated with BSA in various concentrations and the optical density (OD) of the solutions was determined and compared with the OD of a blank. The differences are plotted for each concentration of thalidomide (*Figure 4.6*)

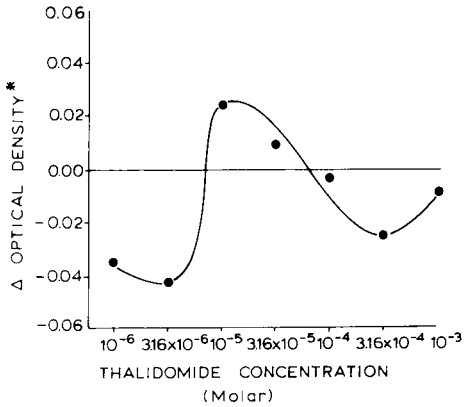


Figure 4.6. Effect of thalidomide on the heat denaturation of bovine serum albumin (from Hastings [18]).

As a result of these experiments, it was found that the intact drug rather than its hydrolysis products is the active moiety, and that it again biphasically stabilizes BSA to heat denaturation to some extent. The drug does not resemble most acidic non-steriodal anti-inflammatory agents in this respect. However, the dose-response relationship in this system suggests a pattern of stabilization

and labilization of proteins by thalidomide which might be responsible for the anti-inflammatory properties of the compound.
(4) The effect of thalidomide on the response of the isolated guinea-pig ileum on various chemical mediators such as histamine, bradykinin and acetylcholine, was studied. Only in high concentrations did the drug nonspecifically depress the responses of the tissue to all three agonists. The results do not support the hypothesis that thalidomide exerts its potent anti-inflammatory effect by acting as an inhibitor of any of the mediators studied.
(5) The stabilization of rat liver lysosomal membranes by thalidomide was tested by measuring a marker enzyme, acid β-glycerophosphatase. 0.003 mM thalidomide, a concentration comparable to that attained in the plasma of humans after administration of a therapeutic dose, significantly stabilizes the lysosomes (*Figure 4.7*). This suggests that at least one site of action of thalidomide is situated in the lysosomal membrane.

There were, however, other authors who were interested in the potential pharmacological activities of thalidomide other than the hypnosedative effect. Brode [145], for instance, conducted experiments to evaluate the possible influence of the drug on the pituitary-adrenal axis. Upon administration of thalidomide in high doses, he found a transient stimulation of corticosterone

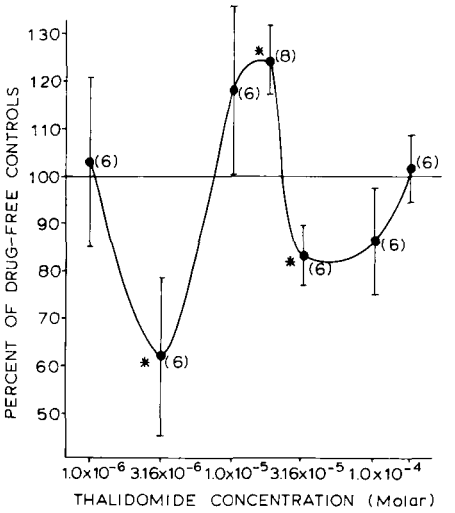


Figure 4.7. Effect of thalidomide on the release of acid β-glycerophosphatase from rat liver lysosomes in vitro. *Bars represent standard errors. Asterisks indicate significant differences from the controls ($P \leq 0.05$) (from Hastings [18]).*

production in rats. The concentration of ascorbic acid in the adrenals was about twice as high as in the controls. However, on administration of the drug over prolonged periods of time, a reduction of adrenal activity was observed. This effect is not caused by a direct attack at the level of the adrenal cortex, but is probably due to an influence on higher centres.

In a second communication, Brode [146] reported experiments directed towards the study of the possible influence of thalidomide and some related compounds on somatotropin-dependent processes. Administration of the drugs (2–50 mg/kg i.p.) to juvenile rats caused significant widening of the tibial epiphyseal cartilage plate. The uptake of ^{35}S-labelled sulphate by the cartilage demonstrates that the sulphation factor in the pituitaries of thalidomide-treated animals was significantly reduced.

Brode [147], in a third paper, studied the influence of thalidomide and a derivative on enzyme induction in connexion with nucleic acid synthesis and detoxication of xenobiotics. *De novo* synthesis in rat liver of tryptophan-pyrrolase and tyrosine-α-ketoglutarate transaminase, which can be induced by corticosteroids, was not inhibited by the drugs. Liver menadione reductase, which is normally induced by DMBA, appeared to be stimulated by thalidomide.

Brode [148] also demonstrated that thalidomide and structurally related cyclic imides can influence some biochemical processes in rat thymocytes. Protein synthesis was stimulated in these cells by the drugs, while nucleic acid synthesis was not. The incorporation of glycine into protein and DNA is much more pronounced under the influence of a congener (CG-601) than by thalidomide itself. These early experiments showed that thalidomide has more than one site of action, some of which might be relevant also for the pronounced anti-inflammatory activity of the drug.

At the molecular level, thalidomide seems to interact specifically with nucleic acids, a phenomenon that has attracted considerable interest, since it provides an explanation of the teratogenic mechanism of action of the drug [8,18, 149–151]. Hastings [18] observed also an interaction of thalidomide with nucleotides *in vitro*. However, it has never been made clear how this sort of binding to biological macromolecules could be connected with the anti-inflammatory effect of the drug.

Hanauske-Abel and Günzler [152] proposed an inhibition of human propyl hydroxylase as the common denominator of the non-sedative effects of thalidomide in man, including the excellent efficacy in the lepra reaction. They explained these effects on a molecular level by assuming a non-sedative metabolite of thalidomide mediating the inhibition of the enzyme, as suggested by steric considerations and correlations of known data. They presented this

metabolite as a model compound for drugs designed for selective fibrosuppression and selective immunosuppression. The theory of proline hydroxylase inhibition has been raised also by others [153–157].

Workers [158] who checked this hypothesis experimentally with thalidomide and some of its congeners, however, arrived at the conclusion that the minor effect of these compounds on collagen biosynthesis cannot explain the induction of foetal malformations. The question of an involvement of propyl hydroxylase in the anti-inflammatory processes remains open.

Speculations of the possible influence of thalidomide on cellular oxygen consumption have been reported [159] with reference to an involvement in brain oxygenation. Indeed, thalidomide increased concentrations of oxygen in the brain of adult guinea-pigs and foetuses by an average of 33 and 200%, respectively. Simultaneously, jugular venous blood partial pressure, arterial-venous difference of oxygen content, and oxygen consumption were decreased, while other parameters were not affected by thalidomide. This interference with intermediary energy metabolism, however, could also have consequences upon inflammatory processes, as it is believed to lead to developmental anomalies in embryonal tissues.

Abreu and Abreu [160] determined ceruloplasmin enzymic activity in the serum of rats treated with thalidomide either orally or parenterally. The levels of the oxidase, which normally plays an important role in the metabolism of biogenic amines (which can also act as mediators of inflammation), were not significantly different from the values obtained in the controls.

THALIDOMIDE IN LEPROSY

Thalidomide is now well documented as an effective and relatively non-toxic treatment for the so-called leprosy reaction or erythema nodosum leprosum (ENL), occurring as a complication of lepromatous leprosy. Despite its teratogenic effect, thalidomide is considerably safer for long-term use than the only acceptable alternative of comparable efficacy in this condition, the glucocorticoids, especially in view of the toxicity of the latter [161]. Thalidomide, therefore, has become the preferred drug for the treatment of ENL, and so it will remain until a better, presumably non-teratogenic, successor has been found.

In this section, first, a brief description of the disease and of the special syndrome for which thalidomide at present is the 'treatment of choice', will be given. This will be followed by a short historical recapitulation of the discovery of the thalidomide's efficacy in ENL and then by a compilation of the clinical reports which have appeared since 1965 up to the end of 1983.

The mechanism of action of thalidomide in ENL (and in other inflammatory conditions of the skin and the mucous membranes) will be summarized and discussed later in the separate section 'Mechanism of action...', pp. 213 ff.

LEPROSY: DISEASE AND COMPLICATIONS [161a]

Leprosy is a chronic infectious disease of man caused by *Mycobacterium leprae*, an acid-fast bacterium isolated and identified in 1869 by Gerhard Henrik Armauer Hansen from Bergen, Norway. The disease was much more important in European countries during the medieval ages than it is nowadays. Today, the highest incidence is in the following order: Asia, Africa, Latin America, Australia and Southern Europe. Although recognized for over 2000 years, the disease is often missed or falsely diagnosed as a different cutaneous lesion, because the incubation period, like the treatment process, is long and complicated [162–167].

Conservative estimates of the global prevalence of the disease are 10–15 million cases at present, another 5 million having been cured during the past 20 years, but with 1 million expected new cases for approximately every 5-year period. The proportion of patients with the disseminated or lepromatous type of leprosy (see below) has been estimated at approximately 2 million and about two-thirds of them are expected to develop a major complication [162,166,168–174]. According to the five-part classification of Ridley and Jopling [175–177], the various types of leprosy are not separate disease entities but merely segments of a spectrum of diseases ranging from a purely localized form (tuberculoid leprosy) on the one hand through a dimorphous stage (borderline leprosy) in the middle to generalized disease (lepromatous leprosy) at the other end of the spectrum (*Figure 4.8*). The degree of acquired immunity decreases from the bottom (complete immunity) or left side (partial immunity), respectively, to the right (feeble to no immunity). It must be noted that external and internal factors, such as physical or psychic stress, intercurrent infections, vaccination, suprarenal insufficiency, sexual episodes (e.g., menstruation, pregnancy, accouchement) or concomitant drug therapy (e.g., sulphone overdosage), can aggravate one or the other condition. There are several reviews which describe in more detail the different clinical forms and their diagnosis, and the reader is referred to the originals, should deeper insight into this subject be desired [163,165,177–182].

The overwhelming majority of people who come into contact with *M. leprae* are apparently able to resist the infection, and only a small percentage go on to develop indeterminate leprosy, which may heal without treatment in many of the cases or may remain undiagnosed. Those who are susceptible to the

infection, however, develop symptoms which can be classified into the spectrum given in *Figure 4.8*. Reactive episodes of the lepromatous form continue to be a serious complication, but the availability of thalidomide to control ENL has markedly improved the prognosis.

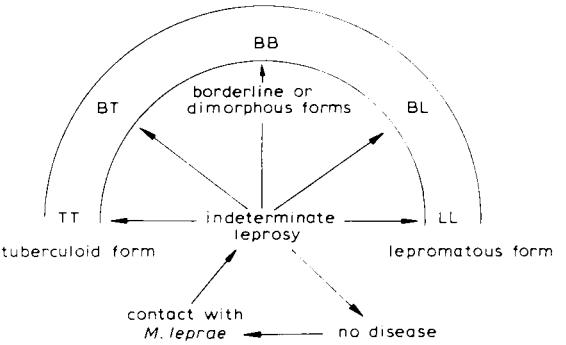


Figure 4.8. Spectrum of the various forms of leprosy (after Ridley and Jopling [175]).

The most common complication of leprosy is the 'lepra reaction'. These episodes are characterized by life-threatening, acute inflammatory states at times, and commonly result in permanent peripheral nerve paralysis, disfigurement of the skin areas involved, and, not rarely, in blindness [183]. The term ENL is now commonly adopted for this singular and independent clinical syndrome with its special histological features.

There are three types of clinical manifestation observed due to the 'lepra reaction'. These are the *erythema nodosum leprosum* (ENL, lepromatous leprosy reaction), the *erythema multiforme* (or polymorphic erythema), and the *erythema necroticans* (or Lucio's phenomenon). The differences are concerned mainly with the appearance and severity of the exacerbation, its spread over either restricted parts of the skin or the total body surface, and the accompanying extracutaneous complications, especially the rather painful sensations. The etiology of the lepra reaction has been discussed in several review articles [89,184–188].

Lucio's phenomenon (Lucio's leprosy) is sometimes considered as a distinct syndrome associated with leprosy, as indicated by a diffuse, waxy infiltration of the skin that is never transformed into nodules, alopecia or total hair loss, absence of fever, leukocytosis and tenderness. This seldom observed complication, which has the worst prognosis, has also been called 'pure and primitive

diffuse lepromatosis'. This syndrome is common in Mexico, Central and South America. A striking difference from ENL is the fact that Lucio's phenomenon normally fails to respond to thalidomide [189].

Rea and Levan [190] report four cases of this rare disease in whom thalidomide-therapy was tried. In one patient, cessation of new acute lesions was achieved, but in the other three, the use of thalidomide (300 mg daily) was associated with the continuing appearance of new acute lesions.

Therapeutic or drug treatment of leprosy, from the basic infection up to all forms of its secondary complications, is a vast field of specialization for physicians, chiefly those who specialize in tropical diseases. There are numerous special reports and review articles available which give a fairly good overview on possible drug therapies [165,166,168,188,191–205].

It has been suggested that the term 'leper' or 'leprosy' should be eliminated from common use and be substituted by the scientific denomination 'Hansen's disease'. However, all the attempts to achieve this have so far failed [206].

DISCOVERY OF THALIDOMIDE'S EFFECTIVENESS IN ENL

In November 1964, Jacob Sheskin in Israel first used thalidomide against lepra reaction. The discovery was incidental. The first (female) patient was admitted to the wards in a state of mania, and thalidomide was administered to calm her down. Overnight there was a dramatic reduction in a widespread nodular eruption from which the patient had been suffering and which later turned out to be ENL [207,208].

The results were overwhelming. The treatment was soon repeated in another six unselected cases of lepromatous leprosy, and again thalidomide produced a dramatic improvement of the painful condition within a few days. All patients had been treated before between 6 months and 12 years with various other drugs, such as sulphone and thiourea derivatives, thiosemicarbazone, prednisone, ACTH, chloroquine, antimony trioxide, and streptomycin, but with unsatisfactory results. Thalidomide was given at 100 mg doses 3–5 times daily. Within 24 h, the severe joint and muscle pains ceased, and the erythematous skin lesions resolved rapidly within 3–5 days [10].

In the following years, Sheskin himself, as well as in collaboration with many others, reported repeatedly further observations with thalidomide in patients showing lepra reactions. In 1965 he reported on another series with 13 unselected patients who were treated successfully with thalidomide (100 mg every 8 h) over a 10-month period, either alone or in addition to the existing regimen (i.e., dapsone, stibophen or prednisone). Dramatic improvement was seen within 12 h after the start of the treatment. No patient suffered from any

serious side-effect. Positive comments on this communication were also published [209].

A second paper reports on 11 patients who were treated in part with thalidomide (400 mg/day) and in part with placebo. In all cases the lepra reaction responded rapidly, both subjectively and objectively, to the drug. By contrast, treatment with placebo was ineffective [210].

A statistically controlled double-blind trial was performed by Sheskin and Convit [211] as early as in 1966 with 58 patients, divided into two equal groups. The result, in brief, was in the thalidomide group 91.76% complete remissions and 8.24% unchanged, while the placebo group showed 27.27% improvement, 50% unchanged and 22.75% in even worse condition than before the experiment. Similar reports were published elsewhere [212,213].

Another group of 24 lepromatous patients, in part, cases with long duration of the disease in whom sulphone treatment had been suspended to prevent further aggravation of the reaction or who had been treated with corticosteroids previously, received thalidomide in doses of 400 mg daily. This restored body temperatures to normal within 48 h and brought about a complete remission of the leprosy syndrome in 4–5 days. The daily doses were reduced gradually to a maintenance dose of 50 mg. This treatment enabled resumption of dapsone therapy also in such patients who were formerly intolerant to sulphones [214].

After 55 months of trials with thalidomide in leprosy patients, Sheskin [215] stated that the drug is an "effective means for lepra reactions in lepromatous leprosy". As an initial therapeutic dose, he recommended 400 mg daily (6 mg/kg body weight) and an optimal maintenance dose of 100 mg per day.

Thalidomide was at that time already considered as firmly incorporated into the arsenal against this complication of Hansen's disease [216].

The idea was taken up by a group of consultants, co-operating with the World Health Organization, who discussed and published the latest developments in the therapy of leprosy, i.e., the therapeutic efficacy and effective dosages of most of the common antileprotic drugs. The potential value of thalidomide in therapy of the lepra reaction was also discussed. A daily dose of 400 mg was adopted as appropriate, but it was emphasized that the teratogenic effect limits the use of thalidomide in man and requires strict medical supervision. Some of the products of thalidomide metabolism might retain the antileprotic properties while losing the teratogenic activity of thalidomide itself. The possibility of using an injectable 'depot preparation' of the drug was also raised [193].

Side-effects of thalidomide treatment, although denied in most of the clinical reports, sometimes pose problems. Sheskin and Sagher [217], in one of their earlier papers, report that out of a group of 40 leprosy patients who were treated with the drug, three developed serious herpetiform dermatitis thereafter.

In another paper, the authors [218,219] point to the unsatisfactory results of thalidomide therapy in a group of 24 patients with various forms of leprosy who were treated with the drug for periods of 3–19 months. In 11 patients the disease process ultimately became worse, eight showed no change, but in five the cutaneous lesions improved after prolonged treatment.

Nevertheless, after 5 years experience, Sheskin and Sagher [220–222] stated that thalidomide treatment of ENL was successful in a total of 91% of the attacks. However, concerning leprosy patients in general, some improvement was seen of the general condition, none in others, and worsening in a third. They arrived at the final conclusion that thalidomide is highly active in the lepra reaction but is inactive as an antileprosy agent.

Sheskin and Sagher [223,224] sent out a questionnaire to 62 different leprosy departments in all five continents. The answers were evaluated, and as a result it was concluded that the response was satisfactory to 99% of lepromatous leprosy patients, whereas they were not good at all in tuberculoid and borderline types of leprosy. A summary of the possibilities of treatment of ENL with special reference to thalidomide has been given by Sheskin at the 9th International Leprosy Congress [225].

Summaries of the continued experiences with thalidomide therapy in leprosy patients were published repeatedly at intervals of a few years. About 10 years after his first report in 1965, Sheskin surveyed a total of 4522 cases from the 62 institutions all over the world mentioned above. He emphasized that a combination of thalidomide with sulphone offers now satisfactory treatment to those patients who have acute ENL, whereas no aid had been available to them previously. In those who had been under steroid therapy before, thalidomide treatment could be started with the whole dosage, while the steroid was slowly reduced. Side-effects are not considered too serious, not even justifying the cessation of therapy. Concomitant contraception in female patients is indispensable, however [226,227].

Again, in a review after 15 years experience with thalidomide in the lepra reaction, Sheskin convincingly reported his success. Thalidomide treatment, according to this, affords the following medical-social advantages [228]:

(a) It shortens the duration of lepra reactions, thus reducing the danger of neural, muscular, osseous and ocular damage as well as deforming sequelae.

(b) It allows the continued administration of antihanseniasis treatment in optimal doses without risk of lepra reaction.

(c) It makes it possible to treat lepra reaction on an outpatient basis, except in woman of fertile age, who should be hospitalized.

(d) It shortens hospitalization, thus reducing hospital costs, so onerous nowadays, and allowing, when necessary, hospitalization in general services. The amounts saved can be used for rehabilitation and research.

(e) It allows patients to return to work earlier, thus lessening the burden on family and society.

THALIDOMIDE IN ENL TREATMENT: CLINICAL EXPERIENCES

After reviewing the appreciable pioneering work of Sheskin and his collaborators in the foregoing section, we turn now to the innumerable communications and comments of other authors on their clinical experiences with thalidomide. In 1968, in an introduction to a working party on thalidomide in the leprosy reaction, Sagher [229] stated that thalidomide seems to represent progress in comparison with cortisone as the only satisfactory treatment of this condition.

Among the earliest presentations, there is a report [230] from 1966 on three leprosy patients who were treated with 300–400 mg thalidomide daily and who experienced spectacular improvement in the acute symptoms within a few days. In the same year, Trimigliozzi [231] registered 15 cases of ENL in Italy who were treated with thalidomide (300 mg/day initially), generally leading to complete remission of the acute phenomenology within 48 h. However, apart from the fact that 12 patients responded satisfactorily, there was also one with doubtful and two with negative results. Shortly after this, Trimigliozzi [232] presented further clinical observations which confirmed the favourable rapid action of thalidomide in the lepra reaction. Again, out of a total of 25 cases, 22 had favourably responded to this treatment.

Also from 1966, a communication [233] from Argentina reports the control of neuralgic manifestations of leprosy in 27 patients. The drug was given at doses of 200 mg daily for 5 days, then at 100 mg for 1 week, and finally at 100 mg every second day. The results were considered by the authors as excellent, mild side-effects appearing in only three cases.

In Brazil, in the same year, Sampaio and Provenza [234] treated eight patients suffering from ENL with daily doses of 250–300 mg thalidomide for 60 days, and they obtained fast symptomatic relief.

These extremely favourable results with the thalidomide therapy of the lepra reaction aroused considerable interest. Under the auspices of the WHO in 1967, a multicentre double-blind study was initiated [235] with thalidomide using acetylsalicylic acid (ASS) instead of a placebo because of its antipyretic and analgesic activity. The result of this study confirmed the previous reports of the efficacy of thalidomide in ENL and, moreover, it indicated that also ASS could be helpful in certain reactions of leprosy.

After this, there was further increase in publications of clinical trials with thalidomide in lepra reaction during the following years. De las Aguas and Duenas [236] presented a first paper in 1966 reporting on six patients who

received relatively small doses of thalidomide (100 mg/day initially, then 50 or 25 mg per day). Within 5–11 days the feverish temperature fell to normal after this treatment. In the following year, the authors reported on 39 patients who were now treated with somewhat higher doses of the drug (100–300 mg daily). The results were substantially similar [237].

In a third paper, De las Aguas and Rostoll [238] now had 122 ENL patients who had received therapy with 100–400 mg thalidomide daily. In all these patients the symptoms disappeared quickly. Again, in 1968, De las Aguas [239] published a paper reporting the results of a clinical trial in 123 patients, most of them with the general symptoms of ENL, but some with additional complications such as neuritis, orchiepididymitis, iridocyclitis and cutaneous exacerbations. The treatment consisted of 100–400 mg thalidomide daily. Especially remarkable was the rapid reduction of fever after this therapy. In 1969, the same author reported 88 cases with essentially the same treatment and results [240].

After 4 years of experience, De las Aguas [241] summarized his results in a review paper: in total, 159 patients with 269 acute incidents had been treated with thalidomide, the doses ranging from 100 to 500 mg daily. The cases included general lepra reactions, acute reactional neuritis, reactional tuberculoid disease, orchiepididymitis, iridocyclitis, and lesions of the pseudo-exacerbational type. In every case, the reaction ceased, and efficiency and rapid action of the drug were noted with respect to fever, neuralgia, and cutaneous lesions. Tolerance of the drug was excellent in all cases.

Other summaries after 6 years published by De las Aguas [242,243] dealt with a total of 165 cases of lepra reactions who were successfully treated with thalidomide.

The efficacy of thalidomide for relief of severe progressive lepra reactions was determined by Cazort and Song [244,245] in 24 patients who had been formerly treated with prednisolone. They all experienced clinical improvement within the 2-weeks trial in that the subcutaneous nodules and the erythema disappeared, pains of muscles, joints and nerves were lessened or completely eliminated, body temperatures returned to normal, elevated white cell counts and sedimentation rates were lowered, and the general condition of the patients improved. However, following withdrawal of thalidomide at the end of the trial, all patients had relapses, which indicated that brief treatment is not likely to interrupt permanently the cycle of recurrent or progressive lepra reactions.

Treatment of seven patients with 500 mg thalidomide daily produced rapid regression of the symptoms. The dosage was reduced to half thereafter, and sulphone therapy was started. With this, the patients were maintained free from inflammatory symptoms, even after suspension of treatment [246].

Prieto [247] used somewhat higher doses of thalidomide (600 mg daily) than

the previous authors, and he observed a rapid fall of fever to normal temperature within 4–5 days. The maintenance doses were also higher (300–400 mg, later on 100–200 mg per day). With this treatment, he achieved 100% cure in eight patients. This dosage is in striking contrast to that of Saul [248], who treated three patients with extremely low doses of thalidomide (50–100 mg/day) initially for 4 days, then 25 mg/day for about a week) with equally rapid fall of fever and complete resolution of the skin manifestations. Later, this author reported 30 cases of ENL which were treated with 200 mg thalidomide daily initially, and with maintenance doses between 25–50 mg per day, yielding good results, too [249].

Tarrabini-Castellani [250] reports 107 cases of ENL who were treated successfully with a preparation containing thalidomide, caffeine, phenacetin and acetylsalicylic acid ('Algosediv'). The initial daily dose of thalidomide was 75 mg, the maintenance dose 37.5 mg daily. He also claims to have obtained 100% cure in all patients.

Further confirmation [251–253] that thalidomide is effective in ENL and that its continuous administration prevents new attacks is provided by reports of the treatment of many patients with daily doses of 100 mg thalidomide.

In Brazil, Netto [254] had ten patients with ENL, among them one case of the tuberculoid form. Daily doses of 100 mg thalidomide brought fast relief of the acute symptoms and subsequently total recovery to all patients. In a Portuguese study [255,256], one case of acute lepra reaction was treated with 100 mg thalidomide daily after various other therapies had failed. However, at this dosage no improvement occurred. As soon as the dose was increased to 300 mg per day, progressive normalization of the reactional state became observable.

A report from Paraguay on the management of the lepra reaction with thalidomide showed that good results were obtained with daily doses of 100 mg already in the first 24–48 h. The maintenance dose was 25 mg [257].

A detailed study of a series of multiform lepra reactions and their successful treatment with thalidomide has been presented by Isla-Carande [258,259]. Mattos and Alonso [260] had 25 leprosy patients treated with daily doses of 100–300 mg thalidomide and observed improvement in all cases. One patient, after 15 months continuous treatment with thalidomide, had, nevertheless, a violent exacerbation of the disease, which was brought under control by increasing the dose to 500 mg per day. This case may indicate that continuous use of the drug can result in decreased effectiveness.

A study of 35 patients of both sexes on thalidomide therapy of ENL with initial doses of 100 or 200 mg reported that fever, general symptoms, and cutaneous lesions disappeared with great rapidity. Tolerance to the drug was good in all patients [261].

A group of six patients with ENL, prescribed 300–400 mg thalidomide daily, obtained relief within 2–3 days [262,263]. Two years later, this research group [264] had 24 patients successfully treated with thalidomide (400 mg/day), while only one person with the dimorphous form of leprosy did not respond to this treatment. Twelve years later, Gatti [265] again stated that thalidomide was the drug of choice in the therapy of ENL when given at dosages of 400–600 mg daily with maintenance doses of 50–100 mg/day until complete restoration.

A first report from Africa in 1968 mentioned 33 cases of lepromatous leprosy who were treated with daily doses of 400 mg thalidomide in cures of 7–14 days [266,267]. Three years later, Languillon [268,269] had 71 patients suffering either from an ENL type of reaction or a reaction equivalent to a neuritis of the cubital nerve with such complications as adenitis, iridocyclitis, orchitis or arthralgia. In all cases, treatment with 400 mg thalidomide daily for 7 days yielded spectacular effects of improvement.

Another single case of leprosy associated with severe furunculosis and accompanied by rhinitis, bleeding from the nose, and arthralgia, has been reported from Africa [207]. Spectacular improvement upon treatment with thalidomide (300 mg/day) was achieved also in this case.

An Italian study [271] of lepra reactions compared various therapeutic agents such as novacain, antihistamines, corticosteroids, benzydamine, hydroxychloroquine, antimony, griseofulvin, indomethacin, and thalidomide. The authors conclude that only steroids and thalidomide were satisfactory. Independently, Baccaredda-Boy and Bertamino [272] reported on their 2 years' experience with thalidomide in 23 leprosy patients who were treated with 150–300 mg/day initially until the acute symptoms ceased (usually within 10–17 days).

At the same time and also from Italy came a report from LaRosa and Casciano [273] on the results obtained with thalidomide (400 mg/day for up to 10 months) in 13 leprosy patients with various forms of the disease. The rapid resolutive action of the drug and its capability of interrupting recurrent reactions was again confirmed.

A double-blind controlled clinical trial of thalidomide vs. placebo has been carried out by Pearson and Vedagiri [274]. Twelve patients who had ENL for at least 10 months and up to 3.5 years previously, were allotted randomly to treatment with 100 mg thalidomide or placebo, respectively three times daily. After 6 weeks the treatment was reversed and continued for further 6 weeks. According to a special scoring system, thalidomide was shown to be significantly superior to placebo. It allowed concomitant reduction of other anti-ENL drugs, and it was clearly preferred by the patients themselves.

Three years' experience of treating 33 leprosy patients in Mexico with

thalidomide (200 mg initially, 25–50 mg maintenance dose) showed that in every case, there was improvement after 1–2 days, especially in fever and general condition [275]. Similar favourable reports have come from Argentina [276], Paraguay [277] and the U.S.A. [278].

Browne [279,280], commenting on the contemporary drug treatment of leprosy at the end of the 1960's, stated that thalidomide is extremely effective in rapidly controlling the acute exacerbations of lepromatous leprosy. He holds clofazimine (B 663, lamprene) and thalidomide as the only promising drugs for the treatment of this disease and its complications, respectively, but he warns against giving thalidomide to females of child-bearing years.

In the following years, clinicians seem to have lost much of their initial enthusiasm for thalidomide therapy for the lepra reaction. During the 1970's, only a small number of reports on clinical trials appeared, as compared with the previous flood of publications on this topic.

In 1971, an internally controlled double-blind clinical trial of thalidomide in severe ENL was undertaken by Waters [281] in ten adult male patients, all of whom were receiving steroids (not less than 15 mg prednisolone daily) or corticotrophin (18 units ACTH per day) concomitantly. The trial was divided into four parts of 4–6 weeks duration, consisting of an initial control period, two trial periods, and a final control period. Throughout the trial all patients received dapsone (100 mg twice weekly) and either thalidomide (300 mg daily) or placebo. As judged from the reduction of their steroid requirement, nine of ten patients in the group showed highly significant improvement. The author concluded from this result that the drug's undoubted beneficial action in ENL also outweighs any risk of side-effects.

Mohr [282] in Germany gave thalidomide to three patients with ENL (400 mg/day initially, 100 mg/day for maintenance) and after 7 days all reactions had subsided.

In Argentina, 80 patients with lepromatous leprosy treated with thalidomide (initial dose 400–500 mg/day, maintenance dose 50–300 mg/day) showed good responses in cutaneous symptoms and fever. The tolerance to the drug was excellent [283].

From India, a successful clinical trial of thalidomide in the treatment of leprosy reaction has been reported. Ramasoota [284] had 20 lepromatous patients with moderate to severe lepra reaction.

A study was made in 1973 in the United States on a long-term ambulatory management of ENL in 22 patients who had been on thalidomide therapy for a year or longer [285]. The efficacy of the drug was amply demonstrated: thalidomide is not only effective in the acute episode, but has also the ability in a high order to suppress recurrent ENL.

Londono and Rueda [286], at a meeting in Norway, reported on their experiences with prolonged treatment of lepra reaction, as well as on possible side-effects. The effect of the drug upon all features of lepra reaction (81 patients) was clear, despite the fact that the patients were receiving dapsone concomitantly. In some of the patients in this study thalidomide was stopped and the specific medication was continued, without there being any relapses. Reports on trials in Mexico conclude that this drug is the best available [287,288].

Iyer and Ramu [289] in India carried out an open trial with clofazimine on the management of recurrent lepra reaction using thalidomide as control. It turned out that the acute phase of the reaction was controlled readily with either of the two drugs. However, thalidomide exerted its action much faster, whereas the bacteriological assessment was superior in the clofazimine group. In the control of ENL, the effects of clofazimine are longer-lasting than those of thalidomide.

In another study, Ramu and Girdhar [290] treated 22 male patients showing a recurrent lepra reaction with a combined regimen of clofazimine and thalidomide and claim that this combined treatment controls the leprosy state more rapidly than monotherapy with thalidomide alone.

In the 1980s, only a few clinical studies have been performed with thalidomide so far. Comparison of the efficacy against ENL of thalidomide (daily dose 100–300 mg) and of chloramphenicol (daily dose 3 g) in 241 and 127 cases, respectively has shown that favourable results were obtained in 97% of those treated with thalidomide and in 82% treated with the antibiotic [291].

DeAlmeida Neto [292] in Brazil compared the efficacy of thalidomide and triamcinolone in 20 patients with ENL and erythema multiforme (Virchowian hanseniasis). According to the excellent results, he believes that both drugs present perfect and undeniable equivalence in the treatment of this syndrome.

In India, Theophilus [293] has been successful in treating patients with thalidomide (100–400 mg/day) who were either dependent on steroids (7 cases) or suffered from recurrent neuritis (13 cases). The results were classified as quite encouraging in both groups.

Spectacular results were achieved in lepromatous leprosy with thalidomide treatment (100 mg at first, later 50 mg daily) [294]. An unusual manifestation of ENL in one female patient, whose clinical history and picture was suggestive of acute systemic erythematous lupus rather than hanseniasis, has been described and commented upon by Petri [295]. Rapid regression of the symptoms was achieved with thalidomide (300 mg/day) in this case, also.

Pfaltzgraff [296], in an editorial, discusses the usefulness of short-term multi-drug chemotherapy of leprosy, which he believes to be a medical contraindication, but he admits that residual neuritis responds extraordinarily well to thalidomide.

Iridoclitis is one of the more severe manifestations of the lepra reaction. Sheskin and Zauberman [297] showed that thalidomide is also beneficial with this symptom. In this context, note must be made on the possibility of peripheral neuritis ('thalidomide polyneuritis') in leprosy patients treated with thalidomide for longer periods of time. The risk was emphasized in 1973 by Crawford [298]. Sagher [299] had earlier mentioned that this complication has not been observed in leprosy patients. Anyway, the suspicion remains, and it has triggered considerable research into this question in more recent years.

Warnings on the potential neurotoxicity of thalidomide were raised also by Berkeley [300]. In a reply, Convit [301] states that a great number of patients under relatively high doses of the drug for extended periods of time did not develop any polyneuritic manifestations. On the contrary, patients suffering from severe physical deterioration, impossible to control with the usual medications, felt a general relief and well-being upon thalidomide therapy.

In order to evaluate the effect of thalidomide therapy upon peripheral nerve inflammation, motor conduction velocity tests were performed at various intervals [302]. It was shown in this manner that the motor conduction test is a reliable indicator not only of the progress of nerve lesions but also of the efficacy of drugs such as thalidomide or prednisone in the lepra reaction. The same authors [303] described six patients suffering from acute ulnar neuritis resulting from leprosy reaction. These patients received therapy and they all showed a remarkable amelioration within a few days.

The condition of the peripheral nerve in leprosy has been studied under various forms of treatment, mainly sulphones, thalidomide and prednisone, in a long-term follow-up trial. All means of evaluation used showed that thalidomide has a more rapid effect than the steroid on the recovery of the neural lesions. Thalidomide also succeeded in preventing recurrence of the reactions [304]. Sheskin and Yaar [305], too, studied the motor conduction velocity of the ulnar nerves in 34 patients with lepromatous leprosy, 26 of whom had active reactions, the other 8 were 'burned-out' controls. All patients received long-term thalidomide treatment (6–13 years) in order to suppress the reaction. In none of the thalidomide-treated leprosy patients did any neurotoxic disturbances occur.

The toxic effect of thalidomide upon the ulnar nerve was studied in 13 patients suffering from lepromatous leprosy over a period of 1 year. The results of motor and sensory nerve conduction examinations were compared with six control leprosy patients receiving sulphones only. No subjective or objective, clinical or conduction findings indicative of a thalidomide neuropathy were detected [306].

Sabin [307] discussed the problem of differential diagnosis of thalidomide

neuropathy, which occurs after prolonged use of the drug in high doses, and leprous neuritis, which is a follow-up of the infection with *M. leprae*. He pointed out that these two forms of neuropathy might sometimes be confused, but there are differences which permit clinical distinction. However, it has already been stated elsewhere that severe ulnar neuritis – a frequent complication in ENL – is less likely to occur in patients receiving drugs like thalidomide [308].

It seems interesting that a recent epidemiological reinvestigation raises the hypothesis that potential neural damage caused by thalidomide might have promoted the entry of poliomyelitis virus into the central nervous system. Thus, thalidomide medication in 1960 in West Berlin might have been responsible for the increased poliovirus infections following vaccination at that time [309].

A few other neural complications of joints and bones are known. During the course of the lepra reaction, an inflammatory arthrosynovitis can sometimes be seen as a further serious complication. Two rare cases of this syndrome, one of which has been treated with 400 mg thalidomide per day, showed good response to this therapy [310,311]. Two other leprosy patients with additional inflammatory arthrosynovitis were treated successfully with thalidomide (200 mg/day) [312,313].

Another complication frequently associated with leprosy is massive osteolysis, which is observed, however, only in those cases in which neuropathy is present, too. Thalidomide is among the various therapies which can prevent or aid the patient in better tolerating its manifestations [314].

Finally, a fatal reaction to dapsone in one single case has been reported in the literature, and the possibility cannot formally be excluded that thalidomide was the agent which precipitated the fatal illness in this case. A patient suffering from ENL received concomitant treatment with dapsone (100 mg/day) plus thalidomide (300 mg/day) together with prednisone (20 mg/day). Three weeks after the start of this therapy he noted malaise, myalgia, rash, soare throat, fever to 40 °C and jaundice. Dapsone and thalidomide were discontinued and prednisone was increased to 60 mg/day until improvement of the condition occurred. The authors believe, however, that this extraordinary phenomenon is an extreme hypersensitivity to dapsone rather than a reaction to thalidomide [315].

With all these reports on thalidomide's clinical investigation in mind, the following conclusions can be drawn briefly:

(a) thalidomide has a unique and rapid suspending effect upon the lepra reaction;
(b) the active daily doses range from 50 to 500 mg;
(c) the administration of thalidomide enables the termination of corticosteroid therapy;

(d) it permits the resumption of specific antileprosy chemotherapy;
(e) thalidomide does not affect *M. leprae* directly;
(f) its mode of action is not yet fully understood.

However, there are several points concerning characteristics of the clinical response to thalidomide which deserve further comment:

(a) thalidomide's action develops slowly, within a few hours; the earliest time after administration at which a definite improvement has been noted is 8 h. Usually, approximately 48 h is required for clinical improvement to start.
(b) the fall of fever is also gradual, and ENL patients do not become afebrile until 48 h of treatment.
(c) the effective dose varies considerably; most authors believe that 400 mg per day in 2–4 divided doses would be optimal. After the ENL has been brought under control, a gradual reduction of the dose, to about 50 mg per day finally, is advisable. It would be wise, however, not to underdose the drug or to discontinue it abruptly, because severe recurrences have been observed, usually with a delay of 5–10 days up to about a month. In contrast, abrupt discontinuation of corticosteroid is followed by a reappearance of the syndrome within 1–2 days.
(d) patients who have been on corticosteroid therapy and concomitantly developed Cushing's syndrome, after switching to thalidomide, rapidly lose their Cushinoid features, while the ENL promptly stops. From this finding, it seems clear that thalidomide does not act in ENL via stimulation of endogenous corticosteroid production.

There are also a few side-effects of thalidomide treatment which must be noted here:

(a) High doses of thalidomide lead unequivocally to sedation, drowsiness and reduced alertness. This must not be forgotten, and patients must be reminded of their reduced ability to drive cars and the like.
(b) Dependent oedema is an often reported side-effect of thalidomide. This oedema is transient and usually disappears despite continued administration of the drug. Its cause is unknown, but it readily responds to a variety of commonly used diuretics [51].

To complete this section, it must be noted that there is also a minority of authors who advanced the opinion that thalidomide should not be used in the therapy of ENL, mostly because of its teratogenic action. The birth rate among leprosy patients is not negligible [316]. In part, they refuse to accept this therapy because they believe that ENL is not that serious and can be managed by alternative measures [298,300,317–320]. This opinion has led to some controversy [307,321]. Moreover, there are also reports of failures of thalidomide therapy. Unsatisfactory results have been reported by a few authors [218,255,322–324].

Finally, thalidomide has attracted considerable attention among the scientific community as a therapeutic agent for treatment of lepra reaction. It has been discussed in a medicinal consilium [325] and in a poster demonstration [326], and it has received wide approval also in numerous review articles and commentaries [179–182,188,192,194,196,197,203,327–343].

THALIDOMIDE IN OTHER SKIN DISEASES

The efficacy of thalidomide in lepromatous leprosy is now well established. It has converted the classical sedative-hypnotic agent into the 'drug of choice' for this condition. Many leprologists are today convinced that thalidomide is superior to any other treatment of the adverse reaction following the infection with *M. leprae*.

However, in the past few years, thalidomide has been shown to be an effective therapy in other inflammatory diseases of the skin and/or the mucous membranes (*Table 4.3*) (for reviews see Refs. 63,207,344). The therapeutic effect of thalidomide on some dermatoses is quite impressive, especially where conventional therapy is often disappointing. There are already a considerable number of reports available in the literature which suggest benefit due to thalidomide in several new fields.

Table 4.3. CUTANEOUS DISEASES IN WHICH THALIDOMIDE HAS BEEN REPORTED TO BE EFFECTIVE

Discoid lupus erythematosus	Pyoderma gangraenosum
Prurigo nodularis Hyde	Weber-Christian syndrome
Actinic prurigo	Postherpetic neuralgia
Aphthous stomatitis, Behcet's syndrome	

In this section, a review will be given based on the present publications on the therapy with thalidomide of various skin diseases other than ENL, except those with lesions in the mucous membranes of the gastrointestinal tract (which will be dealt with in the section 'Thalidomide and the gastrointestinal tract', pp. 212 ff).

Research into this novel application of thalidomide may become a challenging and profitable task for medical research scientists in the future. One can imagine that structure-activity relationship studies will contribute to a better

understanding of the mechanisms of action of the drug itself and/or to the knowledge of the underlying pathology in some of these diseases. It might even happen that further new applications of thalidomide will be found as a by-product of this research and, finally, that a separation of the wanted and unwanted effects of thalidomide will become possible, resulting in the development of a non-teratogenic analogue of higher activity against the inflammatory conditions.

LUPUS ERYTHEMATOSUS

Discoid lupus erythematosus or erythematodes is a skin disease of unclear etiology, characterized by variable, reddish to dark red, flattened efflorescences which are often confluent into large inflamed areas of about a hand's breadth in diameter. The lesions are mainly localized on the face, but other parts of the body may also be involved (disseminated form). Apart from ENL, lupus erythematosus is the most thoroughly investigated skin disease responding well to thalidomide treatment.

Rubio and Gonzalez [345,346] were the first to try the drug in 21 patients with discoid lupus erythematosus, associating anovulatories in 17 females of this group. Treatment was initiated with daily doses of 300 mg. Results were considered to be good, even dramatic in some cases. Side-effects occurred in only one patient, and treatment was stopped in this case. All others showed good tolerance, except for slight sleepiness in some of them. The authors expressed the opinion that the drug exerts its favourable effect through a mechanism of action upon the hypothalamus.

It took about 5 years for clinicians to notice this new indication of thalidomide, and then suddenly a large number of reports on clinical trials of the drug in this disease appeared in the literature. In an open study on thalidomide, 25 patients with chronic lupus erythematosus already resistant to chloroquine and other common drugs were treated [347]. The results varied considerably: in ten cases the lesions healed, six of whom had lasting healthy skin after cessation of the drug, but four relapsed after some weeks. In five cases, the lesions improved significantly, but needed maintenance therapy.

At about the same time, thalidomide was administered to 18 patients with discoid lupus erythematosus at initial doses of 300 mg daily for 15 days, then 100–150 mg per day for another 2 weeks, and finally 25–50 mg daily as maintenance dose. Improvement was obtained within a short period of time. No case of intolerance was recorded requiring withdrawal of therapy. This treatment was useful even in those patients who were resistant to corticosteroids and antimalarials, either systemically or topically. The authors [348]

tried also to verify the presumed immunosuppressive action of thalidomide by means of biohumoral trials in man and two laboratory tests in mice, viz., complement-dependent haemolytic activity and rejection of an allogenic tumour. However, the results obtained did not indicate any immunosuppressive action, but they did confirm the anti-inflammatory action of the drug.

Laugier and Gilardi [349] studied five patients with chronic lupus erythematosus of the upper face involving oedema of the eyelids, also designated as Degos disease or Morbihan disease, and found that no medication appeared as effective in this syndrome as thalidomide given at doses of 100 mg daily for periods up to 1 year. It was recognized that this treatment is effective only when it is given early in the disease; only then is total recovery possible. It was concluded that thalidomide is possibly the only effective remedy for this rare complaint [350,351].

A very extensive study has been performed using thalidomide in 24 patients (8 male, 16 female) with discoid lupus erythematosus (in the females, under concomitant contraception [352]). Thalidomide proved to be highly active, with the therapeutic effect becoming obvious after approximately 4 weeks.

In a second uncontrolled study, the same authors [353] investigated the therapeutic effect of thalidomide on chronic discoid lupus erythematosus in 17 women and 7 men. Complete or substantial regression of the complaints was observed in 19 cases after treatment with thalidomide as before. Side-effects such as somnolence, constipation, exanthema, dry mouth and circulatory disturbances occurred but were not too serious.

Finally, in a third study, on 60 patients with chronic discoid lupus erythematosus who were followed for up to 2 years, the authors [354] found in 90% of cases a complete or marked regression of the disease. But when thalidomide was stopped, 71% of the patients relapsed. Patients undergoing a second and, in part, a third course of thalidomide treatment again responded well. Mild side-effects were common, and 25% of patients complained of slight to moderate polyneuritic symptoms. The authors concluded that thalidomide is an effective drug in lupus erythematosus, but it exerts its effect in most cases only whilst treatment is continued. A warning has been added: because of the danger of developing polyneuropathy, thalidomide's use should be restricted to persons resistant to topical steroids and systemic antimalarials. Several trials with a small number of patients gave good results [326,355–357].

Hasper [358] conducted a clinical trial in 11 patients with severe chloroquine-resistant chronic lupus erythematosus; in this trial, he gave initially 100–300 mg of the drug per day and maintenance doses of 25–50 mg daily; duration of treatment varied from a few days up to 18 months. Seven patients responded with complete remission; in two of them conditions improved

significantly. One patient did not respond well to therapy, and another had to be withdrawn from the trial because of side-effects. Six patients who relapsed after discontinuation of thalidomide therapy were retreated with maintenance drug dosages and achieved good results with no further relapses or exacerbations. In all subjects the side-effects were minor and reversible. Patients with chronic, subacute cutaneous and discoid lupus erythematosus have greatly improved [359–362].

Electromyographic studies in 26 patients under thalidomide therapy for discoid lupus erythematosus point to a mainly sensory, distal and axonal polyneuropathy which correlates in its severity with the total dose of the drug given during the course of therapy [363]. Lastly, Lauret [364,365] points to the usefulness of thalidomide in the management of this skin disease.

PRURIGO NODULARIS

Prurigo nodularis Hyde is an unusual disorder of the skin characterized by extremely pruritic nodules about 0.5 to 3 cm in diameter. The lesions are located chiefly on the extremities, but may involve the trunk as well. They are firm, smooth or verrucous, erythematous or pigmented, and often excoriated. Pathologically, the lesions show marked irregular acanthosis, the dermis contains a nonspecific inflammatory infiltrate. The origin of the disease is unknown and, much worse, most of the classical therapies, such as steroids and antipruritic agents, have been unsatisfactory. Therefore, thalidomide's alleviating action on pruritis nodularis has been unequivocally welcomed [366].

In 1975, in a preliminary communication, Sheskin [367] referred to the good results which he had achieved with 300–400 mg thalidomide daily in three patients suffering from this complaint. In the same year, Mattos [368] reported two cases of prurigo nodularis Hyde which were treated successfully with daily doses of 200 mg thalidomide at first and, later on, with reduced doses of 100 and 50 mg of the drug per day, respectively. The skin lesions improved within 3 weeks and disappeared completely after 4 months.

Five years later Van den Broek [369] confirmed the earlier findings. He reported one case of prurigo nodularis unresponsive to conventional therapy, e.g., topical and systemic corticosteroids, antihistamines, antipruritic agents, dapsone and isoniazide, as well as to psychotherapy and to anticonvulsive treatment (clonazepam), which was successfully treated with thalidomide. Other workers [370–372] have made similar reports.

In another study [373] of five patients suffering from this prurigo, the concentration of iron, copper and zinc in the skin of the patients was measured by diagnostic X-ray spectrometry before and after treatment with thalidomide.

Before treatment, larger skin lesions were associated with significantly higher zinc and iron levels than the smaller ones. Upon treatment, the zinc and iron content in these lesions decreased towards normal range. Copper values were found to be within the normal range before and after thalidomide treatment. A similar result was obtained in an investigation of the skin content of the three metals in patients suffering from lepra reaction. The iron levels were highly elevated in the affected areas. However, in contrast to the fast clinical improvement which followed the treatment with thalidomide, the iron levels did not decrease for prolonged periods [374].

The danger of developing neuropathy after prolonged intake of thalidomide was emphasized again recently [375]. Of six patients treated with thalidomide (50–300 mg daily for 6–22 months) for either prurigo nodularis or discoid lupus erythematosus, four had paraesthesia in the hands and feet, and one complained also of muscular pain and stiffness. Clinical neurological findings in all four patients were normal, but subsequent electrophysiological examination disclosed peripheral neuropathy in five of the six subjects. The neurotoxical symptoms were rapidly reversible after discontinuation of the drug. Once more, this indicates that the use of thalidomide should continue to be very carefully controlled, and it appears to be justified only in case of otherwise resistant and severe forms of this disease.

ACTINIC PRURIGO

Actinic prurigo – synonymous denominations are Hutchinson's disease or summer prurigo, among others [376] – is an evolutionary form of chronic polymorphous light eruptions. Its fundamental clinical characteristics are infiltrated, papular and eczematous lesions of the face, as well as isolated plaques on the exposed areas of the limbs. Cheilitis and vernal conjunctivitis are common. The evolutionary characteristics are onset in childhood and persistence with few changes throughout the life of the patient. The condition is believed to have a genetic origin because the majority of reported cases have occurred in American Indians and persons who have a mixture of Indian blood. It may have an immunological mechanism and a similar pattern to lepra reaction as well [365,377,378].

There are several reports available on this unusual sensitivity to ultraviolet irradiation and its successful treatment with thalidomide, which also in this condition appears to be the treatment of choice at this time [379]. Londono [377] seems to have been the first to use thalidomide in photodermatoses. In 1973, he treated 34 patients suffering from this complaint with an initial dose of 300 mg thalidomide for adults and a proportionate dose for children. This dose was maintained until a significant improvement was observed, afterwards

it was decreased progressively, the smallest dose being 15 mg daily. Patients did not receive any other treatment and were not advised to avoid light exposure. In 32 out of the total number of cases good progress was achieved, one was fair and another one showed no change. Clinical improvement was noticed in a range of 21 to 91 days with an average of 50 days. In all cases when the drug was discontinued, the lesions reappeared [380].

Two years later, Flores [376] treated 25 patients and the results were recorded as excellent or good in 88% and as poor in 4% of cases. Only 32% relapsed after discontinuation, but 25 mg thalidomide daily as a maintenance dose avoided the relapse.

In 1977, Calnan and Meara [381] published their experiences with a total of 51 patients with this skin disease. The efficacy of thalidomide was again confirmed. Very recently, 14 patients suffering from actinic prurigo were reported to have been successfully treated with thalidomide [382]. One patient was unable to tolerate a sufficient dose because of dizziness. Eleven patients, however, showed lasting improvement on 50–100 mg of the drug per day. Two patients relapsed on therapy, despite initial improvement. No major side-effects were observed, although minor reactions were frequent, mainly somnolence.

Attention has been paid to the use of thalidomide in photodermatoses in review articles, and the differential diagnosis of various infectious exanthemata has been reviewed [383].

APHTHOUS STOMATITIS

There are numerous forms of recurrent aphthous ulcerations which may, apart from the buccal mucosa and the lips, involve several organs, most often the genitals and the surrounding area, such as scrotum, glans penis and prepuce, or perineum, vulva and vagina. Sometimes both parts are affected (orogenital or bipolar aphthose, Neumann's syndrome), and in some cases also the eyelids are involved (ocular aphthose, Behcet's syndrome). The disease can be generalized [384], that is, when visceral parts of the skin are associated with one or several of the former (general aphthose), and at least the cutaneous lesions can be accompanied by vascular and articular manifestations, also. An extensive survey on the multiple forms of recurrent aphthous stomatitis, the clinical manifestations, etiological features and therapies was recently published [385].

The exact pathogenesis of recurrent aphthous ulcerations is still obscure, although an involvement of the immune system may be assumed with some certainty. In this context, it is interesting to mention that the latter authors [385] regard contemporary treatment with corticosteroids and immunosuppressants

as very disappointing, but they see remarkable progress in the addition of thalidomide to the therapeutic armamentarium. Particularly in severe attacks, they suggest treatment with 100 mg thalidomide daily, which can be reduced to 50 mg daily or every second day during the maintenance phase. This treatment leads to cessation of the pains within 24–36 h and is followed by rapid regression of the cutaneous lesions during the following days.

Thalidomide was first used on six cases of recurrent and necrotic mucocutaneous aphthae, which were treated successfully with this drug [386]. The same authors [387,388] reported on their experiences with thalidomide in the treatment of recurrent, necrotic and giant aphthosis (nine cases) and Behcet's disease (nine cases) during a 4-year period. The results were equally good in both syndromes and, additionally, satisfactory results were seen also in uveitis, but not in arthritis, thrombophlebitis or neurological symptoms. The recommended dose is 100 mg thalidomide daily for 10 days; higher doses do not appear to give better results. These authors and others [365,389–393] consider thalidomide as the most active medicament, particularly in severe cases with profusion of necrotic aphthae and in controlling the symptoms of Behcet's disease.

Treatment has been described [394] with either thalidomide or colchicine of 25 patients with recurrent oral, mucocutaneous aphthosis, in part with visceral involvement, and with Behcet's disease. Thalidomide was given either alone (200–300 mg/day) or associated with colchicine (2–3 mg/day). Major improvement was noted in all patients, with rapid nealing of mucous lesions and reduction of pain and burning. No new outbreaks were noted on maintenance therapy with 50–100 mg thalidomide and 1 mg colchicine daily.

Roge and Testas [395] described a patient with severe multiple localizations including an intestinal ulceration. The patient was treated with thalidomide (300 mg/day), and he soon experienced general improvement, healing of skin and mucous membrane lesions, except for the instestinal affection, which remained and had finally to be resolved by colectomy.

Bowers and Powell [396] had three patients suffering from vulval and oral ulcers, respectively, who responded well on treatment with thalidomide (200 mg/day initially for 5 days, then reduction to 100 mg/day for several weeks). The ulcers healed completely. After cessation of the drug the ulceration returned, but again under treatment the symptoms disappeared readily.

However, in the same year, some controversial comments on the activity of thalidomide in orogenital ulceration appeared [397]; these ascribed the favourable effects to an immunosuppressive mechanism of action. Efthimou and Spiro [398] criticized the use of thalidomide in skin disease. However, the improvement of the patients with Behcet's disease must have been so impressive that it prompted even headlines in the lay press [399].

OTHER CUTANEOUS DISEASES

In this subsection, favourable effects of thalidomide in other skin diseases will be discussed. These manifestations are either rarely found, or are the subject of only a few cases reported so far in the literature.

One such condition is the so-called Weber-Christian syndrome or non-suppurative panniculitis, an inflammation of the subcutaneous fatty tissue which is regularly accompanied by fever, swelling of the extremities, and pain. Painful skin nodules spread over the limbs and the trunk. The cause of the disease is unknown.

Eravelly and Waters [400,401] report one case of this disease which rapidly improved under thalidomide therapy. The drug was administered at a dose of 300 mg daily, i.e., 100 mg in the morning and 200 mg at night The lesions steadily regressed, and the dosage was reduced to 200 mg/day after 3 weeks, then 100 mg/day after 10 weeks, and stopped altogether after 13 weeks. No relapse occurred after cessation of treatment. Although there is only one case available so far, the benificial effect of thalidomide in this syndrome is nevertheless remarkable.

Another cutaneous condition is pyoderma gangraenosum, which has also been treated successfully with thalidomide in a few cases [350,351]. One case of this disease, associated with chronic myeloid leukaemia, which was treated with 200 mg thalidomide daily, resulted in a spectacular regression of the skin lesions. After 4 weeks, the dose was reduced, and after 13 weeks the drug was discontinued. No relapse occurred, but the leukaemia was decompensated. The use of thalidomide in pyoderma gangraenosum has been suggested also by De Cock [402] and others [391].

A case of extensive pyoderma gangraenosum that occurred in a 3-year-old girl has been reported by Venencie and Saurat [403]. No etiologic factor of the disease could be found. Various medications, including clofazimine, prednisone and minocycline, were tried, but all were ineffective. Administration of thalidomide (150 mg/day initially, then 100 mg/day) led to complete recovery.

Lauret [365], in a recent review, refers to several dermatological affections which have also been treated successfully with thalidomide, although only in very rare cases. This author cites three patients with hidroa vacciniforme, a light-sensibilized dermatitis with red papules. They received 7 mg/kg thalidomide, which gave spectacular improvement within a few days. This result, however, could not be reproduced by the author [365] in another case of this disease.

Lauret [365], in his review article, refers also to one case of disseminated actinic protokeratosis in a postmenopausal woman. The complaint was readily

overcome with 100 mg thalidomide per day. Patients suffering from malignant atrophic papulosis (Degos disease) [404], erythema multiforme [405], and cutaneous sarcoidosis [406,407] have also benefited greatly.

Several years ago, Aitken [89] tried thalidomide in five patients with psoriasis. This chronic, non-infectious skin disease with characteristic scales is often associated with haemorrhages which preferentially affect the head and the extremities. Treatment consisted of daily doses of thalidomide ranging from 25 mg up to 400 mg, given for 3–15 days. However, except for slight somnolence, no effect on the cutaneous lesions could be observed.

In another three cases of psoriatic erythrodermia, thalidomide was administered in small doses (25 mg/day), but for relatively long periods of time (up to 2 months); however, there was no positive effect either. No result was achieved, either, in two cases of atopic eczema [89]. Furthermore, thalidomide was ineffective in a small number of different, but mainly cutaneous, affections such as dermatitis herpetiformis (Dühring-Brock disease), herpes zoster, peripheral arteritis, ichthyosis, as well as in an asthmatic crisis, an adrenopathy, and in collagenosis.

THALIDOMIDE AND THE GASTROINTESTINAL TRACT

GASTRIC ULCERS

The effect of thalidomide in experimental gastric ulcers was studied in rats. Whereas thalidomide inhibited stress-induced ulcers to some extent, it was quite ineffective in phenylbutazone-induced ulcers [408]. Other authors [409] found that the drug at doses of 400 mg/kg, together with an anticholinergic drug, reduced the incidence of the lesions to a much higher extent than single administration of each drug.

An incidental observation of a complete regression of dyspeptic syndrome, together with amelioration of concomitant diarrhoea, in a patient who received thalidomide for treatment of Behcet's disease has been reported in the literature [393]. The antidiarrhoeal acitivity of thalidomide and its analogues may be due to a depression of peripheral ganglionic transmission by these compounds [335].

A clinical trial in a large number of patients of both sexes suffering from gastric and duodenal ulcers and liver diseases had been earlier performed by Luchmann [410,411]. In the group with gastrointestinal disorders, thalidomide was administered at doses between 12.5 and 37.5 mg daily for 4–12 weeks. There was subjective and objective improvement of the condition in the majority of cases.

COLITIS ULCEROSA

This type of colitis is an obstinate inflammatory disease of the large bowel, commonly aggravated by more-or-less extended ulcers, severe abdominal pain, and diarrhoea with blood and mucus in the stool. A related but more serious form which extends also to the upper parts of the gut is Crohn's disease [412].

The first report about thalidomide treatment in this disease, in 1979, concerned a female patient suffering from ulcerative colitis which was resistant to treatment with metronidazole, steroids and sulphasalazine, respectively [413–415]. Thalidomide was started (under contraceptive cover) at a dosage of 300 mg daily, but since there was no immediate response the dosage was increased to 400 mg/day. The patient steadily improved. By 2.5 months, blood in the stool had disappeared and the overall condition seemed to have returned to normal. The dosage of thalidomide was gradually reduced to 200 mg/day without clinical relapse. There was no sign of neuropathy. After 18 months of continuous treatment, the patient was judged to be in remission.

MECHANISM OF ACTION IN INFLAMMATORY CONDITIONS

The lepra reaction and most of the other cutaneous reactions are believed to be a clinical manifestation of an immune-complex or 'Arthus' type of immediate hypersensitivity. There are three types of drug known so far to be effective in such diseases:
the glucocorticoids
cytotoxic immunosuppressants
non-steroidal anti-inflammatory drugs (NSAIDs).

ARTHUS-TYPE MECHANISM OF ACTION

The assumption that the lepra reaction is an Arthus phenomenon finds support from one observation [416] that granular deposits of immunoglobulin and complement were found in the dermis of lesions from patients with ENL. The distribution of these deposits corresponded with the areas of polymorph infiltration. The level of complement in the serum was also elevated.

However, other workers [417], studying the skin lesions in biopsies of patients with various types of leprosy by means of an immunofluorescence technique, did not find any granular immune complexes in or around the vessel walls or in the inflammatory infiltrates. Others [418] proposed that the reversed passive Arthus reaction in the rat be used as a screening model in the search

for more effective anti-inflammatory and antirheumatic agents. They tested various known drugs with this model, among them thalidomide. As expected, thalidomide at 25 mg/kg was found to give good protection against the reaction of about 40%, reaching a peak between 2 and 3 h after administration of the drug.

Concentrating on the lepra reaction, a hypothesis has been put forward which basically considers ENL as an Arthus-type hypersensitivity reaction [99,419]. In this reaction, a series of events could proceed as follows (*Figures 4.9* and *4.10*). Antigens from non-viable *M. leprae* are released from inside phagolysosomes of macrophages in patients developing ENL, and antibodies of the precipitating, complex-fixing type are produced. Then, these antigens and antibodies combine to form an antigen-antibody complex which precipitates in or near blood vessel walls and fixes complement. In some cases, the immune complex may be solubilized, leading to circulating immune complexes. The chemotactic factors released from the complement attract neutrophils to the area of the precipitated immune complex. The accumulated neutrophils phagocytize the immune complex, and in the process they release lysosomal enzymes from their granules which, in turn, lead to tissue destruction with symptoms such as vasculitis, local oedema, necrosis and pain.

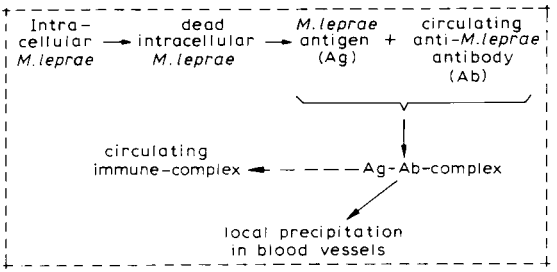


Figure 4.9. Immunologic events in ENL (after Hastings [99]).

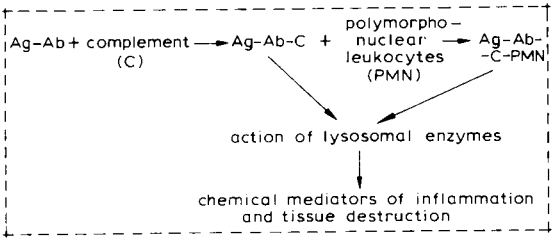


Figure 4.10. Inflammatory events in ENL (after Hastings [99]).

For thalidomide to be effective against this reaction, it reasonably must interfere somewhere in one or more essential steps of this cascade of consecutive processes. Hastings [99] lists the mechanisms or sites of action, respectively, where thalidomide can be effective.

First, there is no evidence that thalidomide influences in any way the viability of *M. leprae*, such as killing the bacteria. The next step, closest to the source of the disease, would be the interference of thalidomide with the release of antigen from non-viable *M. leprae* from macrophage phagolysosomes. A widely known model of lysosomal membrane stabilization was chosen to test this hypothesis; the *in vitro* release of a marker enzyme (3-glycerophosphatase) from partially purified rat and human liver lysosomes. Thalidomide, in concentrations equivalent to those achieved in man by therapeutic doses, indeed stabilizes the lysosomal membrane significantly. Thus, at least part of the drug's action in ENL may involve retardation of the release of antigens.

A third point of interaction of thalidomide could be the antibody formation to antigens of *M. leprae*. This hypothesis was tested in mice immunized with sheep erythrocytes. Briefly, thalidomide inhibits the *de novo* antibody synthesis of the IgM class to the thymic-dependent antigen, but not to the thymic-independent antigen. Since thalidomide-treated ENL patients were found to have significantly reduced IgM serum concentrations, too, it would seem likely that thalidomide interacts in some way with the antibody formation.

Thalidomide could also act at the step of precipitation of released antigen with pre-formed antibody to decrease the amount of immune complex formed. However, no evidence was found for this assumption in a quantitative precipitin test *in vitro* using ^{131}I-labelled bovine serum albumin and purified rabbit antibody to this albumin [99].

Interference of thalidomide with neutrophils is possible, either with their early phases of development or their late function. However, the drug does not cause neutropenia in mice when fed with human equivalent doses for 1–4 weeks.

Thalidomide might also inhibit the release of lysosomal enzymes from intact phagocytizing neurotrophils. However, experiments *in vitro* using purified human neutrophils did not show any decrease in the release of a marker enzyme (β-glucuronidase) from phagocytizing cells when thalidomide was added. On isolated lysosomes from guinea-pig peritoneal exudate neutrophils, thalidomide did not show significant activity in stabilizing the lysosomal membranes, either [99].

A common model for testing of the various steps involved in the post-immunologic, purely inflammatory events in the body is the carrageenin-induced rat paw oedema. This screening procedure has been chosen to estimate thalidom-

ide's potential anti-inflammatory activity, and it was found that the drug antagonizes the oedema in human equivalent doses [99].

Thalidomide also exerts significant antagonism towards prostaglandin F_{2a}, prostaglandin E_2, acetylcholine, histamine and serotonin, but not towards bradykinin on isolated muscle preparations. However, since many non-steroidal anti-inflammatory drugs (NSAIDs), antihistamines and anti-serotonins (methysergide) are not clinically effective in ENL, the antimediator activity of thalidomide is probably not relevant for its mechanism of action in ENL.

On Hasting's judgement, thalidomide appears to have two relevant sites of action in ENL: one is an immunosuppressive site of action involving inhibition of IgM antibody synthesis, and the other is an anti-inflammatory site of action, manifested by the drug's ability to inhibit inflammation *in vivo*, probably due to interference with neutrophil chemotaxis and reduction of phagocytosis by these cells [99].

INTERFERENCE WITH IMMUNOGLOBULIN SYNTHESIS OR ACTION

Investigators into thalidomide's ability to affect the *de novo* antibody synthesis [420–424] used the effect of the drug on antibody response to sheep erythrocytes in mice. Thalidomide significantly inhibited IgM antibody formation when fed to the animals for 5–7 days before immunization with the foreign blood cells. There was also a selective decrease in serum IgM concentrations among leprosy patients being treated with thalidomide for ENL.

Other workers [425], in a study of antibody response in leprosy, confirmed that there was a high frequency of antimycobacterial antibodies in the sera of patients with tuberculoid and indeterminate leprosy. Various strains of *M. leprae* all contained at least one common antigen. Treatment effective in the elimination of symptoms, e.g., thalidomide, however, did not appreciably alter the levels of antibodies in the sera of these patients. Furthermore, no appreciable diminution of active and passive Arthus reaction, cutaneous anaphylaxis, and of antibody production was observed in guinea-pigs [426].

Other workers [427–432] have tried to identify the action of thalidomide on immunological reactions, without much success.

IMMUNOSUPPRESSANT ACTION ON THE CELLULAR LEVEL

The second assumption of Hastings [99], i.e., that thalidomide's action could manifest itself mainly in an inhibition of inflammation *in vivo* through interference with neutrophil chemotaxis and reduction of phagocytosis, found some experimental support. However, over the years [433–451] groups of other workers have failed to obtain positive results to support this idea.

ANTIMEDIATOR OF INFLAMMATION ACTIVITY

From all the possible mechanisms of action mentioned so far, on a molecular level, the possibility to act as an antagonist of one or several of the endogenous mediators of inflammation seems to be most logical and acceptable. Theoretically, thalidomide could either decrease the formation of the natural mediators, increase their catabolism, or block their effects by occupying the specific receptors in either a competitive or a non-competitive manner, or by interacting with the mediator directly in a purely chemical fashion.

Such mediators of inflammation would include compounds like bradykinin, histamine, serotonin, prostanoids and leukotrienes (*Figure 4.11*). Any or several of these mediators may well play a role in the hyperemia, oedema and pain of the cutaneous lesions discussed in detail in the previous subsections. Components of the complement of the antigen-antibody reaction are known to be mediators in their own right. Additionally, the intrinsic clotting mechanism has been implicated in a number of inflammatory states, and activation of kallikrein by the Hageman factor (Factor XII) is well known. Bradykinin is also known to act as a chemotactic agent for polymorphonuclear leucocytes in the inflammatory process.

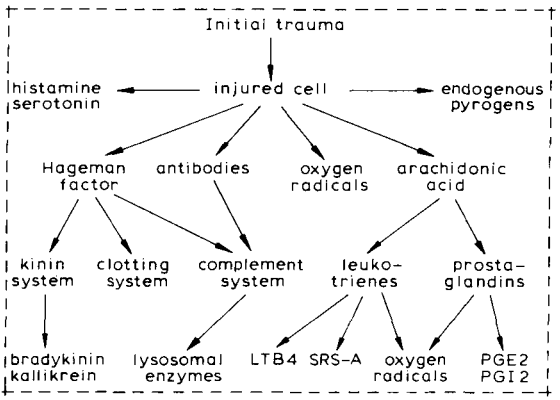


Figure 4.11. Formation of inflammatory mediators.

In an attempt to investigate the characteristic anti-inflammatory effects of several antileprosy drugs, thalidomide was tested [452] in several experimental models. There was a weak inhibition of carrageenin-induced rat acute oedema, as well as of the exudation produced by low-molecular-weight mediators, e.g., of bradykinin. The drug inhibited weakly the emigration of leucocytes when examined by the carboxymethylcellulose pouch method. It weakly stabilized the rat erythrocyte membrane under heat and osmotic shock, and it showed a weak inhibitory effect upon the exsudation into the peritoneal cavity of the mouse. However, in adjuvant-induced arthritis of the rat, thalidomide showed no inhibition at all.

Rea and Taylor [453] found that the lysozyme levels in patients with untreated leprosy were 2–3-times those in healthy controls. Patients with severe lepra reactions and with Lucio's phenomenon had the highest lysozyme patterns. Prolonged sulfone therapy was associated with a fall in serum lysozyme values, but thalidomide therapy, although leading to prompt and dramatic resolution of the acute inflammatory lesions and of neutrophilia, did not change serum lysozyme levels. Thus, the question remains open whether lysozyme does have any role in leprosy reaction. Several years ago, workers [454,455] also found that thalidomide inhibits lipoxidase activity, *in vitro*.

Finally, there is some experimental evidence that thalidomide can reduce ascorbic acid levels in the liver and, thus, interfere with microsomal hydroxylase activity which, in consequence, gives rise to some speculation on the possible mechanism of action of the drug [456].

CONGENERS AND ANALOGUES OF THALIDOMIDE

During the past 20 years, numerous compounds structurally and pharmacologically related to thalidomide have been synthesized and tested for their biological actions, mainly for teratogenicity and/or (in part) for hypnosedative effects. These results have already been reviewed elsewhere [8]. Here, we restrict ourselves to those reports dealing with thalidomide analogues which have been screened for:

(a) anti-inflammatory activity, especially in ENL;
(b) immunosuppressive effect;
(c) potential antineoplastic activity; and
(d) other effects.

THALIDOMIDE ANALOGUES FOR TREATMENT OF ENL

Soon after the discovery of thalidomide's favourable effect in the lepra reaction, a series of analogues and derivatives of thalidomide was studied in an attempt

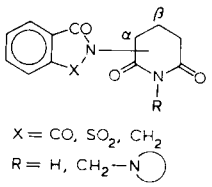


Figure 4.12. Thalidomide analogues. General formula showing some structural variations of the original drug.

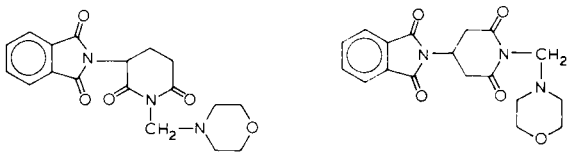


Figure 4.13. Two Mannich base derivatives of thalidomide; 1-morpholinomethyl-3-phthalimidopiperidine-2,6-dione (CG-601) and 1-morpholinomethyl-4-phthalimidopiperidine-2,6-dione (CG-603).

to find novel compounds which have at least the same effectiveness in ENL and, moreover, might be well tolerated and possibly non-teratogenic. The variations in the original drug are summarized in the general formula shown in *Figure 4.12*.

Assuming that the compounds would have an immunosuppressive effect, they were tested in homologous skin grafts in rats and in allergic encephalitis in guinea-pigs. From these efforts, two compounds emerged as candidates for further investigation, since they had shown a certain degree of immunosuppressive action in both models, and low toxicity in the acute and chronic toxicity tests. The two isomeric Mannich bases were given the code designations CG-601 and CG-603, (*Figure 4.13*).

Sheskin and Sagher [458] tested CG-601 and CG-603 clinically in patients with acute lepra reaction. CG-603, which hydrolyzes in aqueous solution to yield 4-phthalimidoglutarimide (isothalidomide), was given to four patients in doses up to 2 g/day and had no effect. CG-601, on the other hand, was used in 13 patients at a dose of 900 mg/day, and this regularly caused improvement within 24–76 h. No untoward side-effect with either compound was noted.

When CG-603 was given to one patient with ENL at doses of 36 mg three times daily, after 8 weeks, the cutaneous symptoms had completely disappeared. Similar results were achieved in another patient who was treated with

thalidomide for comparison. The improvement in both cases has been documented with impressive photographs of the lesions before and after treatment [178].

Sheskin [459,460] tested nine derivatives of thalidomide in patients with lepromatous leprosy. Three of these compounds, all teratogenic, proved to be active, although slightly inferior to thalidomide.

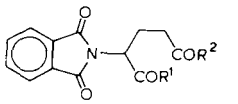
Sheskin [228] points out that in those thalidomide analogues which have been tested for their activity in the lepra reaction, those which were effective were also teratogenic. He supports the idea that the ENL-suppressing property may be linked to the teratogenic factor. Facing the advantages offered by thalidomide treatment, he states that research in this field should persevere in order to find an efficient, rapidly acting medication applicable without reservation to both sexes and to all ages.

Clinical results have been obtained with five thalidomide-related cyclic imides in 42 patients suffering from ENL and 9 patients who were treated with thalidomide for comparison [461]. The test compounds were dosed 400–600 mg daily, while thalidomide was given at daily doses of 400 mg. All compounds were found to be equally active.

A clinical trial with the non-teratogenic thalidomide analogue supidimide (EM-87, CG-3033 *Figure 4.3*) [463] to find out its potential to suppress the lepra reaction showed that the drug was ineffective at all dosages applied. Since supidimide has a sedative action almost identical to that of thalidomide but is devoid of teratogenic effects in animals, the authors believe that it should be possible to dissociate the anti-ENL activity from the teratogenic potential in thalidomide-analogous compounds.

In the United States, Hastings [99] screened numerous thalidomide analogues in two *in vivo* models which, in his opinion, reflect at best the relevant sites of action in ENL of the parent compound. This is, first, the *in vitro* assay of direct plaque-forming cells (PFC) in the spleens of mice 4 days after immunization with sheep erythrocytes as a measure of the ability of the compounds to inhibit IgM antibody synthesis; and, second, the late (6 h) carrageenan-induced rat paw oedema (CRP) as an *in vivo* measure of the ability of the test compounds to inhibit neutrophil chemotaxis.

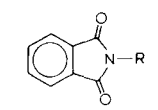
Hastings [99] reports the results which have been achieved with ten phthalimide derivatives screened in both assays. The structures and activities of these compounds can be seen from *Figures 4.14* and *4.15*. Whereas thalidomide exerts high activity in both systems, most of the test compounds are either inactive or are slightly active in only one of them. Nevertheless, the author is optimistic insofar as he concludes from the results that teratogenicity is separable from anti-inflammatory and/or immunosuppressant activity, and



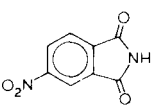
R^1	OH	OH	NH_2
R^2	OH	NH_2	OH

all PFC(+) CRP(−)

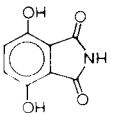
Figure 4.14. Thalidomide metabolites tested for activity in ENL (after Hastings [99]). PFC = plaque-forming cells; CRP = carrageenan rat paw test; (+) = active; (−) = inactive.



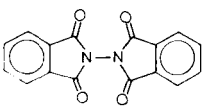
R=H,OH,Et,nBu, Ph, 3 OH—nPr
all PFC(−) CRP(−)



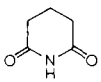
PFC(−)
CRP(−)



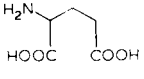
PFC(−)
CRP(−)



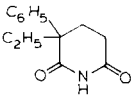
PFC(−)
CRP(−)



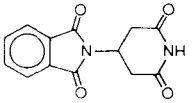
PFC(−)
CRP(−)



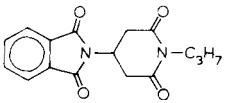
PFC(−)
CRP(−)



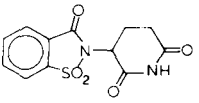
PFC(+)
CRP(+)



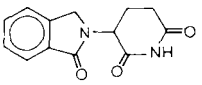
E−350
PFC(+) CRP(−)



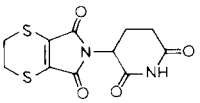
E−565
PFC(−) CRP(−)



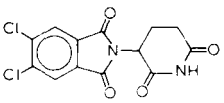
EM−8
PFC(−) CRP(−)



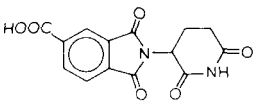
EM−12
PFC(−) CRP(+)



E−35
PFC(+) CRP(−)



E−48
PFC(−) CRP(−)



E−122
PFC(−) CRP(−)

Figure 4.15. Thalidomide analogues tested for activity in ENL (after Hastings [99]). PFC = plaque-forming cells; CRP = carrageenan rat paw test; (+) = active; (−) = inactive.

he raises the hope that a non-teratogenic thalidomide analogue can be found which will also be clinically effective in ENL.

At Carville National Hansen's Disease Center (U.S.A.), as is stated in an overview of this institution's activities [464], approximately 50 thalidomide analogues have been screened so far for their activity relevant to the treatment of ENL. Besides thalidomide only one compound, taglutimide (Biglumide, K 2004, *Figure 4.3*) [465–472], has been brought to clinical trial. In the first three patients with ENL treated with this compound, no anti-ENL activity was demonstrated, while the reaction was controlled by conventional thalidomide treatment. The failure of taglutimide has been explained by a lack of significant anti-inflammatory activity in this drug [464].

ANALOGUES WITH IMMUNOSUPPRESSIVE ACTIVITY

The anti-inflammatory effect of thalidomide and its analogues is closely connected with the (assumed) immunosuppressive activity which has been deduced (mainly) from their ability to prolong skin and organ transplant survival in animals and, in part, also from the alterations induced in cellular and humoral antibody production. These test models have already been considered in the section 'Other actions of thalidomide' (pp. 171 ff). In this present subsection, we refer only to some additional analogous compounds which have been tested in a similar manner.

The two derivatives of thalidomide already mentioned, CG-601 and CG-603, are equally effective in prolonging skin homografts in rats [65] but both compounds, following intraperitoneal injection into mice with skin homotransplants, showed such severe toxicity that no comment could be made on their immunosuppressive action [69].

An inhibition of PHA-stimulated blast transformation in human mixed lymphocyte cultures with CG-601 and CG-603 (10 μg/ml) was observed [91]. The incorporation of thymidine was not inhibited by the same concentration of drugs. The authors believe that an explanation might be that the compounds act by blocking a step in the lymphocyte-stimulation pathway.

The hypothesis that thalidomide exerts its biological activities through interference with collagen biosynthesis, i.e., by inhibition of prolyl hydroxylase, has been checked [158]. Thalidomide itself and six of its putative metabolites, together with two analogues, EM-8 and EM-12, were tested for their ability to inhibit prolyl hydroxylases from three different sources (mouse, chicken embryo, man) and also lysyl hydroxylase (mouse). However, both enzymes were only slightly inhibited, even by high concentrations of the test compounds, and no correlation was found between this effect and the teratogenic potential

of the compounds. *In vivo*, total collagen synthesized during the organogenesis period of rabbit foetuses appeared to be highly depressed by thalidomide treatment (20, 40 and 80 mg/kg b.i.d.) between days 7 and 16 of pregnancy, as it was by treatment with high doses of supidimide (80 mg/kg b.i.d.).

ANALOGUES WITH ANTINEOPLASTIC ACTIVITY

Thalidomide and its congeners have recently aroused considerable interest in medicinal research because of the cytostatic potential which has been ascribed to these drugs, although this has never proved to be of practical consequence for human anti-cancer chemotherapy. Most of the aforementioned thalidomide analogues, as well as some additional compounds, have been screened for cytostatic activity, and these reports will be reviewed here.

The effect of CG-601 and CG-603 on dimethylbenzanthracene (DMBA)-induced experimental tumours in the rat has been compared with that of thalidomide [103,473]. The curative action of the cyclic imides on mammary tumours was more pronounced than that of thalidomide. The efficacy of CG-603 was found to be equivalent to hypophysectomy, and this nourished the impression that endocrine mechanisms play an important part in determining the antitumour action of thalidomide and its congeners. In a consecutive study, CG-603 was fed for a prolonged period of time to rats (concentration in diet 0.25%) carrying DMBA-induced tumours [474]. The compound had significant antitumour effect in this model. Moreover, the activity was enhanced when an androgen, methyldihydrotestosterone propionate, was administered with CG-603 concomitantly. Morphological alterations in the hypophysis and adrenals of such rats have been found [475] by means of light and electron microscopy. This, in the authors' opinion, permits the conclusion that the treatment reduces corticosteroid and prolactin levels.

This effect must have been quite convincing, for a clinical trial with CG-603 was conducted by the 'European Organization for Research on Treatment of Cancer' (EORTC) in 23 patients with advanced breast cancer [476]. The dose was 2 g/day orally, divided into four doses, given over a period of 6 weeks. The result, however, was disappointing: there was only one objective remission. The weakness of the effect could possibly be due to insufficient intestinal absorption of the drug. On the other hand, there were side-effects such as nausea and vomiting occurring in about half of the patients, in some severe enough to necessitate premature arrest of the therapy. The treatment was interrupted in six cases because there was rapid progression of the cancer before completion of the 6-week period of therapy.

De and Pal [477,478] synthesized and tested a series of glutarimide

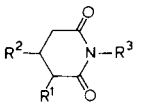


Figure 4.16. Glutarimide analogues tested for antineoplastic activity. R^1 = H or various diacylimido substituents, R^2 = H or aryl, R^3 = H, alkyl, cycloalkyl or aryl.

analogues related to thalidomide (*Figure 4.16*) for antineoplastic activity by means of a computer-aided Hansch analysis. Details on the synthesis and the procedure of biological evaluation in Ehrlich ascites carcinoma have been reported. Results of the computation revealed that the partition coefficients of 38 compounds alone cannot explain the observed activities, but that free-energy-related parameters derived from the Free-Wilson additivity model give fairly good prediction of the intrinsic activities.

Murphy and Stubbins [479] synthesized and tested 11 asparagine analogues of thalidomide of the type shown in *Figure 4.17* with R^1 and R^2 being hydrogen and/or various alkyl, aryl and aralkyl substituents. The idea behind this study was to find an asparagine antimetabolite, since it is known that certain tumour cells require an exogenous source of L-asparagine for their growth. The test was performed in leukaemia cell cultures, and one compound, *N,N*-dibenzyl-asparagine, was found to show significant inhibitory activity.

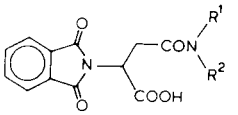


Figure 4.17. Asparagine analogues tested for antineoplastic activity. R^1 = R^2 = H, alkyl, aryl or aralkyl.

A research group from China [480] reported the synthesis of similar derivatives of *N*-phthalylglutamine (*Figure 4.18*) as potential anticancer agents.

An American group [481] extended the research into potential anticancer agents with structural analogy to thalidomide with the synthesis of several

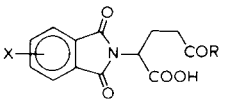


Figure 4.18. N-*Phthalylglutamine derivatives as potential anticancer agents. X = H, NO_2, acylamino; R = H, Cl, OH, NH_2 or arylamino.*

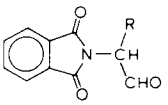
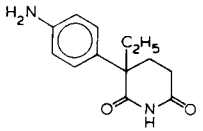


Figure 4.19. 2-Phthalimidoaldehydes as potential anticancer agents. R = CH_3, C_2H_5, C_3H_7 or C_4H_9.

2-phthalimidoaldehydes (*Figure 4.19*) and various semicarbazones and hydrazones of these aldehydes. The biological testing in Ehrlich ascites carcinoma in mice revealed sufficient inhibitory activity in some of the derivatives to justify further evaluation. In general, the semicarbazones and hydrazones were more effective inhibitors of the tumour than the free aldehydes, although they were also more toxic.

When referring to the antitumour effects of thalidomide analogues, it seems worth mentioning that aminoglutethimide (*Figure 4.20*), which is a substituted glutarimide, has been found to be a highly active inhibitor of steroid biogenesis and thus exerts strong anticancer activity, especially in advanced breast cancer [465,482]. Aminoglutethimide, but not its analogues, is also abortifacient [483,484]. Irreversible effects of glutethimide addiction, which must be considered as an expression of the drug's toxicity, have been summarized by Lingl [485].



Aminoglutethimide

Figure 4.20. Aminoglutethimide: inhibitor of steroid biogenesis, anticancer agent and abortifacient.

ANALOGUES WITH OTHER ACTIONS

There are, of course, a few additional pharmacological activities of thalidomide analogues which have sporadically attracted the interest of researchers in medicinal chemistry.

First of all, the efforts have been concentrated on the potential sedative-hypnotic effect of such compounds, since this can be expected in analogy to the parent drug, thalidomide. Koch [465–467] synthesized and screened a large series of non-planar congeners of thalidomide, from which two can-

didates, K 2004 (taglutimide) and K 2604, an oxa-analogue of the former, emerged as non-teratogenic compounds with fairly good sedative properties [486,487]. These two compounds, as well as numerous metabolites and further analogues, have been tested also for their alkylating potential and for anti-inflammatory activity in various *in vitro* and *in vivo* models.

Czejka and Koch [488] tested a series of such analogues, using the well-known 'NBP-test' for potential alkylating activity. None of the active compounds nor any of their simple hydrolysis products reacted positively in this test, whereas some unsaturated carboxylic acid derivatives were strongly alkylating under the same conditions.

Koch and Becker [489] showed in animal tests (rabbit intestine) and in an *in vitro* model (human platelet cyclooxygenase) that thalidomide, taglutimide and K 2604, as well as several open-chain analogues of these compounds, significantly inhibit prostaglandin formation and/or degradation. This effect is believed to be significant for the effectiveness of such compounds in certain inflammatory conditions of the gastrointestinal tract.

The authors [488,489] point to the structural similarity between the main metabolite of taglutimide [490] and the cyclic endoperoxide (CEP) which is the key intermediate in the arachidonic cascade leading from this fatty acid to all those active prostanoids which function as mediators in various inflammatory processes (*Figure 4.21*). CEP is the natural substrate of the enzyme complex which is assumed to be the specific receptor of the NSAIDs; as the NSAIDs bind to this enzyme, they block competitively the formation of CEP and thus inhibit the prostaglandin synthesis. In this manner they exert their anti-inflammatory action.

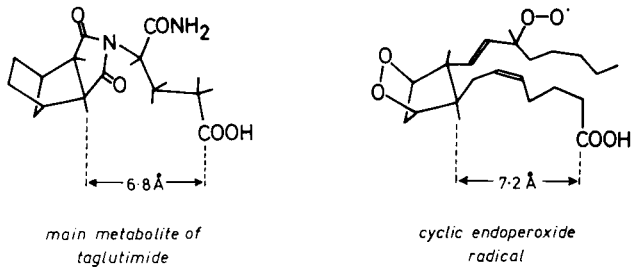
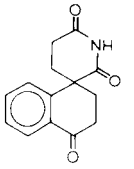


Figure 4.21. Perspectival drawing showing the structural similarity between the main metabolite of taglutimide and the cyclic endoperoxide radical intermediate of prostanoid biosynthesis.

Alonimide, a compound which is structurally related to thalidomide (*Figure 4.22*), has been claimed to possess sedative and hypnotic properties [491].

Italian workers [492–494] prepared a series of compounds structurally related to thalidomide (*Figure 4.23*) in order to investigate their pharmacologi-



Alonimide

Figure 4.22. Alonimide, a sedative-hypnotic compound structurally related to thalidomide.

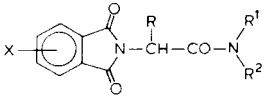


Figure 4.23. Thalidomide analogues showing central depressant and anticonvulsant activity. X = H, Cl, NH_2, NO_2 and others, R and/or R^1 = H, alkyl, R^2 = H, alkyl or acyl.

cal properties. Especially the derivatives of α-phthalimidobutyric acid showed remarkable central depressant and anticonvulsive activity in mice. In part, they were more active than thalidomide itself. Resolution of the compounds with the highest activities into the optical antipodes revealed that these show no marked difference as compared to the racemates.

Several substituted succinimidoglutarimides (*Figure 4.24*) have been synthesized with the aim of finding compounds with anticonvulsant activity. Unfortunately, no mention of the results of the pharmacological screening has been made.

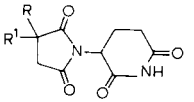
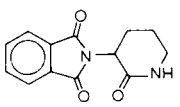
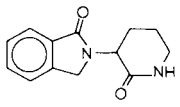


Figure 4.24. Succinimidoglutarimides with potential anticonvulsant activity. R and/or R^1 = H, alkyl or aryl.

Friderichs [496] studied the effects upon the central nervous system of thalidomide and five thalidomide analogues, namely, EM-87 (supidimide, *Figure 4.3*), EM–8, EM-12 (*Figure 4.15*), EM-136 and EM-255 (*Figure 4.25*), in various test systems. All compounds had a qualitatively comparable profile of CNS-depressant actions. Muscle tone and body temperature were lowered, but at relatively high doses. Mydriatic and antidiarrhoeal activity indicate some depression of peripheral ganglionic transmission. Supidimide had also significant influence upon gastric motility and secretion, as was recently reported [410].



EM-136



EM-255

Figure 4.25. Thalidomide analogues EM-136 and EM-255 with central nervous system depressant action.

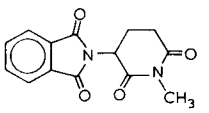
Brode [145–148] investigated the potential influence of CG-601, CG-603 and EM-350, in comparison with thalidomide, on the function of the pituitary-adrenal axis, on somatotropin-dependent processes, on enzyme induction and on nucleic acid synthesis. He found some remarkable effects of thalidomide and also of the congeners. While thalidomide, CG-601 and CG-603 show significant stimulation of some somatotropin activities, E-350 exerts an effect to the contrary. CG-601 and thalidomide do not induce enzymes involved in nucleic acid synthesis, but thalidomide stimulates the activity of liver enzymes. CG-601 also stimulates the incorporation of glycine into proteins and DNA in rat thymocytes, thus showing a certain interference with nucleic acid function.

The endocrine effects of thalidomide and β-thalidomide (EM-350), as well as of their morpholinomethyl derivatives (CG-601 and CG-603), were investigated [497]. The compounds were given to young immature rats of both sexes from the 21st to the 160th day of life in doses of 5–10 mg/kg per day i.p., and they were found to stimulate LTH and ACTH production and/or secretion, and to inhibit FSH, LH and TSH production and/or secretion. These experimental findings, too, point to the conclusion that the endocrine effects of thalidomide and its derivatives are mediated through the hypothalamus.

The inhibition of prolactin release by CG-603 in the rat with a single i.p. injection of 0.5, 1.0 or 2.5 mg/kg significantly and dose-dependently reduced serum prolactin levels as compared with control values in untreated animals [498]. Since prolactin has been shown to be an essential hormone for mammary tumour development, this finding seems to be relevant in connexion with the observed inhibition of DMBA-induced tumours by CG-603.

There is also a considerable analgetic potential in the group of thalidomide analogues. The pharmacologic activity of thalidomide, hexahydrothalidomide and *N*-methylthalidomide (*Figure 4.26*) in the hot plate test in mice showed thalidomide and its *N*-methyl derivative to be active, while hexahydrothalidomide was not [499].

Both thalidomide and *N*-methylthalidomide have been shown to be active in the phenylquinone writhing test in mice, but again not hexahydrothalidomide [500]. The mechanism of this test is not entirely clear, since both narcotic



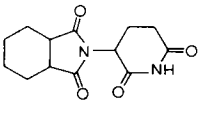


Figure 4.26. Two thalidomide analogues with analgetic activity.

and non-narcotic analgesics, antihistamines and local anaesthetics are also active in this test, but not sedative-hypnotic drugs, ethyl alcohol, chlorpromazine or cortisone. The significance of thalidomide's activity in the phenylquinone writhing test is thus not clear, but it may indicate that it has non-steroidal anti-inflammatory activity similar to that of the salicylates. Since other sedative-hypnotics are inactive, thalidomide's activity in this system seems to be distinct from its CNS effects.

Varma [501] synthesized and screened for antimicrobial activity a series of *N*-substituted phthalimides, for instance the compound shown in *Figure 4.27*. Some of these substances proved to have considerable antibacterial activity *in vitro* (against *E. coli*, *S. aureus*, *Aerobacter aerogenes*).

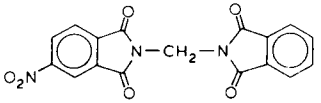


Figure 4.27. Thalidomide analogue with antibacterial activity.

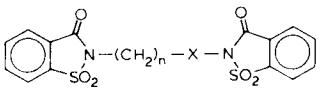


Figure 4.28. Dimeric polymethylene(oxy)saccharine derivatives. n = 1–5, X = CH_2 or O.

A series of similar compounds, *N*-polymethylene and *N*-polymethylene-oxyphthalimidosaccharins (*Figure 4.28*), were synthesized and screened by Hamor and Rubessa [502] to evaluate their pharmacological activity. Preliminary results indicated that these compounds possess no significant anti-cancer, anticonvulsant, diuretic, sedative, cardiovascular or central nervous system depressant activity.

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5 Medicinal Chemistry Research in India*

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* Dedicated to Dr Nitya Anand, doyen of Indian medicinal chemists.

INTRODUCTION

The beginning of modern drug research in India may be traced to the early part of the twentieth century when, in the nineteen twenties, Sir Ram Nath Chopra organized an active centre of research on Indian medicinal plants at the School of Tropical Medicine, Calcutta, and Dr Upendra Nath Brahmachari at the Campbell Medical School, Calcutta, discovered urea stibamine in 1922 and introduced it for the treatment of kala-azar.

Upon independence of the country, the Council of Scientific & Industrial Research, Government of India, realized the significance of drug research and established the Central Drug Research Institute at Lucknow. This was an important event, since the Institute, which has made its own important contributions, has also been instrumental in introducing and developing drug research culture in the country. Later, the CSIR Regional Research Laboratories at Hyderabad and Jammu also became engaged in medicinal chemistry research. The other public undertaking which has emerged recently is the IDPL Research Centre, Indian Drugs & Pharmaceuticals Limited, Hyderabad. Among the university institutions which have significantly contributed to drug research are the Department of Pharmacology, K. G. Medical College, Lucknow, and the Department of Pharmaceutical Sciences, Panjab University, Chandigarh. The prominent private-sector organizations which have made a mark in innovative drug research are the Hindustan Ciba-Geigy Research Centre, Bombay, and the Research Centre, Hoechst Pharmaceuticals Limited, Bombay.

The institutions enumerated above and several other laboratories are today actively engaged in new medicinal chemistry and are producing a respectable number of publications. A survey has recently been made by us and a status

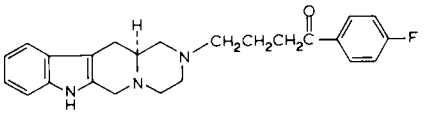
report on Medicinal Chemistry Research in India has been prepared which is to be published by the National Information Centre for Drugs and Pharmaceuticals, Department of Science & Technology, Government of India, Lucknow. The monograph contains twenty chapters and lists around 1200 references. In the present short review, a note is made of the drugs in use and those at different stages of clinical trial, and references are also given to certain promising leads.

DRUGS ACTING ON THE CENTRAL NERVOUS SYSTEM

The work in this area has been quite fruitful, and some worthwhile discoveries have been made in the areas of neuroleptics, antidepressants, tranquillizers, CNS depressants, muscle relaxants and local anaesthetics.

NEUROLEPTICS

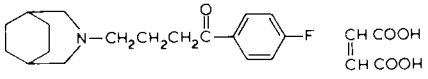
Two drugs which belong to the class of butyrophenones have been developed. During the work on piperazines in a rigid framework at the Central Drug Research Institute, 2-[3-(*p*-fluorobenzoyl)propyl]-1,2,3,4,6,7,12,12a-octahydropyrazino[2′,1′ : 6,1]pyrido[3,4-*b*]indole (1) [1], a potent neuroleptic agent,



(1)

was prepared. It is designated centbutindole. It combines in its structure tryptamine, piperazine and butyrophenone moieties. The drug is in phase II of clinical testing. It has no effect on the cardiovascular system. Structural variations of centbutindole have been found to be less active [2–4].

At the Hindustan Ciba-Geigy Research Centre, there has been designed the butyrophenone 3-[3-(*p*-fluorobenzoyl)propyl]-3-azabicyclo[3.2.2]nonane acid maleate (Go 3315A; nonaperone maleate; Azabiperidol®, 2) (V.P. Arya and J. David, through [5]). The drug is antipsychotic in schizophrenics at doses which do not elicit extrapyramidal side-effects. It also has anxiolytic properties at lower doses. Clinical trials on the drug have been completed and it is ready to be marketed.



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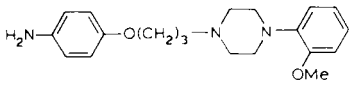


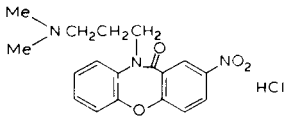
(3)

Centpyraquin, also a butyrophenone, which is a potent antihypertensive agent, discussed later, also has concomitant neuroleptic activity [6].

A mention may also be made of 1-(*o*-methoxyphenyl)-4-[3-(*p*-aminophenoxy)propyl]piperazine dihydrochloride (74/637) (3) prepared at the Central Drug Research Institute, which is a potent neuroleptic and is superior to chlorpromazine in some respects [7, 8].

ANTIDEPRESSANTS

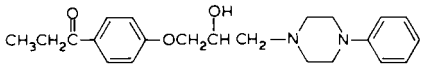
In the field of antidepressants, the most prominent is the drug nitroxazepine hydrochloride (Go 2330; Sintamil®) discovered at the Hindustan Ciba-Geigy Research Centre (K. Nagarajan, through [5]). Chemically, it is 10-[3-(dimethylamino)propyl]-2-nitrodibenz[*b*,*f*][1,4]oxazepin-11(10*H*)-one hydrochloride (4). It was introduced as an antidepressant in 1982, and is indicated



(4)

for the treatment of all grades and types of depression. It is also indicated in nocturnal enuresis.

Centpropazine, 1-(*p*-propionylphenoxy)-3-(4-phenylpiperazinyl)-2-propanol (5), prepared at the Central Drug Research Institute [9], is at phase II of clinical testing. In man, centpropazine appears to have antidepressant activity similar to that of imipramine.



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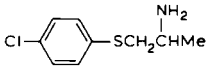
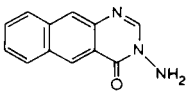


(6)

The other antidepressant compound is 2-amino-1-(*p*-chlorophenylthio)-propane (Go 2998) (6) discovered at the Hindustan Ciba-Geigy Research Centre (M.D. Nair, through [5]). It has shown promising results in clinical trials.

A TRANQUILLIZER

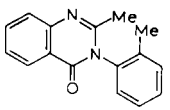
The work on benzo[6,7]quinazolin-4-ones at the Central Drug Research Institute [10] has led to the development of centazolone (compound 65/469), a promising tranquillizer [11]. Chemically, it is 3-aminobenzo[*g*]quinazolin-4-one (7). It is at phase III of clinical testing, and is a potent tranquillosedative having central muscle-relaxant action.



(7)

CNS DEPRESSANTS

From the work initiated on the synthesis of quinazolones at the Department of Chemistry, Lucknow University and later continued at the Regional Research Laboratory, Hyderabad, there emerged 2-methyl-3-*o*-tolyl-4(3*H*)-quinazolinone (8), which was first screened at the K. G. Medical College,



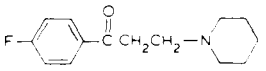
(8)

Lucknow [12]. The drug designated as methaqualone became established as a hypnotic after careful studies in India and abroad. This triggered further work on quinazolinone derivatives, but no compound has reached the clinical stage.

Centpyraquin [13], which has shown significant CNS depressant property along with antihypertensive activity, is discussed later.

MUSCLE RELAXANTS

Some centrally acting muscle relaxants have been obtained at the Central Drug Research Institute. 4′-Fluoro-3-(1-piperidyl)propiophenone (compound CN) (9) [14] is more potent than mephenesin [15,16]. 2*H*-Chromene-3-carboxamide (compound 69/20) (10) is also more active than mephenesin [17,18]. 3-(2-Tri-



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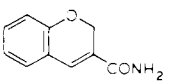
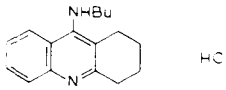


(10)

fluoromethylphenyl)-2-methylthio-4(3*H*)-pyrimidone exhibited marked central muscle relaxant and hypnotic activities [19].

A LOCAL ANAESTHETIC

The most prominent finding in the area of local anaesthetics is the discovery of centbucridine (compound 66/24) at the Central Drug Research Institute. Chemically, the drug is *N*-butyl-1,2,3,4-tetrahydro-9-acridinamine hydrochloride (11) and emerged from work on 4-substituted 2,3-polymethyl-



(11)

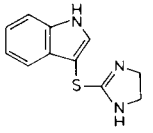
enequinolines [20]. In animal tests it was found to be 5- to 10-times more potent than lignocaine by infiltration and surface application [21]. It exhibited only a mild degree of CNS stimulant, vasopressor, antihistaminic, spasmolytic and antiarrhythmic activities [22]. The drug was found to be safe in chronic toxicity [23] and teratogenicity studies [24]. Evaluation of neurotoxicity potential in rabbits showed that it did not cause any histopathological changes, and it was considered to be as safe as lignocaine as a spinal anaesthetic [25]. On clinical pharmacological studies, the drug was found to be more potent than lignocaine as an infiltration anaesthetic [26]. The duration of anaesthesia following epidural block with the two agents was equal, and in spinal anaesthesia centbucridine had slightly, but not significantly, shorter duration of action [27]. Cardiovascular stability was better in patients receiving centbucridine. It was found to be safe as an intravenous regional anaesthetic

agent [28]. Clinical trials on centbucridine have been completed and the drug is awaiting registration.

ADRENOCEPTOR AGONISTS

A mention may be made of a potent α-adrenoceptor agonist which may prove to be a good nasal decongestant and a catecholamine type β_2-receptor stimulant.

S-(3-Indolyl)isothioureas and analogues [29] show vasoconstrictor, hypotensive and antihypertensive properties [30]. 3-(2-Imidazolin-2-ylthio)indole (Go 7996B) (tinazoline) (12) is a potent vasoconstrictor and is being developed



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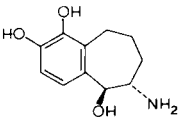


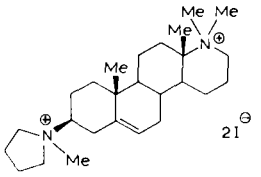
(13)

as a nasal decongestant at the Hindustan Ciba-Geigy Research Centre. It is a potent α-adrenoceptor agonist and does not possess any β-adrenoceptor activity [31]. It has a long duration of action and good central nervous system tolerability. Tinazoline has undergone successful clinical trials and is awaiting registration.

trans-6-Amino-6,7,8,9-tetrahydro-5*H*-benzocycloheptene-1,2,5-triol (13), possessing the catecholamine moiety in a rigid framework, was found to possess marked β_2-stimulant activity [32]. In the guinea-pig tracheal chain preparation, the compound is an agonist of β_2-receptor at low concentration and a non-competitive antagonist at higher concentration.

A NEUROMUSCULAR BLOCKER

At the Panjab University Department of Pharmaceutical Sciences, there have been designed different azasteroidal neuromuscular blockers [33–36]. In the series, a compound of particular interest is chandonium iodide (HS-310) (14)

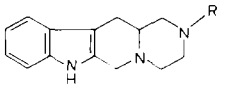


(14)

[34]. It possesses a powerful non-depolarizing neuromuscular blocking activity of short duration and rapid onset, being only slightly less active than the well-known neuromuscular blocker, pancuronium [37,38]. Toxicity studies have been carried out at the Central Drug Research Institute. The drug is under clinical trial and the results are encouraging.

ANTIHISTAMINES

The observed antihistaminic activity among 2-substituted pyrazinopyridoindoles at the Central Drug Research Institute represents a new lead for H_1- and H_2-antagonists. This has been an outgrowth of the work on neuroleptic activity of such derivatives. A systematic study showed compound (15)



(15) $R = p-H_2NCOC_6H_4CONHCH_2CH_2$

(16) $R = (C_6H_5)_2CHOCH_2CH_2$

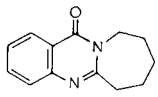
(ethamidindole; compound 76/490) to be the most active H_1-antagonist in the series when tested in different *in vivo* and *in vitro* test models [39,40]. The pharmacological profile of ethamidindole was favourable [41], but it was less potent than mepyramine [40].

A series of other 2-substituted pyrazinopyridoindoles were tested as poten-

tial H_2-antagonists and it was seen that 2-diphenylmethoxyethyl compounds showed promising H_2-receptor blocking activity [42]. The most active compound in the series was diphenethindole (16). It showed selective H_2-receptor blocking activity in cat, rabbit, guinea-pig and rat in different *in vivo* and *in vitro* test models [43]. The blockade was competitive, like that of metiamide. However, diphenethindole had a lower therapeutic index than metiamide.

A BRONCHODILATOR

RLX (17) (Regional Research Laboratory, Jammu) is a synthetic analogue of the alkaloid vasicine (discussed later) which is of interest as a bronchodi-

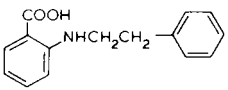


(17)

lator [44]. In *in vitro* and *in vivo* tests, RLX appears to be 6–10-times more potent as compared with aminophylline on a dose basis. Its effect is mediated by its direct effect on the smooth muscles, like that of aminophylline, and not through adrenoceptor stimulation. The ongoing chronic toxicity studies show it to be free from any toxic effects. It holds promise for clinical testing.

ANTI-INFLAMMATORY AGENTS

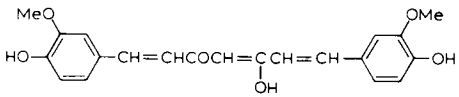
A perusal of research on fenamates (*N*-arylanthranilic acids) at the Regional Research Laboratory, Hyderabad, has led to the discovery of enfenamic acid (RH8), 2-[(2-phenylethyl)amino]benzoic acid (18). The pharmacological studies showed it to be a potent anti-inflammatory drug, comparable in activity with that of phenylbutazone [45]. It has a high margin of safety [46] and has no teratogenic effect in rats and rabbits [47]. The prostaglandin synthetase inhibitory activity is 20-times less than that of indomethacin [48]. Clinical



(18)

study showed the drug to have good gastric tolerance and to lack any side-effects [49]. Detailed clinical trials have confirmed its anti-inflammatory action in rheumatology and soft tissue inflammatory conditions. It is available as a drug under the proprietary name Tromaril®.

Different plant products have been examined for anti-inflammatory activity. Curcumin, a constituent of *Curcuma longa* Linn. (turmeric), chemically diferuloylmethane (19), has been shown to be an effective anti-inflammatory



(19)

agent at the Central Drug Research Institute [50,51]. It is as potent as phenylbutazone in the carrageenan oedema test, but half as potent in chronic tests. It possesses a much lower ulcerogenic index than does phenylbutazone. Curcumin has been tested clinically.

Salai guggal, the oleogum of *Boswellia serrata*, has been investigated at the Regional Research Laboratory, Jammu. It has been shown to possess anti-inflammatory and antiarthritic activities [52,53]. It was shown to be effective in controlled clinical trials in patients suffering from arthritis [54].

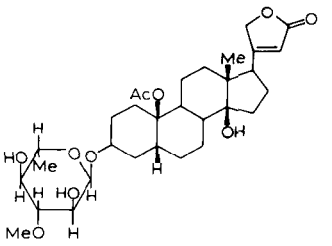
AGENTS WITH CARDIOVASCULAR ACTIVITIES

Different natural and synthetic products have been studied, some possessing cardiotonic, antihypertensive and antihyperlipidaemic activities.

CARDIOTONIC COMPOUNDS

A reference may be made to two cardiac glycosides. The glycoside peruvoside (20) was isolated from *Thevetia peruviana* at the Pharmaceutical Laboratories of Andhra University, Waltair [55,56], and developed in Germany as a cardiotonic agent for use in therapy [57]. Asclepin, isolated from *Asclepias curassavica* [58] and shown to be 3′-*O*-acetylcalotropin (21) [59], has been shown in animal tests to be more potent than digoxin and to have a wider safety margin [60,61].

A mention is made later to coleonol (22) and forskolin (23), which possess positive inotropic and antihypertensive actions.



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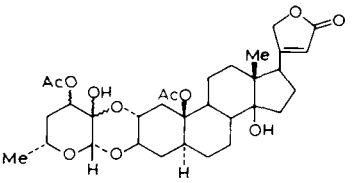

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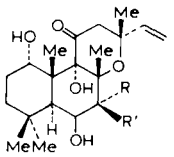
ANTIHYPERTENSIVES

The story of clinical testing of rauwolfia in India [62] as an antihypertensive and subsequent follow-up abroad, resulting in the isolation of reserpine [63] and related alkaloids and their use in therapy, is well known. There have been studies of natural and synthetic products which may have antihypertensive potential.

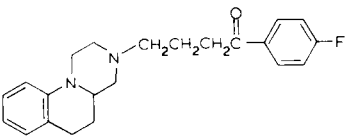
A use of *Coleus* spp. in heart diseases and other disorders has been made by Ayurvedic physicians. A systematic study at the Central Drug Research Institute led to isolation of coleonol (22), a diterpenoid, from *Coleus forskohlii* [64]. Coleonol possesses hypotensive and positive inotropic effects, and it



(22) R = OAc, R' = H
(23) R = H, R' = OAc

exhibits nonspecific spasmolytic activity [65]. This lead was picked up at the Research Centre, Hoechst Pharmaceuticals Limited, and a related principle, forskolin (23), was isolated from the plant [66–68]. It is stated that forskolin differs from coleonol in the configuration of the acetoxyl function at position 7; however, controversy continues about separate identities of these two principles. Forskolin has a potent inotropic, antihypertensive and adenylate cyclase stimulant profile [69]. Structure-activity correlation studies for antihypertensive and positive inotropic properties [70] and adenylate cyclase activation [71] suggest that the optimal structural requirements for the activity of forskolin are similar to those found in the natural product. Some simple derivatives of coleonol have also been prepared, but none reached the antihypertensive activity of coleonol [72]. Forskolin is undergoing clinical studies.

Studies on compounds having a piperazine moiety in a rigid setting led to the discovery at the Central Drug Research Institute of a potent antihypertensive agent in 3-[3-(4-fluorobenzoyl)propyl]-2,3,4,4a,5,6-hexahydro-1*H*-pyrazino[1,2-*a*]quinoline (24) (compound 69/183, centpyraquin) [73,74]. The activity has been confirmed in different test animals [75,76]. The mechanism of its hypotensive action seems to be the blockade of adrenergic neurones, along with direct smooth muscle relaxation. No clinical data are available.



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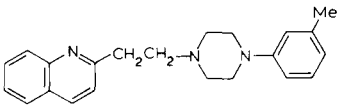

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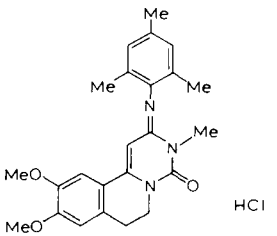


(26)

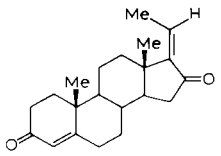
Substituted piperazines are the other kind of compounds which have been studied. Centhaquin, chemically 1-[2-(2-quinolyl)ethyl]-4-*m*-tolylpiperazine (25) is a centrally acting hypotensive developed at the Central Drug Research Institute [77,78]. The drug is at phase I of clinical studies.

At the Research Centre, Hoechst Pharmaceuticals Limited, it was observed that 2-aryliminopyrimido[6,1-*a*]isoquinolin-4-ones showed interesting anti-

hypertensive properties [79]. A prominent compound in the series is 2-mesitylimino-9,10-dimethoxy-3-methyl-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-one hydrochloride (trequinsin, HL-725) (26) [80]. This drug reduces systemic blood pressure in normotensive as well as in hypertensive animals. This is as a result of cardiovascular changes characteristic for a peripheral vasodilator. An inhibition of cyclic AMP phosphodiesterase may also be involved in the mechanism of action. Trequinsin is under clinical evaluation.

AN ANTIHYPERLIPIDAEMIC AGENT

Guggulipid, a fraction of *Commiphora mukul* resin (guggal), has been developed at the Central Drug Research Institute for use as a hypolipidaemic agent [81–83]. It is at phase III of clinical testing. Guggulipid is mainly a mixture of sterols, and compound (27) has been identified as one of the constituents [84].



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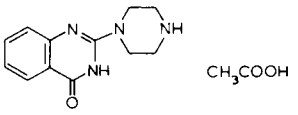
HYPOGLYCAEMIC AGENTS

Several heterocyclic compounds were studied for hypoglycaemic activity at the Central Drug Research Institute [85]. The activity was found to be associated with the cyclic amidine moiety simulated in their molecular structures. A compound of particular interest proved to be 2-piperazinylquinazolin-4(3*H*)-one acetate (68/157) (centpiperalone) (28) [85,86]. It has been found to be at least as potent as tolbutamide in fasted rats. Centpiperalone stimulates insulin release and also enhances (pro)insulin biosynthesis in the beta-cells [87]. The effect of centpiperalone is not potentiated by its combination with either phenformin or tolbutamide [88]. The drug has been dropped after phase I clinical trial.

There is a lead of interest from a plant source (Department of Pharmacology, Banaras Hindu University, Varanasi). The ethyl-acetate-soluble fraction of the ethanol extract of the bark of *Pterocarpus marsupium* Roxb. reversed the alloxan-induced change in the blood sugar level and beta-cell population in the



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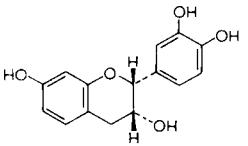
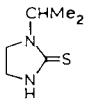


(29)

pancreas in albino rats [89]. The active principle, (–)-epicatechin (29), was isolated from the extract and was also seen to have prophylactic effect against alloxan-induced necrosis of the beta-cell population of the pancreas in rats in doses of 30 mg/kg (i.p.) [90,91]. (–)-Epicatechin was non-toxic in rats in doses of 1 g/kg. The protection by (–)-epicatechin may be due to scavenging of the deleterious and highly reactive hydroxyl radical which is generated by alloxan.

AN ANTITHYROID DRUG

Different systems having the thioamide moiety have been investigated. The notable finding remains that of centimizone (30), which was discovered at the



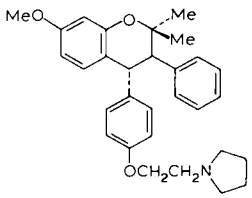
(30)

Central Drug Research Institute. This drug, 1-isopropylimidazolidine-2-thione, was selected from different 1-alkylimidazolidine-2-thiones which were examined for antithyroid activity [92]. Like other well-known antithyroid compounds, this also interferes in the biosynthesis of thyroid hormones [93]. The drug is available for clinical use.

ANTIFERTILITY AND RELATED ACTIVITIES

There have been concerted efforts to design agents which may be effective for control of fertility. The Central Drug Research Institute has made meaningful contributions in the area of non-steroidal compounds.

Several secosteroids [94–97], triphenylethylene-related structures [98–101], tetraphenylethane derivatives [102] and miscellaneous other non-steroidal entities [103–107] have been examined with the objective of obtaining antifertility agents which may be active post-coitum. Successful results accrued from 3,4-diphenyl-chromenes and -chromans [98]. The most significant compound in the series was *trans*-2,2-dimethyl-3-phenyl-4-*p*-(2-pyrrolidinoethoxy)phenyl-7-methoxychroman (centchroman) (31), which was identified

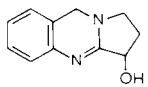


(31)

for detailed study as a post-coital contraceptive. The antifertility effect in animals was promptly reversible [108]. It appeared to exert its antifertility action by virtue of its multiple hormonal attributes such as oestrogenic, anti-oestrogenic and antiprogestational activities. The toxicity studies showed it to be safe for clinical evaluation [109,110], and well tolerated in human volunteers [111]. Trials in female volunteers indicated that its contraceptive effect may be due to its action on cervical mucus and endometrium, affecting sperm transport and implantation [112]. The drug is at phase III of clinical study. Centchroman is stable under standard storage conditions [113].

It has been noted that demethylation of centchroman to the corresponding 7-hydroxy derivative results in a 20-fold increase in binding affinity to uterine cytosol 17β-oestradiol receptors, without any appreciable change in anti-implantation activity [114]. On the other hand, absence of the pyrrolidinoethyl group from the 4-phenyl residue leads to a drop in both receptor binding affinity and anti-implantation activity.

Apart from synthetic compounds, there has been an active interest in plants and isolated plant constituents. A note may be made of the revival of interest in *Adhatoda vasica* and its constituents through recent work at the Regional



(32)

Research Laboratory, Jammu [44,115–120]. Vasicine (32), an alkaloid isolated from the plant, has an abortifacient effect in various species of animals at between 2.5 and 10 mg/kg. It may be acting through the release of prostaglandins. Clinical trials show it to be an effective oxytocic and abortifacient.

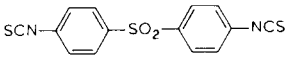
Lastly, a mention may be made of two preparations developed at the Central Drug Research Institute. Isaptent, a cervical dilator prepared from granulated *Plantago ovata* (Isapgol) seed husk [121], is being marketed under the name Dilax-C for medical termination of pregnancy. Consap, a spermicidal cream containing saponins from *Sapindus mukorossi* [122] is at phase II of clinical testing.

ANTIPARASITIC AGENTS

ANTHELMINTICS

The work carried out pertains mostly to agents active against cestode and nematode infections. Several salicylanilides related to niclosamide have been prepared and tested and some have shown good activity in experimental models [123–128]. Among isothiocyanates,4,4′-diisothiocyanatodiphenyl sulphone (centsulphone) (33) showed high oral therapeutic index in mice [129]. Though it showed little activity *in vitro*, it was very active *in vivo*; a dose of 5 mg/kg was sufficient to clear mice infected with *Hymenolepis nana* of all parasites. Tests on naturally infected dogs also showed encouraging results [130]. Certain congeners of centsulphone have been tested without any fruitful results [131].

Considering diethylcarbamazine (1-diethylcarbamoyl-4-methylpiperazine) as a prototype, certain new compounds have been prepared and tested for antifilarial activity. 7-Ethyl-2-methyloctahydro-6*H*-pyrazino[1,2-*c*]pyrimidin-6-one (centperazine) (34), an analogue of diethylcarbamazine having greatly reduced conformational mobility, was synthesized at the Central Drug Research Institute [132] and found to possess marked microfilaricidal activity [133]. Phase I clinical studies show it to be safe and well-tolerated on oral administration; further studies are in progress. Several structural modifications of (34) [134–140] have been carried out, but no better compound has resulted.



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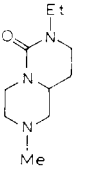

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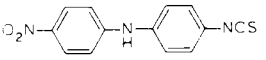
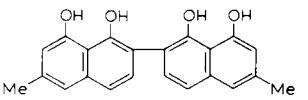


(35)

Out of several hundred isothiocyanates which were tested at the Ciba-Geigy Agrochemicals Division, Basle, and the Hindustan Ciba-Geigy Research Centre, a promising antihookworm compound, 4-isothiocyanato-4′-nitrodiphenylamine (amoscanate; Ancletol®), (35) was discovered [141]. The compound was first prepared at the Basle laboratory and new procedures of synthesis were worked out later [142,143]. Amoscanate is in fact an anthelmintic with an unusual spectrum of activity against intestinal nematodes, filariae and schistosomes in experimental animals [144]. Both adult and immature stages of the hookworm *Necator americanus* are highly susceptible to this compound. It is well tolerated in animals. Clinical trials have been carried out in hookworm patients [145–147]. Permission has been granted for marketing of the drug.

A mention may be made of diospyrol (36), the active constituent of fruits of *Diospyros mollis* Griff. Diospyrol, when tested in hamsters infected with



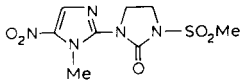
(36)

human hookworm *N. americanus*, showed marked oral antiparasitic activity [148]. Its derivatives have poor or no activity. Diospyrol was also active against the nematode *Nematospiroides dubius* and cestode *Hymenolepsis nana* parasites in mice. Diospyrol was well tolerated when administered orally in various laboratory animals. The disadvantage of the compound is its high susceptibility to aerial oxidation.

ANTIPROTOZOAL AGENTS

In a historical perspective, a significant discovery in the field of antiprotozoal agents has been that of urea stibamine [149–151], introduced for the treatment of kala-azar [149].

Over a decade ago, work began on nitroimidazole derivatives at the Hindustan Ciba-Geigy Research Centre. From the compounds examined, 3-(1-methyl-5-nitro-2-imidazolyl)-1-methylsulphonyl-2-imidazolidinone (satranidazole; C 10213 Go.) (37) [152,153] emerged. It is a potent amoebicide



(37)

both hepatic and caecal infections in the hamster; it is also a trichomonacide, and is claimed to be superior to metronidazole. It is also active against giardial infections. Clinical tolerability and efficacy in patients suffering from amoebiasis or trichomoniasis have been shown [154].

ANTIMICROBIAL ACTIVITY

A number of compounds obtained from natural sources or synthesized have been studied for antimicrobial activities. However, only the antifungal hamycin has shown promise. Hamycin is an antibiotic discovered at the Research Laboratory of the Hindustan Antibiotics Limited, Pimpri. It is a polyene produced by *Streptomyces pimprina* Thirum., an actinomycete isolated from Pimpri soil [155]. The antibiotic is active against *Candida albicans* [156,157] and the protozoon *Trichomonas vaginalis* [158,159]. Hamycin has been administered topically and by mouth in a variety of fungal infections including candidiasis, tinea, blastomycosis and madura foot.

CONCLUDING REMARKS

It is not widely known that the hypnotic methaqualone (8) was discovered in India but developed abroad. Urea stibamine was another early discovery and the drug continues to be used in the treatment of kala-azar. Other synthetic

drugs which have been discovered and are in clinical use are the antidepressant nitroxazepine hydrochloride (4), the anti-inflammatory agent enfenamic acid (18), and the antithyroid centimizone (30). Drugs due to be marketed are the neuroleptic, nonaperone maleate (2), the local anaesthetic, centbucridine (11), the nasal decongestant, tinazoline (12), and an antihookworm drug, amoscanate (35). Many other drugs are at different stages of clinical testing.

Among the natural products, the antifungal-antibiotic hamycin is in use. A cardiotonic glycoside, peruvoside (20), discovered in India, has been promoted abroad. Salai guggal, an oleogum, is of use in arthritis patients. Curcumin (19) has been tested clinically as an anti-inflammatory agent. Coleonol (22) and forskolin (23), the diterpenoids, constitute new leads, and forskolin is being tested clinically as an inotropic and antihypertensive agent. Guggulipid, a natural resin fraction, is being tested clinically as a hypolipidaemic agent. The alkaloid vasicine (32) is being developed as an oxytocic and abortifacient. Isaptent (Dilax-C) is marketed for medical termination of pregnancy, and consap is a saponin-containing spermicidal cream.

These are the highlights so far of medicinal chemistry research in India. Today, more than hundred papers are published on medicinal chemistry every year from Indian laboratories. This short account is selective in its coverage and several other fields which continue to be explored have not been included. The monograph on Medicinal Chemistry Research in India being published by the National Information Centre for Drugs and Pharmaceuticals will adequately project the status of research in different areas.

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6 The Riddle of Cholinergic Histamine Release from Mast Cells

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INTRODUCTION

The activity of histamine (2-(4-imidazolyl)ethylamine) was discovered in the course of an investigation on ergot, showing that its extracts, the synthetic substance and the base produced by splitting off carbon dioxide from histidine (by bacterial action or by chemical means), produced, in minute dose, tonic contraction of the uterus [1].

The activity of acetylcholine was also identified in certain specimens of ergot and its extracts, with an action which appeared to be of the muscarinic type [2]. Thus, the history of histamine shows several close parallels with that of acetylcholine, since both compounds were first detected as uterine stimulants in extracts of ergot, from which they were subsequently isolated.

Beyond the common historical origin, the early work carried out on the biological activities of histamine and acetylcholine showed a striking similarity between their physiological actions. In the cat, both acetylcholine and histamine increase the intestine volume and leg volume and produce a profound hypotensive effect, transient in duration with acetylcholine and more long-lasting with histamine [2,3]. Besides the intense hypotensive and vasodilator action, both compounds stimulated a host of smooth muscles, ranging from the isolated uterus of guinea-pig and rat, the isolated intestine of cat and rabbit, to the musculature of the bronchioles in the pithed guinea-pig [2,3]. Both compounds cause a flow of saliva, tears and pancreatic juice in the cat [2,3]; in these early experiments, the heart is the only organ endowed with different sensitivity, reacting to histamine with an increase in the strength of contraction and to acetylcholine with cardiac standstill [2,3].

More than half a century after the early observations, the cholinergic system is clearly separated from the histaminergic system in terms of biosynthetic pathways, storage, release, receptor-mediated effects and termination of the physiological actions. However, a link between the cholinergic system and tissue histamine stores has been established by a variety of experimental evidence, showing that parasympathetic nerve stimulation or the use of cholinergic agonists produces an enhancement of antigen-induced release of histamine from sensitized tissues and the secretion of histamine from tissues and isolated cells, even in the absence of previous sensitization. If such a relationship between the parasympathetic nervous system and one of the major autacoids (histamine) were established, two significant objectives would be reached: (a) to place histamine in a more physiological context; (b) to gain some insight into certain diseases (urticaria; asthma; ulcerative colitis; Chron's disease; coeliac disease) in which an interaction between the two systems is suspected. Our present effort is to describe the state of knowledge on the interaction between cholinergic and histaminergic systems.

HYPOTHESES

Mast cells are the main repository of tissue histamine and are present in human lung and skin in concentrations of $(1-10) \times 10^6$ cells/g [4], comprising 2% of the alveolar tissue [5]. Because of their number, the characteristic secretory response to a specific ligand interaction on their membranes and their positioning where potentially noxious materials are likely to enter the body, the mast cells probably represent a physiological system [6]. The possibility that the mast-cell system could be linked to the autonomic sections of the nervous system, with particular emphasis on the cholinergic domain, represents the core of our hypotheses.

As in the case of any other secretory cell, the homeostatic link with the environment is accomplished through receptors present on the mast-cell membrane. Indeed, recent and past information provides experimental evidence that mast cells are endowed with a variety of specific receptors for physiologically occurring substances. The most thoroughly studied mast-cell receptor is certainly the immunoglobin E (IgE) receptor. The physicochemical characteristics of the IgE receptor on rat mast cells have been critically analyzed and recently reviewed [7]. This receptor is about as large as the Fc regions of IgE, i.e., approx. 8×10^4 g/mol [8]. The receptor *in situ* is uncluster-ed, mobile and univalent, and its aggregation into dimers triggers degranulation. There is evidence [9] that the receptor is a glycoprotein composed of two subunits, an alpha chain and a beta chain, with different molecular weights. There are $(1-5) \times 10^5$ receptors per mast cell, of which about 10% are occupied *in vivo* [10]. The receptor has an apparent M.W. of 45,000–60,000 and has been purified from rat mast cells [11]. The IgE receptor on the mast cells can be altered by isolation procedures, as shown by Coutts, Nehring and Jariwala [12] who demonstrated that IgE-specific receptors on rat mast cells are either shed or inactivated by sedimentation through BSA, Ficoll or Metrizamide, and that the loss of these receptors is prevented by occupying them with IgE.

A second class of receptors which have been recently identified on mast cells are the histamine H_1 and H_2 receptors. Rat peritoneal mast cells bind [^{3}H]pyrilamine specifically, rapidly and reversibly in a saturable fashion, and to a receptor of the H_1 type, as shown by (i) specific binding of the H_1-antagonists, while [^{14}C]cimetidine fails to bind appreciably; (ii) a more efficient inhibition of pyrilamine binding by H_1-receptor antagonists, in comparison with histamine; (iii) selective H_1-agonists' inhibition of pyrilamine binding, in a more efficient fashion than the H_2-agonists. Mast cells have a homogeneous population ($(4-5) \times 10^6$ receptors per cell) of low affinity receptors, of uncertain chemical structure; so far, these receptors have not been isolated in a purified form [13].

H_2-Receptors have been reported to be present on the mast cells of guinea-pig [14,15] but are apparently absent or non-functional in rat mast cells [16,17]. However, specific binding sites for [^{3}H]cimetidine have been demonstrated in rat mast cell-membrane preparations, Lineweaver-Burk analysis revealing a homogeneous population of specific binding sites, with a K_d of 1.75×10^{-8} M,and a B_{max} amounting to 105 fmol/mg protein [18]. Conclusive evidence of H_2 receptors on mast cells must await experiments carried out using a more selective antagonist, [^{3}H]tiotidine, since it has been reported that the low affinity of the traditional H_2-antagonists for the H_2-receptors may hamper their use for labelling these receptors [19,20].

A third class of receptors probably recognizing endogenous mediators has been identified in the cellular membranes of rat peritoneal mast cells and in their perigranular membranes. On these preparations, β-adrenergic receptors have two binding sites, each cell containing 120×10^3 high-affinity binding sites and 720×10^3 low-affinity binding sites [21]. Only high-affinity binding sites have been reported on the same preparation by different authors [22]. There does not appear to be a significant number of α-adrenergic receptors on the isolated rat peritoneal mast cells, since the binding of [^{3}H]dihydroergocriptine is negligible [21]. However, an α-adrenergic agonist, phenylephrine, is capable of promoting the secretion of histamine in isolated mouse neoplastic mast cells [23].

The neurotransmitter peptides, neurotensin, substance P, endorphins and enkephalins, also possess separate and specific receptors on mast-cell membranes. Neurotensin releases histamine from human peritoneal mast cells *in vitro* [24] and binds to rat peritoneal and pleural mast cells, with a reported K_d of 154 mM for iodinated neurotensin [25]. Notably, neurotensin induces histamine release chiefly from a subpopulation of pleural rat mast cells, thus confirming mast-cell heterogeneity in response to the same physiological stimuli [26,27]. Studies of histamine release by neurotensin analogues indicate that the C-terminus is the biologically active portion of the molecule [28,29]. The secretion of histamine by isolated mast cells has been also reproduced with the tetradecapeptide hormone, somatostatin [30]. Johnson and Erdös [31] showed that another physiologically occurring peptide, substance P, which is contained in sensory nerves [32], releases histamine from rat peritoneal mast cells. Substance P receptors in rat mast cells have been extensively characterized [33,34], indicating that substance P released from sensory nerves acts on mast cells in and around the vessel walls to release histamine which, in turn, causes vasodilation. Less clear-cut is the evidence for the presence in mast cells of specific receptors for naturally occurring peptides with morphine-like pharmacological activity. Using an immunological model of [^{14}C]serotonin release

from rat mast cells [35], it has been demonstrated that β-endorphin and Met-enkephalin dose-dependently reverse the inhibition by PGE_1 of serotonin release. This anti-PGE_1 action is blocked by naloxone and is thought to be mediated through opioid receptors. The type of opiate receptors on rat mast cells remains to be clarified, in terms of their relevance to μ, δ, σ and κ types.

Two other natural systems regulating the inflammatory process influence mast-cell function presumably through a receptor mechanism, that is, the prostaglandin and the purinergic systems. On rat mast cells, PGE_1 inhibits immunological histamine release corresponding to its ability to increase the level of cellular cyclic AMP [36]. On the same cells, but using a different secretagogue (compound 48/80), PGE_1 inhibits the release of histamine, although at rather high concentrations [37]. Some discrepancies appear in the literature as far as the prostaglandin modulation of histamine release is concerned. The results presented by Ennis, Robinson and Dollery [38] show that low concentrations of PGD_2 and PGE_1 are without significant effect on the immunological release of histamine from rat peritoneal mast cells. The data are in agreement with those reported from different authors [39] and in contrast to the inhibition of anaphylactic histamine release with low doses of PGD_2 reported by Truneh [40]. In addition, it has been shown that high concentrations of PGD_2 inhibit histamine release only in combination with aminophylline [41].

ATP is considered to be the neurotransmitter of purinergic nerves and is capable of releasing histamine in isolated purified rat serosal mast cells [42]. Adenosine, formed intracellularly from the ATP pathway, potentiates the release of histamine by isolated mast cells, the effect being blocked by theophylline [43]. The potentiation of histamine release by adenosine and phenylisopropyladenosine has been repeatedly confirmed in isolated mast cells challenged with immunological and non-immunological stimuli [44–46].

Taken together, these observations envisage the mast cell as a cell loaded with preformed mediators (histamine and serotonin; chemotactic factors for neutrophils and eosinophils; lysosomal enzyme; heparin) and capable of producing secondary mediators (prostaglandin D_2, leukotrienes C,D,E, platelet-activating factor, superoxide anions), clustered near richly innervated vessels and provided with a variety of different receptors on its membrane. These receptors recognize amines (histamine; noradrenaline), peptides (neurotensin, somatostatin, substance P, opioid peptides), prostaglandins and adenosine. Upon the recognition of the physiological signals, the secretion of mast-cell mediators is either inhibited (as in the case of histamine, noradrenaline, prostaglandins) or enhanced (as occurs with peptides and purines [47–49]). It is therefore conceivable that mast cells, being capable of recognizing

endogenous transmitters through specific receptors, might be considered as 'paraneurons' in spite of their preferential mesodermic rather than ectodermic origin [50] (*Figure 6.1*). In this context, the presence of cholinergic receptors on mast-cell membranes would strengthen this hypothesis.

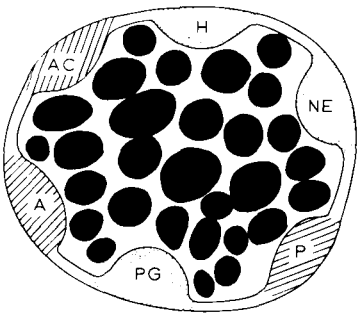

Figure 6.1. Mast cells as 'paraneurons'. The dotted areas indicate inhibitory receptors and the hatched areas excitatory ones. Receptors for: H, histamine; NE, noradrenaline; P, peptides; PG, prostaglandins; A, adenosine; AC, acetylcholine.

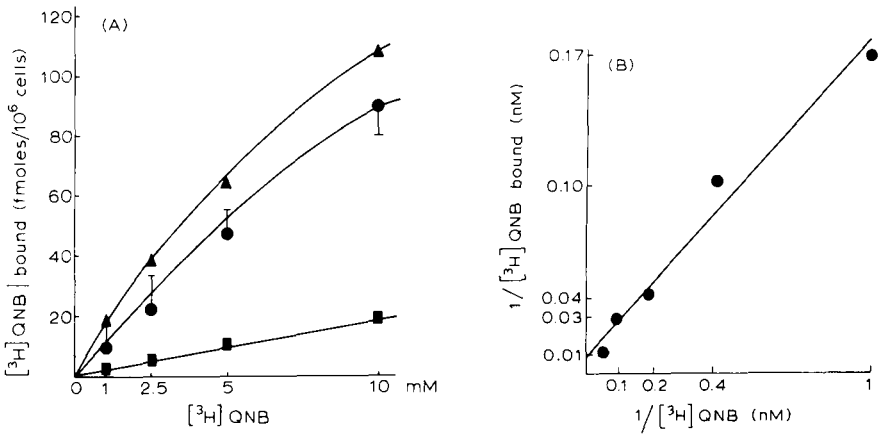

Figure 6.2. (A) Total (▲——▲), specific (●——●) and unspecific binding (■——■) of [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB) to rat mast cells with increasing concentrations of [^{3}H]QNB. The results are the means ± S.E. of four experiments performed in triplicate. (B) Lineweaver-Burk analysis of the saturation data of specific binding [83]. $r^2 = 0.93$; $K_d = 17$ nM; $B_{max} = 100$ fmol/10^6 cells.

The riddle of cholinergic histamine release starts from the demonstration of specific binding sites for cholinergic antagonists on mast cells. In fact, Donlon, Hunt, Catravas and Kaliner [21] have studied the binding of [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB) to whole cells and isolated granules, trying to assess muscarinic binding sites in the presence of the specific competitor, oxotremorine. The conclusion of their study indicates the lack of any muscarinic cholinergic receptors on isolated rat peritoneal mast cells. However, on the same preparations, specific [^{3}H]QNB binding was shown to be present (*Figure 6.2*) by other authors [51]. The binding is saturable, time- and temperature-dependent, and selectively inhibited by atropine and by oxotremorine, indicating that rat peritoneal mast cells probably possess cholinergic muscarinic receptors. Tentatively, the reasons for these discrepancies may lay in the difference in the incubation temperature (5°C [21] against 37°C [51]).

In the following sections, the hypothesis of a cholinergic link between the parasympathetic nerves and the mast-cell system will be substantiated, with the aim of achieving new insights into the physiopathology of these fascinating cells.

RELATIONSHIP BETWEEN THE CHOLINERGIC NERVOUS SYSTEM AND HISTAMINE RELEASE

IN IMMEDIATE HYPERSENSITIVITY

Cholinergic stimulation with carbamylcholine has been found to enhance the immunological release of histamine from human lung tissue passively sensitized with atopic serum and challenged with specific antigen [52]; in the same preparation, enhancement of histamine release was observed with the physiological mediator, acetylcholine, in concentrations of 10^{-7}–10^{-10} M [53]. Stimulation of sensitized nasal polyps with the cholinomimetic agent, carbamylcholine, likewise produced a significant increase of the immunological release of histamine [54]. The cholinergic influence on immunological release of chemical mediators from human tissues has been extensively reviewed by Kaliner and Austen [55]. However, no enhancement of IgE-mediated histamine release was observed from human peripheral blood leucocytes [56]. In further animal studies, it was found that low concentrations of carbachol (10^{-10} M) enhanced antigen-induced histamine release from sensitized bovine lung [57] but not from bovine leucocytes [58]. The enhancement of histamine release induced by these cholinomimetic agents is prevented by pretreatment

of the tissues with atropine and thus appears to be effected through a muscarinic receptor.

Therefore, clear-cut evidence of muscarinic receptors facilitating the immunological histamine release was obtained in sensitized tissues of different animal species, except for the white blood cells, in which such a prosecretory rôle of cholinomimetic compounds seems to be lacking.

IN UNSENSITIZED TISSUE

The vagal mechanism of insulin-induced gastric secretion has been known for many years and insulin is used as a test for completeness of vagal section. Interestingly, insulin and reserpine (which is also capable of activating a centrally derived vagal stimulation) provoke gastric acid secretion and produce gastric lesions in the rat, while lowering the stomach histamine content; all these effects are prevented by vagotomy [59–61]. These data strongly suggest that vagal stimulation leads to a mobilization of gastric histamine. In cats, histamine is considered to be the mediator of the parasympathetic stimulation of the submandibular gland, since the demonstration that prolonged stimulation of the chorda tympani reduced to about 50% the content of [^{3}H]histamine in the stimulated gland; the effect was shared by pilocarpine and associated with a concomitant increase in the flow of saliva [62]. In dogs, after intraarterial injection of acetylcholine, the histamine content in the saliva of the submandibular gland was found to represent a concentration about 6-times higher than that in plasma [63]. When rats were stressed by restraining at 4°C, animals treated with saline exhibited a significant decrease in mast-cell number in the gastric mucosa, submucosa and muscle. Atropine treatment antagonizes this stress-induced decrease in mast cell counts in mucosa and submucosa, but not in muscle, implying that the effectiveness of atropine against stress-induced gastric mast cell degranulation involves a cholinergic-mediated mechanism [64]. Moreover, in isolated rat serosal mast cells, the release of histamine induced by compound 48/80 was significantly enhanced by carbamylcholine [65].

The relationship between cholinergic and histaminergic systems may be bidirectional. As it is conceivable that vagal stimulation leads to release of tissue histamine, it has also been shown that histamine stimulates afferent cholinergic nerves called 'irritant receptor sites' located along the bronchial mucosa, with resultant efferent vagal discharge producing bronchospasm in experimental animals [66] and man [67]. In fact, asthmatics are said to be 100- to 1,000-times more sensitive to the bronchoconstrictive properties of inhaled acetylcholine than are non-asthmatics [68]; in the guinea-pig, vagotomy

reduced by 75% the bronchomotor response to anaphylaxis [69]. It thus appears evident that, in different experimental models (gastric mucosa, salivary glands, tissue mast cells and isolated mast cells), vagal excitation or the use of cholinergic agonists is somehow related to the release of histamine.

THE RELEASE OF HISTAMINE BY ACETYLCHOLINE

IN VIVO EXPERIMENTS

Evidence of a closer relationship between the parasympathetic nervous system and tissue histamine stores may arise from studies on the release of histamine evoked by acetylcholine or by the direct stimulation of parasympathetic nerves. In the dog, the administration of acetylcholine by the inhalation route is associated with bronchoconstriction and with a significant increase in plasma histamine levels [70]. This effect is differentially influenced by vagotomy, which does not prevent the rise in plasma histamine concentrations although it abolishes the bronchomotor response [71]. Indirect evidence of histamine release by acetylcholine was also provided by experiments carried out in rats severely poisoned by irreversible acetylcholinesterase inhibitors, in which a highly significant increase in plasma histamine levels was recorded (G.D. Bloom and P.E. Alm, personal communication).

Obstructive airway disease is obtained in man when challenged with an acetylcholine aerosol; under these circumstances, a net increase in plasma histamine concentration has been measured [72]. The increase in plasma histamine levels after acetylcholine and methacholine challenge by the respiratory route appears to be strongly potentiated in asthmatic subjects [73].

IN VITRO EXPERIMENTS

Information is scanty about the release of histamine evoked by acetylcholine in isolated organs. In the isolated guinea-pig heart perfused at constant flow, a spontaneous overflow of histamine is detectable. When acetylcholine (10^{-7} M) was added to perfusates recirculated at constant rate through isolated guinea-pig heart, a significant increase in the histamine overflow was observed [74]. In isolated strips of rat small intestine, the spontaneous output of histamine appearing in the perfusates was increased in a concentration-dependent fashion when acetylcholine was added to the perfusion fluid, after preincubation with DFP [75].

In the aforementioned experiments, either *in vivo* or *in vitro*, it is difficult to

ascertain whether the apparent release of histamine associated with the administration of acetylcholine is a primary event (i.e., the liberation of histamine from tissue stores by acetylcholine) or an epiphenomenon of the multiple physiological responses evoked by acetylcholine (such as the increase in airway resistance and the consequent hypoxia, the negative inotropic and chronotropic effect, or the contraction of intestinal smooth muscles), which may cause unspecific mast cell damage, leading in turn to histamine release. A tentative answer to the question may be provided by experiments carried out on isolated mast cells in the presence of acetylcholine.

EXPERIMENTS WITH ISOLATED MAST CELLS

Some contradictory data concern the histamine-releasing properties of acetylcholine from isolated mast cells. It has been repeatedly demonstrated in some laboratories that acetylcholine possesses the ability to stimulate mast cells to secrete histamine at concentrations as low as 10^{-12} M [74,76]. An intact glycolytic and oxidative metabolism is required for the acetylcholine-induced histamine secretion. The secretion is calcium-dependent and is inhibited by removal of extracellular glucose, by hypoxia, and by cyanide and monoiodoacetate. The secretion of histamine has the characteristic features of the sequential exocytosis: it depends on the extracellular H^+ concentration and is blocked when the cells are exposed to sodium-deficient media. The order of potency of cholinergic agonists in evoking the secretion of histamine is oxotremorine > acetylcholine > choline > carbamylcholine > nicotine. Atropine competitively blocks the acetylcholine-induced histamine secretion, indicating the presence of cholinergic muscarinic receptors on mast cells. The cholinergic histamine release is inhibited by dibutyryl-cyclic AMP, adrenaline and histamine H_2-receptor agonists, tentatively indicating a feedback regulation through β- and H_2-receptors, mediated by an increase in cyclic nucleotide contents [18,21,76–78,80,81]. As in the case of dextran [82], a heterogeneous pattern of mast cell sensitivity to acetylcholine has been identified, ranging from a full reaction to nanomolar concentrations of acetylcholine to a virtual lack of response [83]. The same variability in the response to acetylcholine was reported by Schmutzler, Poblete-Freundt, Rauch and Schoenfeld [84] in human mast cells: some pools of freshly isolated but otherwise untreated human mast cells responded with a distinct dose-dependent histamine release when exposed to acetylcholine, while cells from other pools did not react.

However, of all the suspected neurotransmitters, only ATP degranulates isolated rat mast cells, acetylcholine being ineffective at concentrations ranging

from 2×10^{-7} to 2×10^{-2} M [85]. Several investigations have failed to demonstrate any cholinergic histamine release from isolated mast cells of the rat and guinea-pig [42,49,86,87], even when mast cells have been isolated from rats injected with *Nippostrongylus brasiliensis*, and bearing a high titre of mast cell-bound IgE (F. Pearce, personal communication). Therefore, cholinergic histamine release is a matter of controversy, a possible solution perhaps lying with genetic disposition and active or passive sensitization. It is unlikely that the lack of response to acetylcholine may be accounted for by the fluctuation in the number of muscarinic receptor sites on mast-cell membranes. In this respect, it has been demonstrated that rat mast-cell membranes exhibit high-affinity binding for [^{3}H]QNB, which was found to be identical in membranes obtained from cells reacting and those not reacting to acetylcholine, therefore excluding the variability in response being due to a heterogeneous distribution of muscarinic cholinergic receptors [83]. It seems likely that muscarinic cholinergic receptors are always present in rat mast cells, but capable of triggering the exocytotic response to acetylcholine only when activated by the presence of IgE. Interestingly, unstimulated mesentery mast cells from sensitized dogs show a prosecretory state with increased surface area, formation of microfilaments and conversion of some granular matrices to fine granular structures [88].

THE POSSIBLE ROLE OF IgE IN CHOLINERGIC HISTAMINE RELEASE

A rôle of IgE in modulating cholinergic histamine release from isolated mast cells and from tissue histamine stores is strongly envisaged from experimental results. In the experiments reported by Poblete-Freund, Rauch, Schonfeld and Schmutzler [89], a striking difference was found in the behaviour of isolated mast cells from sensitized and non-sensitized individuals. In human mast cells, acetylcholine caused a dose-dependent histamine release only when the cells were capable of reacting to anti-human IgE. Acetylcholine caused a significant histamine release from guinea-pig mast cells only when the cells were isolated from actively sensitized tissues. Acetylcholine was ineffective on mast cells from normal rats, but induced histamine release from mast cells from actively sensitized rats. The effect could be blocked by atropine, and the acetylcholine sensitivity became detectable about 12 days after active sensitization, reaching a maximum at about 30–40 days and decaying at about 60 days [84]. Similar results were obtained using isolated mesenteric mast cells from normal dogs in which acetylcholine was ineffective and in mast cells isolated from actively

sensitized animals in which acetylcholine caused a significant release of histamine, even at low concentrations (10^{-12}–10^{-10} M) [88]. The authors suggest that active sensitization may cause mast cells to acquire a particular functional state of susceptibility for stimuli and of readiness for secretion, so that the cholinergic release of histamine may be taken as an indicator for a particular reactive state of actively sensitized mast cells and possibly as a test for the atopic condition [90]. In this context, it is of interest that the spontaneous histamine release was higher in mast cells isolated from actively sensitized dogs [88]. This observation is consistent with the ultrastructure of sensitized cells showing altered granules and some kind of degranulation [88]. It is also consistent with the release of histamine evoked by threshold concentrations of compound 48/80, which was more effective in the actively sensitized than in the normal cells [88]. However, it is worth recalling that Sydbom, Karlsson and Uvnäs [91] have demonstrated a diminution of the histamine release by compound 48/80 in rat serosal mast cells bearing an excess of IgE as consequence of passive sensitization.

A difference in the rôle of IgE on cholinergic histamine release stems from active and passive sensitization. In fact, passive sensitization did not induce any increased acetylcholine susceptibility in isolated human adenoidal mast cells [90] and in rat isolated serosal mast cells [84]; this fact is taken as evidence that acetylcholine supersensitivity after the immunostimulation of the

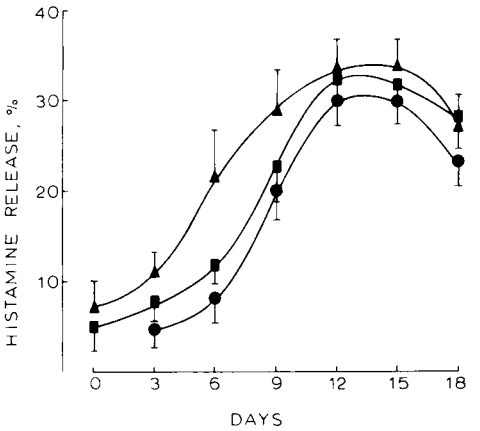

Figure 6.3. Histamine release induced by acetylcholine and specific antigen in purified mast cells isolated from actively sensitized Brown Norway rats at different times (days) after sensitization. The values are the means ± S.E. of four experiments in triplicate. ■, 10^{-8} M acetylcholine; ▲, 10^{-6} M acetylcholine; ●, 20 μg ovalbumin.

organism (either by infection or by active sensitization with a protein antigen) is not due simply to the fixation of IgE to the mast cells. Active sensitization of Brown Norway rats with ovalbumin and *Bordetella pertussis* vaccine induces the serosal mast cells to release histamine in response to different concentrations of acetylcholine, reaching a maximum within 12–18 days after the sensitization (*Figure 6.3*). However, acetylcholine supersensitivity was likewise obtained after incubation of serosal mast cells isolated from Wistar albino rats with sera obtained from actively sensitized Brown Norway rats at different times after sensitization (*Figure 6.4*). These results indicate that not

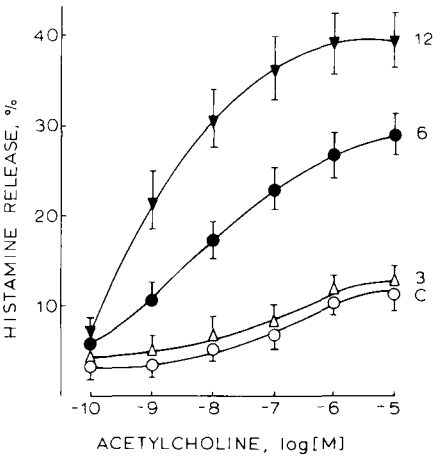

Figure 6.4. Histamine release induced by acetylcholine in isolated Wistar albino rat mast cells, preincubated with serum of actively sensitized Brown Norway rats at different days (figures on right) after sensitization. The values are the means ± S.E. of four experiments in duplicate. C, control serum.

only active, but also passive, sensitization can reproduce the supersensitivity of rat mast cells towards the cholinergic stimulus. Further evidence of a cholinergic histamine release triggered by passive sensitization is provided by the experiments reported in *Figure 6.5*: serosal mast cells isolated from normal Wistar albino rats do not react to acetylcholine, but acquire supersensitivity to acetylcholine as a function of the concentration of IgE (mouse IgE against ovalbumin or DNP-lysine) in the incubation medium. The cholinergic histamine release produced in passively sensitized mast cells is blocked by atropine (*Figure 6.6*), thus indicating again the presence of a muscarinic cholinergic receptor. The prosecretory effect of passive sensitization is clearly shown by

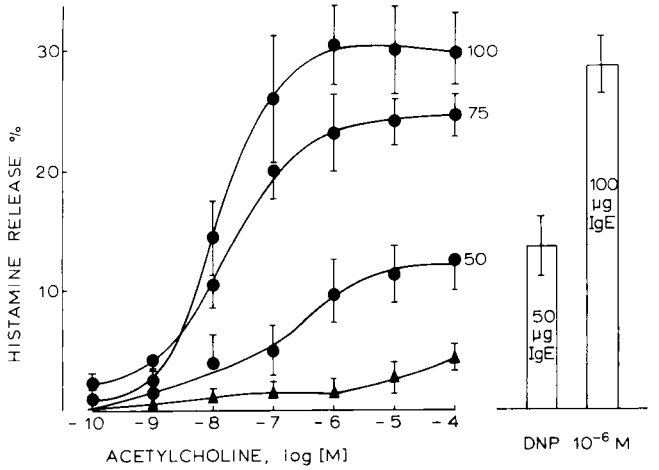

Figure 6.5. Histamine release induced by acetylcholine in isolated Wistar albino rat mast cells preincubated with different concentrations of IgE (figures at ends of traces, in µg IgE/10^6 cells; ▲, control). The values are the means ± S.E. of six experiments in duplicate. The IgE was raised in mice against $(DNP)_2$-lysine.

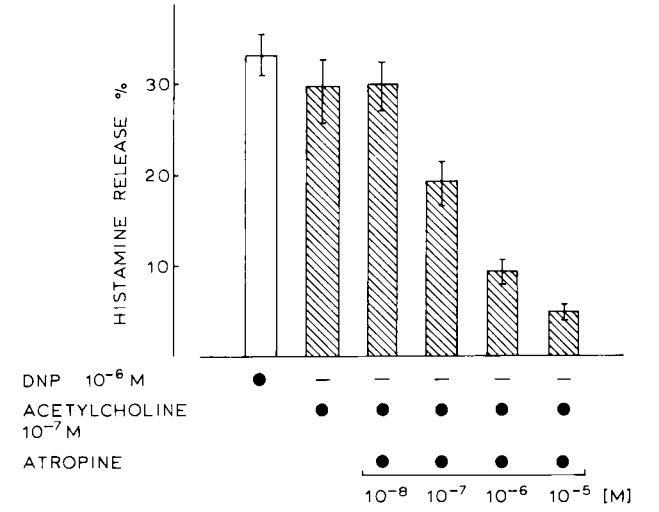

Figure 6.6. Effect of atropine on acetylcholine-induced histamine release in isolated Wistar albino rat mast cells preincubated with IgE (100 µg × 10^6 cells). The values are the means ± S.E. of four experiments in duplicate. The IgE was raised in mice against $(DNP)_2$-lysine.

an increase in calcium-45 uptake in passively sensitized mast cells isolated from Wistar albino rats (*Figure 6.7*). The effect of verapamil blocking the acetylcholine-stimulated calcium-45 uptake and histamine release in isolated passively sensitized Wistar albino rat mast cells is an interesting pharmacological annotation in keeping with a predictive use of calcium antagonists in the therapy of type I hypersensitivity reactions (*Figure 6.8*).

Since the only difference between sensitized and non-sensitized mast cells is the relevance of the IgE concentration on the cell membrane, it is conceivable

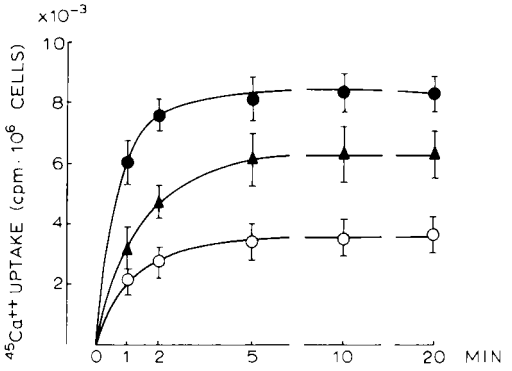

Figure 6.7. Rates of ^{45}Ca uptake in isolated Wistar albino rat mast cells treated with 10^{-7} M acetylcholine (○——○); in mast cells preincubated with IgE (▲——▲); in mast cells preincubated with IgE and treated with 10^{-7} M acetylcholine (●——●). The values are the means ± S.E. of three experiments in triplicate. The IgE was raised in mice against $(DNP)_2$-lysine.

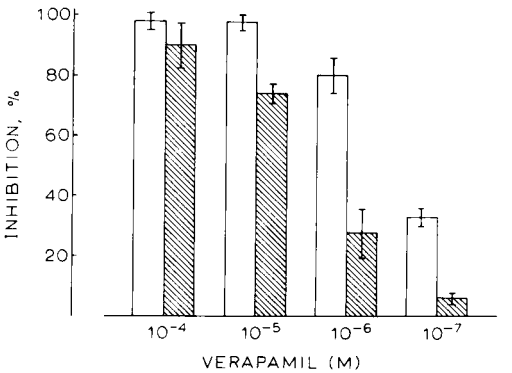

Figure 6.8. Effect of verapamil on acetylcholine (10^{-7} M) stimulated ^{45}Ca uptake (□) and histamine release (▨) in isolated rat mast cells preincubated with IgE. The values are the means ± S.E. of four experiments in quadruplicate. The IgE was raised in mice against $(DNP)_2$-lysine.

that the binding of IgE to specific IgE receptors on cell membrane may in turn evoke a cholinergic supersensitivity. A tentative explanation envisages that an extra binding of IgE to the mast-cell membrane activates silent cholinergic receptors.

THE RELEASE OF HISTAMINE BY PARASYMPATHETIC STIMULATION

In isolated vagally innervated guinea-pig auricles, vagal stimulation leads to a standstill of both contraction and rate, to vagal escape, and to a consistent rebound phenomenon at the end of the stimulation, characterized by a net increase in rate and amplitude of contraction; such events are potentiated by eserine and fully blocked by atropine. In the perfusates collected during the vagal stimulation, acetylcholine overflow is detectable. Together with acetylcholine, histamine appears in the perfusates collected during vagal stimulation with a time-course which is consistent with the physiological changes. Treatment with atropine decreases, in a dose-dependent fashion, the histamine overflow, while eserine extends the time-course of histamine release. The number of degranulated mast cells has been found to be significantly higher after vagal stimulation of guinea-pig auricles [92]. Similar experiments have been carried out on isolated strips of rat small intestine in which the parasympathetic nerve endings have been stimulated by specific field stimulation: the contractile response was prevented by atropine and potentiated by eserine. In the perfusates, a resting output of both acetylcholine and histamine was detected; field stimulation increased both acetylcholine and histamine output; atropine antagonizes, and eserine potentiates, the release of histamine evoked by field stimulation of parasympathetic nerve endings [75]. It appears from these experiments that parasympathetic stimulation of different organs of different animal species and endowed with heterogeneous mast-cell populations (connective tissue mast cells and mucosal mast cells) responds in the same way, releasing acetylcholine in response to the vagal stimulus which in turn causes the release of histamine, presumably from tissue mast cells. In these experiments, cholinergic histamine release is achieved even in the absence of any rôle of IgE. However, the experiments on the release of histamine evoked by vagal stimulation have been carried out on non-germ-free animals, which certainly bear a definite number of IgE molecules on the mast-cell membrane. Therefore, the contrasting evidence can be reconciled assuming two types of cholinergic histamine release. A first type is a physiological release of histamine induced by acetylcholine acting upon mast cells bearing a small number of IgE molecules on their membrane, subserving physiological events (in the heart, the

control of vascular permeability and of inotropic and chronotropic effects; in the gut, the modulation of peristalsis and of exocrine secretions). A second type is a pathological process, produced by acetylcholine acting upon mast cells loaded with IgE molecules, increasing the severity of type I hypersensitivity reactions or even producing a neurogenic pseudoallergic reaction even in the absence of the specific challenge (for example, exercise-induced asthma, cholinergic urticaria). Both these models necessitate the assumption that mast cells are classically innervated or are in close contact with nerve terminals.

THE INNERVATION OF MAST CELLS

An intimate association between nerve fibres and a variety of non-neuronal cells has been repeatedly demonstrated. Cauna and Cauna [93] have shown a close relationship between nerve fibres and plasma cells in human nasal respiratory mucosa. Ultrastructural studies of avian and mammalian gut and pancreas have revealed a close contact between different types of neurones and endocrine cells (islet cells) [94], basal-granulated cells of the foetal duodenum [95] and enterochromaffin cells [96]. In a strict sense, nerve terminals being close to non-neuronal cells does not imply a synaptic connection. However, true synapses have been demonstrated between boutons containing numerous small, clear and some large, dense core vesicles and enterochromaffin cells of the rat ileal mucosa [97]. In a broad sense, it has been stated that peripheral autonomic neurones can exert their action on effector cells by transmitter release even without true synapses, provided that the distance of the 'autonomic gap' does not exceed 100 nM [98]. In this context, it is relevant to note that only about 5% of the axonal varicosities from which the aminergic transmitter would appear to be released are associated with post-synaptic neurones. The remainder lack the characteristics of a classical synapse (cellular opposition, subsynaptic web and post-synaptic membrane thickening), implying that the majority of amine-containing varicosities release neurotransmitters which diffuse to remote receptors and that receptors for released amines are distributed further afield than in classical junctions, even on non-neuronal cells [99]. This type of transmission, which is intermediate between the specific synaptic travelling of nervous signals and the more diffuse broadcasting of the endocrine secretion, implies a nervous control of the release of the secretory products from these cells.

As an example, vagal nerve stimulation induces a neurogenic release of serotonin from enterochromaffin cells of the cat small intestine [100], and the vasodilatation induced by histamine is dependent on innervation [101]. As far

as mast cells are concerned, histamine release and mast-cell degranulation by sympathetic stimuli have been reported in the rat, in which stimulation of the splanchnic nerve led to an increased number of altered mast cells in the tissues [102]. Suggestive evidence of the physiological and morphological relationship between nerve fibres and mast cells has been described. The proportion of degranulating mast cells is significantly increased in the rat dermis following antidromic stimulation of the common trunk of the great auricular and lesser occipital nerves [103]. From the morphological standpoint, other workers [97] have described a relationship between mast cells and the vegetative end-formation of postganglionic neurones ('plexiform synapse on distance'), and a direct relationship between mast cells and unmyelinated preterminal nerve fibres has been reported by Heine and Förster [104] in human subcutis and dog myocardium. In a sublingual salivary glomus tumour of man, a population of mast cells was found in direct contact with nerve fibres either with each cell body or by means of lomellopodia or broader cytoplasmic protrusions in a way which is reminiscent of a classic synaptical organization [105]. In the mucosa specimen of rat terminal ileum, many of the mucosal mast cells contained numerous nerve terminals or boutons seemingly in direct contact with their plasma membranes. The terminals had a mixed composition of organelles (small and large dense core vesicles), the boutons with many small vesicles representing adrenergic and/or cholinergic terminals while the boutons with mainly large dense core vesicles belonged to peptidergic neurones. The membrane specializations which have been observed are highly suggestive of a true synapse [106].

The concept of possible innervation of mast cells implies neural control in the liberation of histamine or other secretory products. This is in keeping with the proposed rôle of substance P, which is released from sensory nerve endings and plays a part in the triple response of skin to injury by means of the release of histamine from mast cells ('neural inflammation' [107]). We propose a similar mechanism, as far as cholinergic histamine release is concerned.

CLINICAL IMPLICATIONS

The clinical implications of cholinergic histamine release link with the work of McFadden, Luparello, Lyons and Bleecker [108], who showed that 50% of asthmatic subjects developed an increase in airway resistance when they thought they were breathing a bronchoconstrictor, even when they were not, and that this response was blocked by atropine. An endogenous release of acetylcholine may result from physical exercise, emotional stress, or exposure

to heat and cold [100]. In our hypotheses, acetylcholine presumably acts directly on the mast-cell membrane to initiate mast-cell degranulation and mediator release. The behaviour of rat peritoneal mast cells after nonspecific stimulation strengthens this hypothesis: using the cold-restraint technique as a stressing factor, a significant proportion of mast cells were found degranulated [110]. On the other hand, bronchoconstriction obtained in dogs sensitized with aerosols of nematode (*Toxocara canis* and *Ascaris suum*) recalls the results of previous observations in human asthmatic patients in which vagally mediated reflex bronchoconstriction probably plays a critical rôle in acute antigen-induced asthma [111]. Thus, physical exercise, emotional stress or exposure to heat or cold may stimulate airway receptors, leading to a vagal reflex which in turn produces bronchoconstriction (and the other pathobiological events occurring in asthma [112]) both through the cholinergic stimulation of traditional muscarinic receptors and through the cholinergic-mediated mast cell degranulation and mediator release. Assuming the release of histamine as a marker of mast-cell secretion, it is worth noting that resting plasma histamine is usually higher in asthmatic than in normal subjects, that a correlation has been found between basal plasma histamine values and the severity of asthma, and that in acute asthma some authors have found raised plasma histamine [113]. The assumption that mast cells are paraneurones revives the rôle of mast cells and their mediators in the pathogenesis of asthma, and challenges the view that asthma has to be considered as an entirely immunological disease. There is no question that, before the discovery of the phenomenon of anaphylaxis in 1902, asthma was considered a disease of excessive irritability involving the nervous system [114]. Evidence to support the concept of cholinergic histamine release from IgE-bearing mast cells as a crucial event in the pathogenesis of asthma includes the effectiveness of cromolyn sodium as both an inhibitor of mast-cell secretion and a therapeutic agent in the treatment of exercise-induced asthma [115], and of the hyperreactive response of the bronchi of subjects with asthma to challenge with methacholine. In addition, 30% of patients with exercise-induced asthma benefit from the inhalation of ipratropium bromide, an anticholinergic agent [115]. The lack of antihistamine effectiveness in asthma may in part reflect insufficient pharmacological blockade, since it is unlikely that systemically administered antihistamines are able to achieve local concentrations in the lungs high enough to counteract the massive release of histamine (over 10 μM). Limitation of the dosage is due to the intense sedative side-effect. In this respect, the new H_1-antihistamines devoid of depressant effects on the central nervous system should be worth a further trial [116].

In 1978, Blumberg [117] reported a case of asthma associated with

cholinergic urticaria. Three out of seven subjects who developed cholinergic urticaria running on a treadmill in a plastic occlusive suit also experienced asthmatic attacks [118], extending to the lung the manifestations of a condition which was previously thought to be limited to the skin. Cholinergic urticaria has been proposed as a nosological entity since the classical paper by Duke [119] in 1924. Cholinergic urticaria, which has been estimated to occur in 5–7% of patients with urticaria, is a form of physical urticaria precipitated by stimuli that increase core body temperature, such as hot showers, physical exercise and episodes of pyrexia. The clinical aspects and the pathogenesis of cholinergic urticaria have been extensively reviewed [120–125]. The eruption can be induced by any stimulus that causes autonomic discharge of acetylcholine, and is distinctive, consisting of tiny pruritic wheals surrounded by extensive areas of macular erythema. The level of serum histamine in subjects with experimentally induced cholinergic urticaria rises from normal values before challenge to peak levels at 20 min after challenge, with a return to baseline levels by 40 min [109,118]. Of relevance to our hypothesis is the case reported recently by Shelley, Shelley and Ho [126]. A patient suffering of cholinergic urticaria is described in whom skin biopsies of exercise-induced urticarial lesions showed degranulated mast cells. Moreover, papular wheals were induced by acetylcholine chloride in threshold concentration, and a patch test to pure metallic copper elicited no response *per se*, while showing multiple wheals when the patient was challenged with exercise or when the sites were injected locally with acetylcholine in subthreshold concentration. The study shows that cholinergic urticaria can be associated with a specific subclinical immediate-type hypersensitivity, since the clinical symptoms were not apparent until elicited by autonomic cholinergic discharge. This case report is strongly reminescent of the experimental results obtained with acetylcholine, preferentially releasing histamine from IgE-bearing mast cells.

The pathogenesis of inflammatory bowel disease remains obscure, despite many years of intensive research. However, recent evidence has been provided concerning the possible rôle of mast cells in ulcerative proctocolitis and in Chron's disease. A significant increase in the number of intestinal mast cells was found in patients with active ulcerative proctocolitis. The mast cells observed while the disease was active contained varying numbers of altered granules, suggesting the release of mediators. Reduction in the number of mast cells and improvement of their morphological appearance were observed in association with the clinical response to disodium cromoglycate treatment [127,128]. A morphological study of Chron's disease has indicated mast-cell degranulation to be a prominent feature. Interestingly, the mast cells were adjacent to and within proliferating autonomic nervous system elements of the

gut [129]. A patient with Chron's disease was found to respond to disodium cromoglycate, obviating the need for resection [130]. After the demonstration of synapses between mast cells and autonomic nerve fibres in the gut of experimental animals [106], participation of cholinergic mast-cell degranulation and mediator release in the pathogenesis of some inflammatory bowel disease in man may be envisaged.

Finally, a beneficial effect was found by sectioning the vidian nerve in patients suffering of severe allergic rhinitis. After the nerve resection, a significant diminution both in mast-cell number and histamine content in the mucosa was found, suggesting further indirect evidence of a relationship between parasympathetic nerve terminals and tissue mast cells (P.F. Mannaioni, personal observation).

GENERAL REMARKS

A relationship between sympathetic nerve terminals and the mast-cell system has been repeatedly suggested. In animal studies, post-sympathetic vasodilatation has been shown to consist of passive and active components, the passive component being simple withdrawal of sympathetic tone and the active component being due to a local histaminergic mechanism operating under sympathetic neuronal control [131–133]. An increase in sympathetic tone arising from the hypothalamus causes vasoconstriction through α-adrenergic stimulation but it also affects the mast cell membrane, causing the release of histamine and vasodilatation. H_2-receptors located on the sympathetic nerve terminals are stimulated by the released histamine, leading to the inhibition of noradrenaline release [134]. A deficiency in such a histaminergic vasodilating system should be responsible for the sustained vasoconstriction in response to cold exposure or emotional stress, which is typical of the Raynaud's phenomenon [135].

In the present paper, we have tried to substantiate a relationship between the parasympathetic nervous system and the mast-cell system. In this case, complete neuronal control of the secretion of histamine and other mediators could be visualized. The neuronal control of mast-cell secretion would be relevant in physiological terms, balancing the local vascular tone and permeability, modulating exocrine secretion, and influencing the cardiac rate and inotropism and the contraction of smooth muscles. The neuronal control of mast-cell secretion would be also relevant in pathological terms, leading to excessive mast cell degranulation, which occurs when the number of IgE molecules present on the mast cell membrane is significantly enhanced. The

mechanism would clarify many of the puzzling features of diseases such as exercise-induced asthma, cholinergic urticaria, ulcerative proctocolitis, Chron's disease and allergic rhinitis.

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7 New Approaches to Bronchodilator and Antiallergic Drug Therapy

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INTRODUCTION

"I have no magic cure to report"

Reply of Maimonides to the asthmatic son of Saladin, Sultan of Egypt, in the 12th Century.

There is still no cure for asthma and many other allergic diseases, although a variety of therapies have been developed to treat the symptoms of these disorders. The search for a cure awaits greater knowledge of the basic mechanisms involved in these complex disease processes.

Bronchial asthma is a common disease affecting both children and adults. However, a precise definition has proved elusive and consequently the prevalence and incidence have not been well characterized. It has been suggested that 5% of adults and 7–10% of children in the U.S.A. and Australia have the disease [1]. Etiologically, asthma is a heterogeneous disease and the different types of asthma are often defined by the stimuli that provoke acute episodes. Seven major categories of stimuli exist: infections, exercise, antigens, occupational stimuli, environmental causes, pharmacologic stimuli and emotional stress [2].

Airways responses are controlled by the sympathetic and parasympathetic nervous systems on the smooth muscle of the tracheobronchial tree. The parasympathetic-cholinergic motor supply to effector cells in the lung predominates and results in release of acetylcholine which constricts the major airways, increases mucus secretion from goblet cells and dilates the pulmonary vessels. The sympathetic innervation of the lung is sparse and affects primarily the blood vessels, parasympathetic ganglia and mucus glands [2]. However,

the bronchial musculature has a high density of adrenergic receptors. In addition to airway relaxation, particularly in the peripheral lung, stimulation of β-adrenoceptors relaxes pulmonary vasculature, facilitates ion and water transport to the airway lumen and stimulates glandular secretion [3,4]. Airways relaxation to β-adrenoceptor agonists is mediated almost entirely by β_2-adrenoceptors, whereas relaxation in response to sympathetic nerve stimulation is mediated predominantly by β_1-adrenoceptors [3]. This is consistent with the view that circulating catecholamine regulates β_2-adrenoceptors and noradrenaline release from sympathetic nerves regulates β_1-adrenoceptors.

Adrenergic α_1- and α_2-adrenoceptors are also present in high density in the lung, especially in submucosal glands, airway epithelium and vascular smooth muscle [5]. Their stimulation may result in airway constriction and glandular secretion.

There is growing evidence for a third nervous system in the lung, referred to as the peptidergic or non-adrenergic inhibitory system [6]. The neurotransmitter mechanism of this inhibitory nervous system may be vasoactive intestinal polypeptide (VIP). The peptidergic system, rather than the sympathetic inhibitory system, may oppose parasympathetic excitation.

Airway responsiveness in asthmatics is greatly increased, and this phenomenon, often referred to as airway or bronchial hyperreactivity, is a cardinal sign believed by many to be the primary pathogenetic event in the development of the disease. Several mechanisms have been proposed to explain the pathophysiology of bronchial hyperreactivity [7]: (i) a decrease in baseline airway calibre; (ii) an elevation in the responsiveness of the bronchial smooth muscle; (iii) an imbalance in the autonomic nervous control of the airways; and (iv) a breakdown of airway defences, allowing an increase in accessibility of allergens or nonspecific stimuli to mast cells, sensory nerve endings or bronchial smooth muscle.

These mechanisms have been reviewed in detail by others, and it is apparent that airway hyperreactivity does not depend on just one factor [8–10]. Therapeutic modulation of airway hyperreactivity is clearly highly desirable; however, most bronchodilator agents, perhaps with the exception of disodium cromoglycate (DSCG) and the corticosteroids, do not appear to affect the underlying pathophysiology of hyperreactivity [7]. The complexity of this phenomenon makes this goal exceedingly difficult to achieve at present.

In many asthmatics, atopic status is also important. In these patients, immunological triggers including airborne allergens induce the formation of immunoglobulin E (IgE) antibodies which subsequently coat mast cells in the lung and also circulating leucocytes. Bronchospasm is produced when allergen interacts with these IgE-coated cells, resulting in release of numerous

bronchoactive substances including histamine, prostaglandins, leukotrienes and chemotactic factors. Release of the latter mediators is also thought to be involved in asthma involving non-immunological events and, consequently, the formation and/or release of these substances appears pivotal in most forms of asthma.

CURRENT THERAPIES IN THE TREATMENT OF ASTHMA

During the last 15 years, a variety of new drugs for the symptomatic treatment of asthma have been developed [4,11]. The judicious use of inhalation technology with β-adrenoceptor agonists, corticosteroids and anticholinergics has made possible more effective therapy with fewer side-effects. Improvement in our knowledge relating to optimal dosage for older drugs such as the methylxanthines has also provided safer therapy.

Table 7.1. outlines the major groups of drugs currently used to control asthma and includes many of the limitations still associated with these compounds. The effectiveness of these drugs is variable, some patients responding erratically and exhibiting undesirable side-effects.

Multiple drug therapy in asthma is generally accepted and is mandatory for severe disease. Combinations of drugs working through different mechanisms to produce bronchodilation would be expected to improve results of the same drugs administered alone. This may be additive or even synergistic. There is also an opportunity to lower the concentrations of both drugs and consequently reduce side-effects.

Clinically, there are many problems associated with the assessment of bronchodilator combinations, e.g., patient responsiveness, routes of administration, pecularities with individual drugs, and the important question as to whether a second drug will improve the bronchodilation achieved with one drug alone is often unanswered. Nevertheless, a variety of studies have been performed, the majority involving anticholinergics and β-agonists, and β-agonists and methylxanthines [12]. As predicted, there is usually an additive bronchodilator effect between the anticholinergics and β-agonists when administered by inhalation. A similar advantage is found with the β-agonist-methylxanthine combination. However, concern has been expressed concerning the concomitant use of oral β-agonists and methylxanthines in the light of the enhanced cardiac toxicity noted in animals given this combination [13]. Confirmation or refutation of this concern will await the completion of clinical studies to determine whether the animal findings have relevance for the asthmatic.

Table 7.1. MAJOR CURRENT CLASSES OF BRONCHODILATOR-ANTIALLERGIC DRUGS USED IN THE TREATMENT OF ASTHMA AND THEIR MAJOR LIMITATIONS

PO, oral; INH, inhalant; IV, intravenous; SC, subcutaneous

Class	*Examples*	*Routes*	*Major limitations*
(1) β-Adrenoceptor agonists	albuterol (salbutamol)	PO, INH	Skeletal muscle tremor (especially PO)
	fenoterol	INH	Tachycardia (minor)
	terbutaline	PO, SC	Reduction of arterial O_2 tension
			Tolerance (questionable)
(2) Methylxanthines	theophylline	IV, PO	Variable bioavailability pharmacokinetics.
	aminophylline	IV, PO	Side-effects related to serum level:
			<20 μg/ml: uncommon
			20–35 μg/ml: nausea, vomiting, diarrhoea, headache and irritability
			>30–35 μg/ml: hyperglycemia, hypotension, cardiac arrhythmias, seizures, brain damage, death
(3) Corticosteroids	beclomethasone dipropionate	INH	Local: oropharyngeal candidiasis, dysphonia. Systemic: suppression of HPA function, deaths due to adrenal insufficiency after transfer from systemic to aerosol steroids. Long-term side-effects of inhaled steroid unknown
	triamcinolone acetonide	INH	
	budesonide	INH	
(4) Antiallergics	disodium cromoglycate	INH	Coughing, wheezing, nasal irritation (minor). Prophylactic usage only
(5) Anticholinergics	ipratropium	INH	Weak efficacy in allergic asthma
	atropine methonitrate	INH	

Further clinical combinations, such as methylxanthine and anticholinergic, oral β-agonist with inhaled anticholinergic, and drugs from all three groups administered together appear intriguing [12]. More prolonged assessment of these combinations will also be necessary in order to establish this therapeutic technique firmly.

Increasing knowledge of the pathophysiology of asthma, particularly at the biochemical level, and further understanding of the mechanism of action of currently used antiasthmatics, assists the pharmacologist and medicinal chemist in their combined search for more effective and safer therapies for asthma. The remainder of this review will address several of the directions currently being pursued in attempts to achieve the latter goal.

MODULATION OF IgE SYNTHESIS AND BINDING

Immunotherapy attempts to modify the interaction of allergen with IgE usually by increasing allergen administration to allergic patients, thus producing a state of tolerance to that allergen [14]. However, more favourable results have been obtained in the treatment of rhinitis rather than asthma. Numerous problems also exist concerning the preparation and standardization of allergen extracts and the frequency of injection. Nevertheless, treatment of respiratory allergy may lie with immunotherapy.

There is no unitary hypothesis of how immunotherapy works. Increases in circulating specific immunoglobulin G (IgG), the 'blocking' antibody, have been observed after immunotherapy with ragweed and grass pollen, but this is not always correlated with clinical improvement [15]. Furthermore, increases in both specific IgG and immunoglobulin A (IgA) antibodies occur in secretions from the respiratory tract, although these changes are once again not correlated with clinical improvement [16]. The suggested rôle of IgG as a protective antibody is complicated by the fact that it is also capable of sensitizing target cells for mediator release [17].

Denatured allergens, essentially non-allergic but capable of stimulating suppressor T cells, thus preventing IgE production, can occur in animals and probably in man [18]. Poly(ethylene glycol)-substituted allergens or allergens linked to copolymers of D-glutamate or D-lysine have also been used to achieve this goal [14]. Allergoids, or chemically treated (formaldehyde or glutaraldehyde) allergens, may also reduce the ability to cause clinical reactions whilst suppressing IgE production [19].

The regulation of IgE biosynthesis has been the subject of much recent

research. Mechanisms exist for both enhancement and suppression of IgE production, although their interactions remain ill-defined [20–22].

The differentiation of precursor B lymphocytes to IgE-forming cells is under the control of T cells which possess helper and suppressor functions. Isotype-specific regulation of the IgE response involving two binding factors derived from T cells has recently been described [20,21]. One of the IgE-binding factors potentiated the IgE response (IgE-potentiating factor), whereas the other IgE-binding factor suppressed the IgE response (IgE-suppressive factor). Both substances bind the Fc domain of IgE and have molecular weights of 13,000–15,000, but they differ in their carbohydrate content. They regulate IgE production by binding to IgE-bearing B cells through surface IgE. Elaboration of either enhancer or suppressive factors by T cell lines *in vitro* is dependent on substances that modulate protein glycosylation. Increased glycosylation leads to stimulation of IgE-enhancing activity, whereas substances that inhibit glycosylation lead to production of IgE-binding factors with suppressive activity [20]. Inhibition of protein glycosylation may thus provide a means to turn off IgE production. The endogenous glycosylation-inhibiting factor appears to be a fragment of phosphorylated lipomodulin (see later section on Phospholipase inhibition). The factor may correspond to macrocortin and may exert its function through inhibition of phospholipase on or in lymphocytes. By contrast, phospholipase activation in lymphocytes may enhance IgE production.

Little attention has been paid to the possibility of interfering with IgE-target cell (mast cell, basophil, eosinophil, lymphocyte and macrophage) interaction, although this approach is potentially one of the most specific ways of interfering with allergic reactions. Significant advances have been made in our understanding of the molecular nature of IgE receptors (Fc R) in mast cells and basophils, and studies on the tertiary and quaternary structure of the Fc R have begun to emerge [23]. However, efforts to obtain a smaller fragment containing the binding structure have proved difficult. Hamburger [24] described a synthetic pentapeptide (Asp-Ser-Asp-Pro-Arg) corresponding to a segment found in the human-chain of IgE, which he claimed competitively inhibited the binding of IgE to its cell receptor. However, these claims were later refuted by others [25]. Hamburger later suggested an alternative mechanism such that the human IgE pentapeptide (HEPP) binds to unoccupied receptors, thus saturating the cell surface and triggering membrane receptor turnover [26]. HEPP is currently under clinical evaluation in allergic rhinitis [27]. Further advances in this area coupled with a knowledge of primary structure of Fc R may well allow development of selective inhibitors of IgE binding.

MODULATION OF CELLS IMPLICATED IN ASTHMA

The cellular involvement in asthma and related allergic conditions is unequivocally a critical component of the pathogenesis of this class of pulmonary disorders. Evidence to support this concept includes the identification of various inflammatory cell types in asthmatic lung, the capacity of these cells to synthesize and release allergic mediators, and the ability of these soluble factors to induce various facets of the asthmatic response.

It is beyond the scope of this review to describe the biology of over 40 cell types that have been identified and classified in the lung. A detailed analysis of the various pulmonary cells has been provided by the review of Breeze and Wheeland [28]. Instead, it is intended to discuss the cellular mechanisms involved in the pathogenesis of asthma and describe the probable involvement of the major cell types in the synthesis and release of allergic mediators.

INVOLVEMENT OF MAST CELLS AND BASOPHILS

Ever since the identification of mast cells by Paul Ehrlich nearly 100 years ago, the mast cell has been intensively studied by various investigators. The presence of large amounts of histamine in mast cells [29] and the ability to synthesize other bioactive mediators in response to appropriate stimuli have placed the mast cell in a central rôle in the pathogenesis of asthma. Mast cells are found in the connective tissue of the lung and are often situated close to the microvasculature, lymphatics, bronchial lumen, submucous glands and throughout smooth muscle bundles [30]. They do not circulate, and thus the consequence of mast-cell activation depends largely on the existing distribution. Activation of intraluminal and intraepithelial mast cells by specific antigen leads to mediator release which can relax the tight junctions to permit antigen to penetrate into the lumen.

Mast cells participate in IgE-mediated reactions and cross-linking of IgE receptors causes the release of the biologically active mediators. The isolation of purified populations of human lung mast cells indicated that mast cells do not consist of a single population but can vary substantially in structure and function [31]. For example, small mast cells contain less histamine than large mast cells, and the maximum histamine release after immunological stimulation varies with mast-cell size.

Although basophils share several notable properties with mast cells, such as being able to bind IgE and release histamine [32–35], mast cells and basophils are not identical [36]. Basophils, unlike mast cells, differentiate and mature in the bone marrow, are present in the circulation and are not normally found in

the connective tissues. Nonetheless, it appears that similar underlying biochemical mechanisms may operate during activation of mast cells and basophils.

The complex biochemical processes that follow IgE activation of mast cells and basophils have recently been partially elucidated [37–39]. It has been proposed that perturbation of the membrane IgE receptors generates a signal (perhaps mediated by serine esterases) that activates two methyltransferases to convert phosphatidylethanolamine to phosphatidylcholine. Subsequently, it is believed that phosphatidylcholine is cleaved by a phospholipase A_2 (PLA_2) to lysophosphatidylcholine which, together with increases in membrane fluidity, promote Ca^{2+} entry and 3′5′-(cyclic)adenosine monophosphate (cAMP) generation. In addition, it has been proposed that free arachidonic acid released through the action of PLA_2 from phosphatidylcholine is converted to prostaglandins or leukotrienes which can modulate histamine release, since lipoxygenase products such as 5-HETE (see later in *Scheme 7.2*) have been shown to increase histamine release [40].

Since phospholipid methylation and increases in cAMP occur almost immediately after receptor bridging, it had been difficult to assess the temporal relationship between these processes. There is some evidence to suggest that phospholipid methylation precedes adenylate cyclase activation, since 3-deazaisobutyryladenosine, an inhibitor of methylation, blocked the synthesis of cAMP [39]. Conversely, the rise in cAMP may feedback on the phospholipid methylation process and, therefore, act as a negative feedback mechanism to shut off histamine release.

Mast cell activation may also occur through the 'phosphatidylinositol' (PI) cycle. PI breakdown is a very early cellular event [41,42] and recent studies using mast cells have indicated that there are close similarities between the profiles for PI breakdown and the Ca^{2+} signal [43,44]. It has been suggested that the Ca^{2+} signal is functionally independent of the normal Ca^{2+} influx and is maintained by the balance between the increased Ca^{2+} influx and active Ca^{2+} influx across the cell membrane. However, it is not known whether PI breakdown precedes the Ca^{2+} signal or an activation of PI phosphodiesterase.

Regardless of the signal after receptor activation, mast cells are known to release several classes of mediator into the extracellular milieu. In general, these mediators may be preformed substances that are released from intracellular stores and these would include histamine [29,45], eosinophil chemotactic factors [46–49] and neutrophil chemotactic factors [50–52]. Alternatively, newly formed mediators such as prostaglandin D_2 [53,54], leukotrienes [55,56] and platelet-activating factor (PAF) [57], may also be synthesized and released after IgE activation. A variety of enzymes and proteoglycans such as neutral

proteinases [58,59], lysosomal enzymes [60], heparin [61] and chondroitin sulphate [62] has also been demonstrated in mast cells.

Preformed and newly generated mediators have the capacity both individually and in concert to mediate the asthmatic attack. Biogenic amines that are secreted with PGD_2, PAF and leukotrienes enhance vascular permeability and plasma exudation, while various chemotactic factors can direct the infiltration of eosinophils and neutrophils into the lung [63,64].

Recently, it has been recognized that the asthmatic response consists of an early and late phase. Increasing interest has been focused on the late-phase reaction [65,66] since it has been suggested that the occurrence of a late-phase reaction may be central to the development of bronchial hyperreactivity [67]. The late-phase reaction may be due to the influx of circulating inflammatory cells into the lung. Typically, the late response is unresponsive to β-agonists and the administration of cromoglycate is only effective prophylactically and does not affect ongoing late reactions [68]. It has been suggested that the mechanism of action of cromoglycate *in vivo* is to inhibit inflammatory mediator release by stabilizing mast cells [69,70], although studies with human mast cells and basophils have failed to support this mode of action [71–73]. The reader is asked to refer to the review of Suschitzky and Sheard [74] for a critical assessment of this drug and a variety of other 'mast-cell stabilizers'. The exact mechanism of action of cromoglycate is still unclear and its relative inactivity in inhibiting human mast cell or basophil mediator release leaves open the question of whether other agents that stabilize animal mast cells may also lack the ability to influence human mast cells or basophils.

Anti-inflammatory steroids are widely used as antiasthmatic agents and steroids are effective against the late-phase reaction rather than the early-phase response. Cellular studies show that steroids inhibit histamine release in human basophils but do not inhibit histamine release from human mast cells [75] and would suggest that basophils may be more important in the late-phase reaction. Since steroids have been proposed to work through the induction of an antiphospholipase protein (see section on Phospholipase inhibition), this raises the possibility that steroids inhibit selectively certain lung phospholipases but not lung mast-cell phospholipases. Furthermore, it would suggest that cells other than mast cells participate in the late-phase reactions.

Although pharmacological modulation of late-phase reactions is attractive [76,77], it is still premature to predict that agents that inhibit late-phase reactions will be more efficacious in asthmatics. However, there is sufficient evidence to suggest that the late-phase reaction reflects lung tissue inflammation which is caused by the influx of neutrophils, eosinophils and monocytes in response to chemotactic mediators such as eosinophil or neutrophilic chemotactic factors and arachidonic acid metabolites.

INVOLVEMENT OF EOSINOPHILS

Eosinophils are believed to be recruited into the lungs by the release of soluble chemotactic factors from degranulating mast cells [78,79] and a variety of mast-cell-derived factors has been shown to be chemotactic for eosinophils. Amines [80], acidic peptides [81] and lipoxygenase products such as mono-HETEs and the leukotriene LTB_4 [82,83] are chemotactic for eosinophils at low concentrations. It has also been demonstrated that eosionophils possess IgE receptors [84] and the interaction of anti-IgE with eosinophil-bound IgE leads to mediator release [85].

Although eosinophils have been clearly identified in asthmatic lungs, their precise rôle in asthma remains to be determined. There is some evidence for believing that eosinophils may have a 'dampening' effect on mast cell mediators. For example, eosinophil major basic protein can inactivate heparin [86], and eosinophil-derived enzymes such as histaminase and arylsulphatase can degrade histamine and leukotrienes, respectively [87,88]. More recently, eosinophil peroxidase was demonstrated to inactivate the leukotrienes LTB_4, LTC_4 and LTD_4 via an oxygen–free-radical mechanism [89]. Eosinophils also contain phospholipase D, which may inactivate PAF [90]. Although eosinophils possess the necessary mechanisms to reduce the effects of mast-cell degranulation, they have also been shown to release inflammatory mediators which can potentiate the asthmatic reaction. For example, major basic protein has been shown to damage respiratory epithelium [91], which leads to an increased penetration of antigen through the bronchial lumen. There is also evidence to suggest that eosinophils may, in fact, have a greater capacity to synthesize leukotrienes than mast cells or neutrophils [92]. Proteolytic enzymes may also lead to epithelial cell damage, resulting in the opening of tight junctions. These studies, therefore, suggest that eosinophils may be proinflammatory and could potentially be injurious to the lung. It would appear that modulation of their activity is desirable in asthma, but until the rôle of eosinophils in asthma is more firmly defined, pharmacological intervention remains at the level of inhibiting mediator release. For example, there is no reason to believe that an agent such as a lipoxygenase inhibitor which inhibits leukotriene synthesis in other inflammatory cells will not also act on the eosinophil-leukotriene generating system. Furthermore, by blocking the synthesis of leukotrienes, a situation might be created whereby the leukotriene-degrading enzymes of eosinophils can aid in the total removal of these potent bronchoconstrictors.

INVOLVEMENT OF MACROPHAGES AND NEUTROPHILS

Phagocytic cells such as alveolar macrophages and neutrophils play a major rôle in the immunological processes of the lung [93–95]. Their host defence capabilities are critical for maintaining normal pulmonary antimicrobial defences. It is, however, also evident that these cells possess the ability to injure tissue when their production of mediators is uncontrolled during an inflammatory or allergic reaction. Increasingly, it is recognized that these 'professional' phagocytes synthesize and release a variety of mediators which participate in the inflammatory response [96,97].

Both neutrophils and macrophages secrete lysosomal enzymes and neutral proteinases during phagocytosis. Ingestion of non-digestible particles such as asbestos results in the chronic release of these potentially lethal substances [98,99]. Proteinases are, at present, considered the most likely causative agent of the destructive changes reported in emphysema [100,101] and it is interesting to note that macrophages from smokers secrete higher levels of these enzymes than do normal macrophages [102]. In addition, neutrophils and macrophages can release highly reactive oxygen free-radicals that can damage tissues [103]. Clearly, in light of these observations, both cell types have the potential for severe tissue injury.

Neutrophils and macrophages are also active producers of arachidonate metabolites [104]. LTB_4, a potent chemoattractant, is released during neutrophil or macrophage activation [105], and this suggests that these cells could contribute to the overall production of leukotrienes in the lung. PAF has also been shown to be released from phagocytes [106]. In light of the air-tissue location of alveolar macrophages, it is possible that they may be the first cells to be exposed to inhaled antigen and secrete arachidonate metabolites.

Despite the observations favouring the participation of phagocytes in allergic reactions, it is still not clear whether these cells play a critical rôle in asthma *per se*. Alveolar macrophages have IgE receptors [107] and are activated by anti-IgE or specific antigen to which asthmatic patients have been sensitized [108]. Mast cells in the lumen of the peripheral airways may trigger macrophages indirectly and mast-cell-derived chemotactic factors may attract circulating neutrophils into the interstitial spaces of the lung. It is possible that phagocytes may be initially recruited to inactivate or remove the inciting agent but, due to abnormal control mechanisms, these cells may continue to produce inflammatory mediators that lead to tissue destruction. One can further speculate that macrophage and neutrophil participation in the asthmatic reaction may signal the progression of the asthmatic reaction to a more inflammatory condition where tissue destruction occurs at the epithelial layer,

thereby allowing more antigen to penetrate deeper into the submucosal region. Thus, the modulation of these cells or their mediators, as in the case of mast cells, is desirable and could possibly lead to better strategies for pharmacological intervention.

INVOLVEMENT OF EPITHELIAL CELLS

Obstruction of the airways by mucus is a common feature associated with asthma [109,110]. Intrinsic changes in mucus and decreased mucus clearance by the mucociliary apparatus combine to induce plugging of the airways. Mucus secretion from submucosal glands is under cholinergic nervous control [111], whilst goblet cells secrete their mucus content directly into the airways [112,113]. Under normal conditions, the expulsion of mucus is through the concerted beating action of the ciliated cells.

Recent findings have suggested that mediators released from neighbouring cells may influence the secretion and clearance of mucus [114]. In addition to reflex mechanisms, arachidonic acid metabolites such as prostaglandins and leukotrienes [115,116], HETEs [117] and histamine [118] have all been shown to increase airway mucus secretion. There is considerable increase in volume and quality of the secretions during an acute attack of asthma. Interestingly, in the *Ascaris*-sensitive dog, FPL 55712, a leukotriene antagonist, prevented the decrease in mucus clearance after the antigen-induced bronchoconstriction [119], suggesting that leukotrienes may be a major mediator in mucus secretion.

Epithelial cells also serve as a protective barrier towards the diffusion of potent mediators and antigen into pulmonary tissue. Tight junctions between epithelial cells have been described in airways [120,121] and morphologic abnormalities of airway epithelium tight junctions have been demonstrated in asthma [122–124]. In ozone- and virus-induced models of bronchospasm, reversible airway epithelial damage was correlated with transient airway hyperreactivity [125–127]. Studies have also described the relaxation of tight junctions between epithelial cells after exposure to methacholine and histamine [128]. Thus, epithelial damage could cause airway hyperreactivity by increasing airway permeability, allowing greater penetration of inhaled materials to reach effector tissues. Because of the anatomical arrangement of nerve fibres and smooth muscle, agents that cause reflex bronchoconstriction may require only relaxation of tight junctions to reach sensory receptors, whereas inhaled direct smooth muscle agonists may need other mechanisms to penetrate deeper into the target tissue. However, it should be noted that mediators released by intraluminal secretory cells (e.g., mast cells) may have direct access to smooth muscle. Changes in epithelial permeability could be important only

for high-molecular-weight substances such as antigens and other proteinaceous materials, whereas histamine and other lipid mediators (e.g., leukotrienes and PAF) may have the capacity to overcome such physical barriers.

INVOLVEMENT OF LYMPHOCYTES AND LYMPHOID TISSUE

The relevance of cell-mediated immunity in bronchial asthma is unclear. There is clear evidence to suggest that IgE production is strongly influenced by T-lymphocytes [21,22,129]. Changes in lymphocyte subset populations have been observed in the peripheral blood of asthmatics after antigenic provocation, although such changes were subsequently shown to be absent in methacholine-induced asthma [130]. It is most likely, therefore, that non-immune asthma is unrelated to changes in lymphocyte populations and that lymphocytes do not play a major rôle in this type of asthma. Nevertheless, the manipulation of lymphocytes that modulate the IgE response will possibly be useful in immunotherapy of certain types of allergic asthma and may provide further insight into the pathogenesis of antigen-induced bronchospasm.

Bronchus-associated lymphoid tissue (BALT) consisting of both lymphocytes and macrophages [131] exists as aggregated, follicular and solitary forms throughout the lung. BALT can selectively sample antigens in the lumen of the respiratory tract and may be the first site in the lung in which particles may be deposited [132]. Interestingly, granulated basophil-like cells have been demonstrated in association with BALT and these cells appear to proliferate in response to an aerosol deposition to antigen. Thus, it seems likely that basophils or mast cells may be influencing T-cell activity. Despite these observations, there is no direct evidence to suggest that BALT is involved in asthma. One can perhaps speculate that exposure of BALT to antigen may influence the ultimate hyperproduction of IgE that precipitates allergic asthma

MODULATION OF AIRWAY SMOOTH MUSCLE RECEPTORS

ADRENOCEPTORS

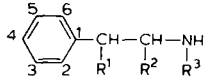
Biological responses to catecholamines are mediated by α- and β-adrenergic plasma membrane receptors which have been subdivided into α_1, α_2, β_1 and β_2 subtypes. It appears that different organs, including the lung, have mixtures of functional β_1- and β_2-adrenoceptors. A 1:3 ratio ($\beta_1 : \beta_2$) has been reported in the lung [133]. A recently developed approach for delineating the structure of β-adrenoceptors is the covalent incorporation of photoaffinity probes derived

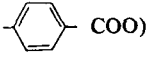
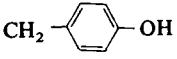
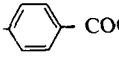
from high-affinity, radiolabelled β-adrenergic antagonists [134]. Photoaffinity labelling of mammalian β_1- and β_2-adrenoceptors in both particulate and purified preparations indicate that these receptors are predominantly if not exclusively polypeptides of molecular weight 62,000 to 64,000. Unfortunately, β_2-receptors from various tissues seem to be similar, probably ruling out the development of organ-specific β_2-agonists [135].

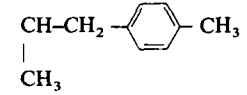
The currently used β_2-agonists have advantages over the mixed β_1- and β_2-agonists such as isoprenaline (isoproterenol) and are very effective in the treatment of asthma. Their development and therapeutic importance has been reviewed in detail elsewhere [4,11,136]. β-Agonists reduce airways obstruction by relaxing bronchial smooth muscle, and they also stimulate mucociliary transport, reverse bronchial venoconstriction and inhibit release of mediators from mast cells. The short duration of action of isoprenaline predisposes it to overuse and, because of this and other limitations, safer and more selective and effective drugs have been sought. It is worth briefly recounting some of the major structure-activity relationships uncovered in the development of the newer β_2-agonists (*Table 7.2*). The majority of the older agents, including isoprenaline, contain the catechol nucleus and the ethanolamine side-chain. Potency is generally reduced when the catechol nucleus is altered. In addition, susceptibility to enzymatic degradation by catechol-*O*-methyltransferase (COMT) or sulphatase is also due to the catechol, and this precludes oral use for these drugs. However, esterification of the catechol hydroxyl groups as in bitolterol mesylate or substitution of a resorcinol (metaproterenol) or saligenin (albuterol) nucleus, produced orally effective drugs that have a longer duration of action since they are unaffected by COMT. Modification of the ethanolamine side-chain also results in dramatic changes in the pharmacological profile, particularly relating to receptor sensitivity.

Great interest has been generated with the development of terbutaline prodrugs. Terbutaline, a polar molecule suffering from slow and incomplete absorption in the gastrointestinal tract, undergoes first-pass conjugation in the gut and liver [137]. Lipophilic terbutaline esters have been developed to improve absorption and prolong duration by slow hydrolysis after distribution to different tissues [138]. One of the first terbutaline esters, ibuterol, had improved absorption, but was accompanied by a reduced duration of action and increased first-pass metabolism [138]. However, bambuterol, the bis(*N*,*N*-dimethylcarbamate) of terbutaline, and the cascade ester of terbutaline, D2438, are effective bronchodilators in animals with prolonged duration of effect compared with that of terbutaline [138]. Orally administered bambuterol displayed improved hydrolytic stability, partly by inhibition of its own hydrolysis and has been shown to survive first-pass hydrolysis in the dog. Compound

Table 7.2. EXAMPLES OF BRONCHODILATOR β-ADRENOCEPTOR AGONISTS



	Ring	R^1	R^2	R^3	*Receptor specificity*	*Ref.*
Catechols						
Adrenaline	3-OH, 4-OH	OH	H	CH_3	α, β_1, β_2	4
Isoprenaline	3-OH, 4-OH	OH	H	$CH(CH_3)_2$	$\beta_1 = \beta_2$	4
Isoetharine	3-OH, 4-OH	OH	CH_2CH_3	$CH(CH_3)_2$	$\beta_2 > \beta_1$	4
Bitolterol						
	3,4(CH_3  COO)	OH	H	$C(CH_3)_3$	$\beta_2 > \beta_1$	4
Resorcinols						
Metaproterenol	3-OH, 5-OH	OH	H	$CH(CH_3)_2$	$\beta_2 > \beta_1$	4
Fenoterol	3-OH, 5-OH	OH	H	CH-CH_3 	$\beta_2 > \beta_1$	4
Terbutaline	3-OH, 5-OH	OH	H	$C(CH_3)_3$	$\beta_2 > \beta_1$	138
Ibuterol	3,5-$(CH_3)_2CHCOO$	OH	H	$C(CH_3)_3$	$\beta_2 > \beta_1$	138
Bambuterol	3,5-$(CH_3)_2NCOO$	OH	H	$C(CH_3)_3$	$\beta_2 > \beta_1$	138
D2438	3,5-($(CH_3)_3CCO$  $COO)_2$	OH	H	$C(CH_3)_3$	$\beta_2 > \beta_1$	138

Miscellaneous						
Albuterol	3-CH_2OH, 4-OH	OH	H	$C(CH_3)_3$	$\beta_2 > \beta_1$	4
Ephedrine		OH	CH_3	CH_3	α, β_1, β_2	4
Carbuterol	3,5-(NH_2CONH), 4-OH	OH	H	$C(CH_3)_3$	$\beta_2 > \beta_1$	4
Clenbuterol	3,5-Cl, 4-NH_2	OH	H	$C(CH_3)_3$	$\beta_2 > \beta_1$	4
Tolbuterol	6-Cl	OH	H	$C(CH_3)_3$	$\beta_2 > \beta_1$	145
Formoterol	3-OH, 4-HCONH	OH	H		$\beta_2 > \beta_1$	146

D2438 was designed to undergo first-pass hydrolysis and conjugation at the *p*-pivaloyloxybenzoic acid moiety and was effective in dogs orally and after inhalation in guinea-pigs, although it possessed a shorter duration of action than bambuterol in these species. These drugs are undergoing phase I and II clinical trials. It is likely that new β-agonist prodrugs will be developed in an attempt to improve duration of effect of this therapeutic class.

It is conceivable that chemically distinct agents will be further developed with β_2-agonist properties; however, they are unlikely to avoid completely the tremorogenic effects of the current β_2-agonists, since these appear to be mediated by β_2-adrenoceptors within skeletal muscle [139].

In conscious man, the selectivity of β_2-agonists for airways is not as great as was initially predicted. The low selectivity may result from stimulation of cardiac β_2-adrenoceptors or from cardiac stimulation following activation of vascular β_2-adrenoceptors [140]. This suggests that a further improvement in β_2-selectivity will not necessarily increase airways selectivity in man. It may nevertheless be appropriate to develop β_2-agonists with greater bronchodilator efficacy in an attempt to overcome cases of severe bronchoconstriction.

Recently, pharmacologically inert blocked amino acid and peptide carriers have been conjugated with β-agonists and shown to retain β-adrenergic activity [141]. This approach might also be adopted to develop new bronchodilator agents. Corticosteroids have been reported to increase the number of lung β-adrenoceptors, suggesting another means to improve β-agonist therapy [142].

β-Adrenergic subsensitivity in asthma has been reported on many occasions; however, whether this is a result of the disease itself or is due to prolonged adrenergic medication is in question [143]. The presence of autoantibodies to β_2-adrenoceptors in the plasma of atopic patients with a corresponding reduced β_2-responsiveness suggests the abnormality may be due to an autoimmune mechanism [144].

In addition to reduced β_2-responses, an increased α-adrenergic responsiveness was reported [143]. A number of α-blockers, including indoramin, phentolamine and thymoxamine, have been shown to be effective in the treatment of specific types of asthma [5]. However, their rôle in asthma remains controversial, in part because some of these agents have other pharmacological actions that could account for their bronchodilator effects.

HISTAMINE RECEPTORS

Histamine is a major mediator of immediate-type hypersensitivity reactions and is a powerful bronchoconstrictor in man, particularly in asthmatics [147].

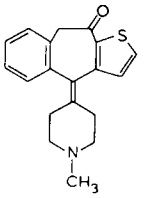
It has been implicated as a mediator in allergic and exercise-induced asthma. Two types of histamine receptor exist in the airways: H_1-receptors, which are blocked by classical antihistamines such as mepyramine and mediate bronchoconstriction, and H_2-receptors, which are blocked by antagonists such as cimetidine and mediate bronchodilation. The presence of H_2-receptors in human airways is contradictory; indeed, H_2-receptor antagonists do not potentiate bronchial responses to antigen or exercise [148]. Nevertheless, results obtained with human basophils suggest that histamine by interaction with H_2-receptors may regulate its own release by a feedback inhibition [149].

The clinical use of H_1-antihistamines in asthma has generally been disappointing, despite their effectiveness in animal models of bronchospasm and in some types of urticaria and hay fever, atopic dermatitis and allergic rhinitis. This is partly due to the failure to achieve a sufficient concentration of the drug at receptor sites in the lung and the central sedative effects that preclude administration of sufficiently high doses. However, several antihistamines, including thiazinamium, chlorpheniramine and clemastine, have been shown to inhibit antigen- and exercise-induced bronchospasm [147].

A variety of newer H_1-antihistamines, many claimed to be non-sedative, have been developed and shown to possess beneficial bronchodilator-antiallergic polypharmacy (Ref. 150 and *Table 7.3*). These agents, perhaps administered by inhalation to achieve high local concentrations, may overcome the problems faced in many previous studies with this class of compound.

Table 7.3. NEWER H_1 ANTIHISTAMINES WITH THEIR ANTIALLERGIC POTENTIAL

Compound No.	*Name*	*Additional profile*	*Sedative properties*	*Ref.*
1	ketotifen	mast-cell-stabilizing (high concentrations only)	+	151
2	mequitazine	anticholinergic anti-LTC_4	0	152
3	SCH 29851	long lasting	0	153
4	oxatamide	antiserotonin anti-SRSA mast-cell-stabilizing	+	154
5	terfenadine	–	0	155
6	astemizole	long lasting	0	156
7	azelastine	long lasting	0	157
8	BW 825c	–	0	158



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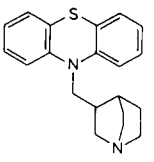

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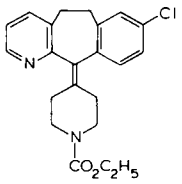

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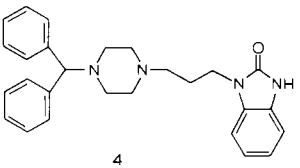

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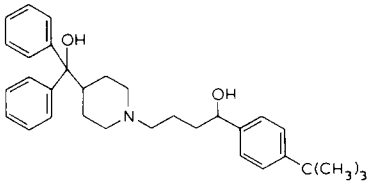

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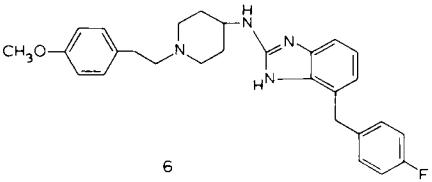

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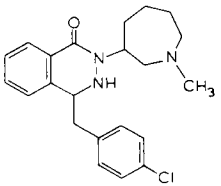

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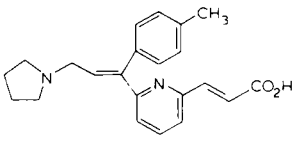


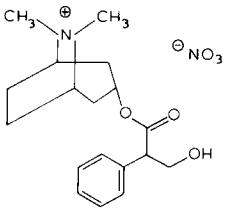
8

CHOLINERGIC RECEPTORS

Before the widespread use of adrenergic bronchodilators, aerosolized atropine methonitrate (9) was a major therapeutic agent for asthma and related disorders [159,160]. However, at low doses there was little bronchodilation, and at higher doses unwanted side-effects, including dry mouth, urinary retention, loss of

visual accommodation and tachycardia, appeared. There was also concern that atropine produced drying of respiratory secretions, which would become more viscous and difficult to expectorate.

There has been a resurgence in the use of anticholinergic bronchodilators with the development of a number of quaternary ammonium compounds that are highly polar and consequently poorly absorbed across biological membranes [161]. These drugs are bronchoselective when administered by aerosol and are remarkably free of side-effects. Ipratropium bromide (Sch 1000) (10), an isopropyl homologue of atropine, has been the most thoroughly investigated of these drugs [162]. It is twice as potent and has a longer duration of action than atropine. Bronchodilation following ipratropium develops more slowly



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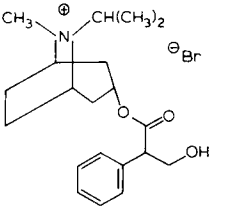


10

than β_2-agonists, reaching a maximum after 60–120 min, but it lasts longer (at least 3 h). The side-effects of ipratropium and other quaternary ammonium compounds when administered parenterally are similar to those observed with tertiary ammonium compounds such as atropine, except that CNS effects are reduced. However, inhalation of these quaternary ammonium compounds at therapeutic and even greater concentrations results in very few side-effects other than dry mouth and bitter taste in the case of ipratropium.

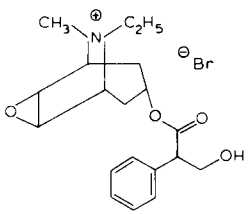
Indeed, anticholinergics do not adversely affect either sputum viscosity or sputum volume as initially feared [161]. Anticholinergic agents do not produce tremor. Furthermore, prolonged administration of ipratropium does not produce tolerance to its bronchodilator effect as is sometimes the case with β_2-agonists [163].

The inhaled anticholinergics fully protect against cholinergic agonists and partially protect against histamine and $PGF_{2\alpha}$ [161]. However, they are often less effective as bronchodilators than β-agonists using stimuli such as inhaled antigens, cold air and exercise. They do counteract bronchospasm resulting from β-adrenergic blockade and psychogenic factors and effectively augment bronchodilation achieved by adrenergic agents. They do not apparently interact unfavourably with either β-agonists or methylxanthines.

It is suggested that inhaled anticholinergics may be useful adjuncts in asthmatics whose symptoms are poorly controlled with β-adrenergic agents, who poorly tolerate the side-effects (e.g., tremor, tachycardia), who have intrinsic asthma or who are older [161]. Their principal rôle may be in the management of patients with chronic bronchitis and emphysema.

The bronchodilator effect of anticholinergics is primarily on large airways, whereas β-agonists affect the peripheral airways [161]. Indeed, muscarinic receptors are more plentiful in the trachealis muscle than in peripheral lung tissue [164]. About 10% of the metered dose of ipratropium is likely to reach the lower airways, since a majority of inhaled drugs is deposited in the upper airways and mouth and is swallowed [165]. The bronchial selectivity of ipratropium cannot be interpreted on the basis of selective binding to muscarinic receptors in the airways, but is ascribed to the pharmacokinetics of the compound after inhalation.

In addition to ipratropium, several other quaternary ammonium compounds have been reported to possess clinical bronchodilator efficacy after inhalation. Atropine methonitrate (9) possesses a more prolonged bronchodilator effect than does aerosolized atropine sulphate [166]. Oxytropium bromide (Ba253Br) (11) possesses a greater potency and longer duration than does ipratropium [167]. Ba598Br (12) in animal studies demonstrated more potent and longer lasting activity than did atropine [168], and it also possessed transient antihistaminic activity. Diphemanil methylsulphate (13) possesses direct smooth relaxant properties and protects against acetylcholine- but not histamine-induced brochospasm in asthmatics [169]. Glycopyrrolate (14) was used orally to control gastric acidity and parenterally as an antisialogogue and neuromuscular blocker. Recently, it produced bronchodilation when administered by aerosol in exercise-induced asthma without atropine-like side-effects [170]. Thiazinamium chloride (15), a quaternary analogue of promethazine, possesses potent anticholinergic and antihistaminic properties in animals [171]. Several years ago, a similar compound, thiazinamium methyl-



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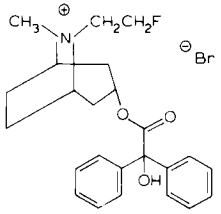

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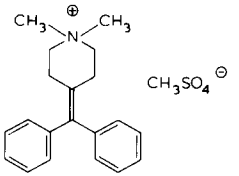

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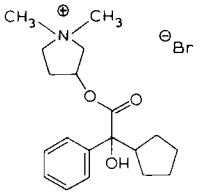

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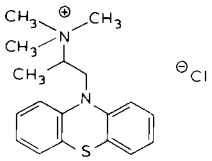

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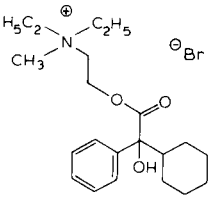

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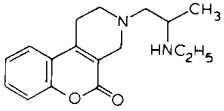

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sulphate (Multergan), proved effective for the treatment of a variety of obstructive lung diseases [172,173]. However, this methylsulphate salt was used as an intramuscular injection and resulted in irritancy at the site of administration and consequently did not attract widespread usage. A recent report of clinical efficacy of the inhaled thiazinamium chloride has appeared [174]. Oxyphenonium bromide [175] (16) and CI-923 [176] (17) are both orally effective anticholinergics; the latter is claimed to be broncho-selective.

The majority of bronchodilator anticholinergics are competitive antagonists at the acetylcholine muscarinic receptor. In contrast, β-agonists are functional antagonists and, consequently, modulate many different bronchoconstrictor agonists. Recently, ipratropium was shown to inhibit the formation of thromboxane (TXA_2), a mediator of bronchospasm, in isolated guinea-pig lung stimulated by histamine or SRS-A [117]. Furthermore, atropine, ipratropium and oxytropium all prevented guinea-pig airway constriction and concurrent release of TXA_2-like substances into the circulation induced by histamine, bradykinin and LTC_4, suggesting that these drugs may prevent the release or activity of PLA_2 and hence make arachidonic acid unavailable for TXA_2 synthesis [178]. Thiazinamium chloride also inhibits arachidonic acid metabolism in rat alveolar macrophages [179]. This drug is also a potent antihistaminic and antiserotoninergic *in vivo* and is capable of inhibiting the bronchoconstrictor and platelet aggregatory effects of PAF [180].

With the development of receptor binding techniques, complexities have been found with the muscarinic receptors once thought to be homogeneous. Multiple agonist-binding sites of muscarinic acetylcholine receptors have been

suggested [181]. The presence of multiple binding sites in the lung has not yet been systematically studied, but there is an indication of multiple binding sites in human airways [182]. There is also a surprising reduction in the number of binding sites in lung tissue from patients with chronic obstructive lung disease [182].

The currently available inhaled anticholinergics thus seem to be therapeutically acceptable in terms of potency and absence of side-effects and toxicity. Improvements in duration of effect may be desirable. However, anticholinergics with additional beneficial pharmacological properties may prove to be more efficacious in the treatment of asthma.

PEPTIDERGIC RECEPTORS

Several regulatory peptides have been demonstrated in the respiratory tract of man and other mammals [183–185]. They may influence pulmonary function and consequently play a rôle in a number of respiratory diseases, including asthma. These peptides, originally found in the brain and/or gut, are localized in autonomic-sensory nerves and endocrine cells and may play the rôle of neurotransmitters in the lung.

Substance P

Substance P (SP) is an 11-amino-acid peptide found in sensory nerves within the bronchiolar epithelium and around blood vessels [186]. It produces an atropine-resistant bronchial smooth muscle contractile response, possibly by releasing PGs or leukotrienes [187]; in addition, SP is neurally released from the hilum bronchus of guinea-pigs, suggesting that it may have a rôle in neurogenic contraction [188, 189]. SP elicits non-cytotoxic histamine release from rat peritoneal mast cells *in vitro*, possibly via mast-cell receptors for SP [190]. A recent hypothesis suggested that a noxious stimulus might stimulate impulses in neuropeptide-containing C-fibres to release SP from peripheral terminals [191]. This would result in vasodilation and increased permeability of the regional microcirculation as well as activation of mast cells to release histamine, chemotactic peptides and leukotrienes. This would amplify the initial inflammatory response, including further release of SP, by activating terminals of C-fibres directly.

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

Substance P

A variety of substance P-related peptides have been shown to possess agonist and antagonist properties [188,192]. Several octapeptides, including DPro-Gln-Gln-DTrp-Phe-DTrp-DTrp-DPhe-NH_2, are substance P antagonists, while a number of related undecapeptides are potent contractile stimulants of the airways through their histamine-releasing properties [193]. However, several problems exist with these antagonists; notably, they do not have good potency, often possess partial agonist properties and have histamine-releasing activity. Some of the current antagonists exhibit neurotoxicity and it has been speculated that substance P may be a trophic agent influencing survival of some neurones [194]. Thus the development of substance P antagonists for use primarily as analgesics and secondarily as agents that might be effective against bronchospasm could prove problematic. Aerosol administration might obviate certain of the problems and the evaluation of such agents is awaited with interest.

Vasoactive intestinal peptide (VIP)

VIP is a 28-amino-acid peptide with potent vasodilating, airway smooth muscle relaxant and secretory properties. It is localized in autonomic nerves found in close association with blood vessels, seromucosal glands and smooth muscle, particularly in the upper respiratory tract of animals and man [195]. Specific VIP receptors have been demonstrated in membrane preparations of mouse, rat, guinea-pig and human lungs [195].

A physiological rôle for this neuropeptide as a mediator of non-adrenergic, non-cholinergic airway relaxation has been suggested [196]. Such a rôle would be of major consequence, since the non-adrenergic, non-cholinergic component of airway relaxation is probably the dominant relaxant component in a number of species, including man [6]. Other potential rôles for VIP in the lung include the modulation of secretions from bronchial glands and a relaxant effect on pulmonary vasculature.

Intravenous infusion of VIP caused bronchodilatation in asthmatics and ameliorated the effect of histamine-induced bronchoconstriction in volunteers [197]. However, this may not be due to direct effects on smooth muscle but to nonspecific reflex effects or catecholamine release [198]. Inhaled VIP was without effect in asthmatics [199], although aerosolized VIP was effective against histamine- and $PGF_{2\alpha}$-induced bronchospasm in anaesthetized dogs [200].

It remains to be seen whether other VIP agonists will be prepared and investigated for bronchodilator activity.

Other peptides

Peptide histidine isoleucine (PHI) is a 27-amino-acid peptide with distribution and localization similar to those of VIP, suggesting that they originate from a common precursor [201]. Bombesin, a 27-amino-acid peptide, is localized in mucosal endocrine cells of the bronchial epithelium and is capable of contracting airways smooth muscle [202]. Physalaemin and eledoisin are structurally related to SP and also produce contractions of guinea-pig lung strip [190]. Neuropeptide tyrosine (NPY) is found in nerve fibres in the adventitia of blood vessels and airway smooth muscle of a variety of mammals, including man [203]. Low concentrations of calcitonin, choleocystokinin, ACTH and somatostatin are also detected in the respiratory tract, although their localization and function are unknown [184].

METHYLXANTHINES AND ADENOSINE RECEPTORS

The dimethylated xanthine, theophylline, has enjoyed widespread use in the treatment of asthma for over 50 years, although only in the last 10 years has an understanding of its pharmacodynamics and pharmacokinetics permitted its use with optimal efficacy and safety [204,205]. Theophylline has a short half-life and a narrow therapeutic range after oral administration and therefore considerable attention has been placed on sustained-release formulations to alleviate these problems. Advantages that have been claimed include reduced frequency of dosing, increased patient compliance, prevention of overnight subtherapeutic levels and lower patient-to-patient variations. Recently, inhaled methylxanthines, including theophylline, demonstrated mild bronchodilator activity in asthmatics, but were extremely bitter in taste [206].

Numerous theophylline 'salts' have been marketed, including aminophylline, the ethylenediamine salt. Since theophylline is a weak acid at high pH values, it is largely un-ionized at physiological pH values and therefore not stable in 'salt' form during clinical usage [205]. Other substituted xanthines also possess bronchodilator properties, although the majority do not appear to have therapeutic advantage over theophylline [205]. However, the relaxant effects of a series of xanthines in carbachol-constricted guinea-pig trachea showed that substitution in the 1- and 3-positions of the xanthine nucleus improved relaxant potency [207]. Unsubstituted xanthine and all 9-methylxanthines were weakly active.

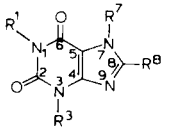
The mechanism of xanthine-induced brochodilation is not clearly established. It was thought that phosphodiesterase (PDE) inhibition, resulting in reduced degradation of cAMP, was important, but this occurs only at *in vitro*

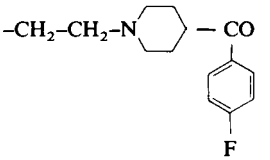
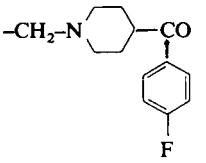
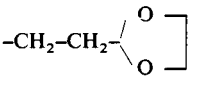
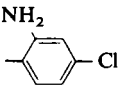
concentrations (above 10^{-4} M) 10-fold greater than therapeutic levels associated with bronchodilation in asthma (5×10^{-5} M free theophylline) [208]. Theophylline may also act as a prostaglandin antagonist, may alter intracellular calcium ion distribution, and may inhibit release of catecholamines from the adrenal medulla [205]. Theophylline also induces T suppressor cells in asthmatic children, thus restoring to normal the immune imbalance in these patients [209]. However, recent interest has centred on the ability of theophylline to inhibit adenosine (Ado) receptors [210]. In isolated tissue, including airways and cell preparations, theophylline at therapeutic concentrations competitively antagonizes the actions of Ado. The rôle of Ado and its receptors in asthma is unclear; however, Ado can cause bronchoconstriction in asthmatics which is antagonized by inhaled theophylline [206]. It is capable of both relaxing and constricting isolated guinea-pig, monkey and human airways [211]. Ado is also capable of modulating intracellular levels of cAMP following interaction with membrane receptors. However, both A_1- and A_2-receptors exist; A_1-receptors have a high affinity for Ado and are inhibitory towards adenylate cyclase (R_1 site effect) whereas A_2 receptors have a low affinity for Ado and stimulate adenylate cyclase (R_a site effect [212,213]). To add to this complex situation, receptor-binding studies suggest heterogeneity among A_1-receptors [214]. Both A_1- and A_2-receptors are blocked by the methylxanthines. In contrast, an intracellular P site which is activated by high (10^{-5} M) concentrations of Ado and results in inhibition of adenylate cyclase is insensitive to methylxanthine blockade [216,217].

Modulation of mast-cell and basophil histamine release by Ado are inconsistent. In guinea-pig lung [218] and rat mast cells [219], Ado interacts with A_1- and A_2-receptors and potentiates immunologically induced histamine release. In contrast, Ado is capable of both inhibiting [220,221] and potentiating [221] immunologically induced histamine release from human basophils. The latter responses are dependent on the time of Ado administration and are mediated through stimulation of A_2-receptors [221]. Recently, Church, Holgate and Hughes demonstrated that P_1 site inhibitors such as 2′,5′-dideoxyadenosine are also capable of inhibiting immunologically induced histamine release from human basophils [222]. Of the series of alkylxanthines evaluated for Ado receptor binding, 1,3-dipropyl substituents enhanced binding potency compared with that of the 1,3-dimethyl substitution in theophylline [223]. The last compound shown in *Table 7.4* possessed a K_1 for Ado A_1-receptors of 22 pM, being 70,000-times more potent than theophylline [223]. The bronchodilator activity of these compounds has not yet been reported.

However, the bronchodilator methylxanthines lack universal Ado antagonism and it has been suggested that Ado antagonism is neither necessary nor

Table 7.4 EXAMPLES OF BRONCHODILATOR XANTHINES



Name	R^1	R^3	R^7	R^8	*Ref.*
Theophylline	$-CH_3$	$-CH_3$	$-H$	$-H$	205
Dyphylline (Diprophylline)	$-CH_3$	$-CH_3$	$-CH_2-\underset{\displaystyle OH}{\underset{\vert}{CH}}-CH_2-OH$	$-H$	205
Etophylline	$-CH_3$	$-CH_3$	$-CH_2-CH_2OH$	$-H$	205
Proxphylline	$-CH_3$	$-CH_3$	$-CH_2-\underset{\displaystyle OH}{\underset{\vert}{CH}}-CH_3$	$-H$	205
Fluphylline (Sgd 19578)	$-CH_3$	$-CH_3$		$-H$	229
Fluphylline (Sgd 14480)	$-CH_3$	$-CH_3$		$-H$	229
Enprofylline	$-CH_3$	$-C_3H_7$		$-H$	225
Doxyfylline	$-CH_3$	$-CH_3$		$-H$	227,228
1,3-Dipropyl-8-(2-amino-4-chlorophenyl)-xanthine	$-C_3H_7$	$-C_3H_7$	$-H$		223

a desirable property of xanthine antiasthmatics [224]. Enprofylline, a 2-propylxanthine (see *Table 7.4*), is an example of a bronchodilator xanthine with negligible activity to antagonize Ado that is also a weak cAMP-PDE inhi-

bitor [225]. It has 5-times the bronchial potency of theophylline, but seems to be devoid of theophylline-like CNS stimulatory, tremorogenic and diuretic side-effects [226]. It is thus conceivable that inhibition of Ado receptors by theophylline may be associated with the latter side-effects rather than bronchodilation. Enprofylline is well absorbed orally and has a high renal clearance, in contrast to theophylline, which required substantial metabolism (90%) [226]. Variable elimination of theophylline within individuals may result in toxic theophylline levels due to unpredictable, long half-lives [205]. However, enprophylline produces headache and nausea in asthmatic patients, which may preclude its widespread usage [226].

Doxofylline (*Table 7.4*) is another new bronchodilator xanthine with few cardiovascular or CNS properties; moreover, the mechanism of action does not appear to depend on Ado receptor blockade [227,228].

Future research will probably result in the development of a bronchodilator xanthine with an improved therapeutic ratio over theophylline. Whether such a compound will possess selective Ado receptor inhibitor activity will depend on further elucidation of the importance of Ado receptors in the airways. The discovery of enprophylline has clearly led to a re-evaluation of Ado receptor antagonism as a primary mechanism of action of bronchodilator xanthines. It further suggests that the mechanism of action of xanthine bronchodilation remains a mystery, hopefully to be solved in the near future.

REGULATION OF SMOOTH MUSCLE CONTRACTION AND MEDIATOR RELEASE BY CALCIUM

As with other types of smooth and striated muscle, the contraction-relaxation cycle of airway smooth muscle is dependent upon changes in the concentration of intracellular free Ca^{2+} ions. The source of activator Ca^{2+} for contraction may be extracellular or intracellular and is dependent upon the agent which is producing the contraction [230]. For example, contraction due to cholinergic-muscarinic receptor stimulation is thought to occur via influx of Ca^{2+} through potential-independent calcium channels (receptor-operated channels; ROC) and/or release of Ca^{2+} ions from intracellular storage sites [231–233]. However, low concentrations of cholinergic agonists may stimulate Ca^{2+} entry through a potential-dependent Ca^{2+} channel (PDC). On the other hand, leukotriene-mediated contractions may be more dependent upon release of intracellular Ca^{2+} ions [234]. The sources of Ca^{2+} ions for contractions by other agents (such as histamine and prostaglandins) are concentration-dependent (as with acetylcholine) and mostly extracellular [235].

Mechanistically, increased intracellular Ca^{2+} levels may stimulate smooth muscle contractile protein activity at several loci. By far, the most prevalent hypothesis concerning the Ca^{2+}-dependent regulation of contractile activity in airway (and other) smooth muscle involves Ca^{2+}-calmodulin-mediated phosphorylation of the 20 kDa light chain (phosphorylatable or P-light chain) of the myosin thick filament [236–239]. In this proposed scheme (*Figure 7.1*), intra-

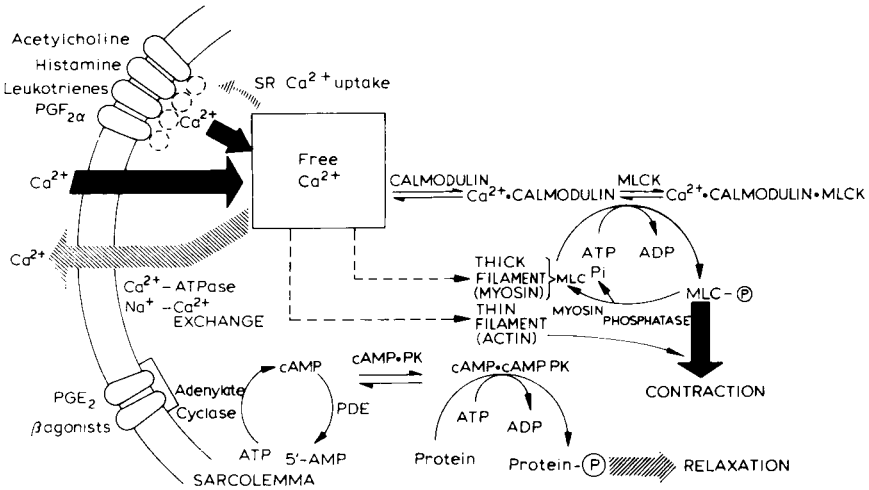


Figure 7.1. Diagramatic representation of intracellular mechanisms of Ca^{2+} ions and cAMP which regulate contractile activity in airway smooth muscle. Excitation through receptor-binding of various mediators results in Ca^{2+} influx and/or release of Ca^{2+} from intracellular stores. Increased intracellular free Ca^{2+} binds to calmodulin, forming Ca^{2+}-calmodulin complexes which bind to and activate myosin light chain kinase (MLCK). Activated MLCK catalyzes the phosphorylation of myosin light chains (MLC) which leads to contraction; myosin phosphatases dephosphorylate MLC with a return to the non-contracted state. In addition to regulating MLC phosphorylation, free Ca^{2+} may also directly affect thick or thin filament function. High levels of intracellular free Ca^{2+} are regulated by extrusion through the sarcolemma primarily by a Ca^{2+}-ATPase, but $Na^{+}-Ca^{2+}$ exchange may also be a factor. Also, reuptake into sarcoplasmic reticular-like (SR) vesicles may occur. Cyclic AMP is formed from ATP by activation of membrane bound adenylate cyclase after receptor binding by agents such as β-agonists or PGE_2. Cyclic AMP activates a different protein kinase, cyclic AMP-dependent protein kinase (cAMP PK), which catalyzes the phosphorylation of phosphoprotein substrates which are directly involved in the regulation of Ca^{2+} flux or contractile protein function. Phosphodiesterases (PDE) catalyze the degradation of cAMP to the inactive 5'-AMP.

cellular free Ca^{2+}, which increases during excitation, binds to the ubiquitous Ca^{2+}-binding protein, calmodulin. The Ca^{2+}-calmodulin complexes bind to and activate myosin light chain kinase (MLCK), which is a substrate-specific

protein kinase. The MLCK-Ca^{2+}-calmodulin complex catalyzes the phosphorylation of the P-light chain, with a resultant increase in the rate of actin-myosin interactions. Myosin phosphatases have been identified [240,241] which can dephosphorylate the P-light chain, thus reducing the rate of actin-myosin interactions. Additional sites of Ca^{2+} regulation have also been suggested; these include the thin filament [242,243] and Ca^{2+} binding to myosin [244,245].

There is substantial biochemical evidence that implicates myosin P-light chain phosphorylation in the Ca^{2+}-dependent regulation of smooth-muscle actin-myosin interactions [236–239]. However, in intact smooth muscle, the relationship between the extent of isometric force development and the extent of P-light chain phosphorylation is not straightforward. In rabbit and bovine tracheal smooth muscle [246,247], the phosphate content of the P-light chain increases during the initial development of isometric force, and then declines during the subsequent maintenance of such force. Initial force development by a variety of pharmacologic stimulants is always accompanied by, and seemingly correlated with, varying extents of P-light chain phosphorylation [248]. The rate and magnitude of the transient phosphorylation is dependent upon the contractile stimulus and may reflect gradual decreases in the concentration of intracellular free Ca^{2+} during maintained contractions [248,249]. Shortening velocity in tracheal smooth muscle [246] is also transient and follows the same time-course as myosin P-light chain phosphorylation. A 'latch bridge' hypothesis has been proposed [250] which suggests that isometric force may be maintained in smooth muscle with low cross-bridge cycling rates. Thus, P-light chain phosphorylation may be necessary to initiate force development, while a second site of Ca^{2+}-dependent regulation may be necessary for the maintenance of force. Others [251], however, have reported sustained levels of P-light chain phosphorylation during maintained isometric contractions.

Another potential mechanism for Ca^{2+} regulation in smooth muscle involves activation of phospholipase C, hydrolysis of phosphatidylinositol 4,5-biphosphate, production of inositol 1,4,5-triphosphate and diacylglycerol, and ultimate activation of protein kinase C, a unique Ca^{2+}-dependent kinase which also requires phosphatidylinositol and diacylglycerol for full expression of activity [252]. However, further research is needed to identify the physiologically relevant substrates for this kinase and to demonstrate a physiologically significant rôle for it in the regulation of smooth muscle contractile activity.

Release of mediators such as histamine and SRS-A from mast cells is also dependent upon influx of extracellular Ca^{2+} [235,253–256]. Influx of Ca^{2+} is tightly linked to the magnitude of mediator release. However, it is not clear whether this influx occurs through PDCs and/or ROCs. Factors such as the

type or source of cells and the agent producing the stimulation (e.g., antigen) have to be considered. In this regard, histamine release by compound 48/80, protamine, somatostatin or other polypeptides is relatively insensitive to extracellular Ca^{2+} ions [235]. Similarly, calmodulin has been implicated in mediator release [257]; the mechanism by which calmodulin effects release and the rôle of microfilaments or microtubules in this process remain to be further elucidated.

CYCLIC AMP

Relaxation of airway smooth muscle by agents such as β-adrenergic agonists, the methylxanthines and PGE_2 (*Figure 7.1*) has been correlated to increases in intracellular cAMP formation [258–260]. The levels of intracellular cAMP are controlled through the opposing actions of membrane-bound adenylate cyclase and soluble cyclic nucleotide phosphodiesterase. Beta-adrenoceptor and PGE_2 receptor coupling to adenylate cyclase activity has been determined, while the methylxanthines have been identified as nonspecific phosphodiesterase inhibitors [261]. However, it is also clear that the methylxanthines may have other actions independent of cAMP which promote relaxation and contribute to their bronchodilatory effectiveness (see subsection on Methylxanthines and adenosine receptors).

Mechanistically, the intracellular mediator of cAMP in eucaryotes is cAMP-dependent protein kinase [262–264]. Partial purification and characterization of this enzyme from bovine tracheal smooth muscle was initially reported [265], while activation of this protein kinase in response to β-adrenoceptor stimulation was initially demonstrated in bovine coronary arterial smooth muscle [266]. Subsequently, similar correlations between relaxation and cAMP protein kinase activation were demonstrated in isolated canine trachealis muscle [267].

Activation of the cAMP-dependent protein kinase initiates the physiological effects of cAMP by stimulating phosphorylation of specific protein substrates which are important in the regulation of contractile activity. Thus, effects of cAMP and its protein kinase on Ca^{2+} flux and contractile protein function have been proposed. Studies at the National Institutes of Health [268,269] have shown that gizzard smooth-muscle MLCK is a substrate for cAMP-dependent protein kinase and that phosphorylation of MLCK in the absence of bound Ca^{2+}-calmodulin results in a marked reduction in affinity of MLCK for Ca^{2+}-calmodulin. Similar results are apparent in studies with MLCK purified from aortic [270] or tracheal [271,272] smooth muscle. In contractile proteins purified from mammalian vascular smooth muscle [273,274], cAMP and cAMP protein kinase inhibit Ca^{2+}-dependent myosin light chain phos-

phorylation and actin-myosin interactions by depressing the Ca^{2+} sensitivity of both processes. Inhibition is attenuated in the presence of maximal levels of calmodulin. Similar results are apparent in experiments with skinned (membraneless) smooth-muscle preparations [275,276]. In intact tracheal smooth muscle, pretreatment with isoprenaline inhibits carbachol-mediated contraction and simultaneous P-light chain phosphorylation [247]. Thus, phosphorylation of MLCK by cAMP-dependent protein kinase might be expected to result in less Ca^{2+}-calmodulin bound to the enzyme, decreased phosphorylation of the myosin light chains, and ultimately, relaxation.

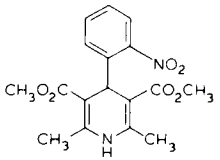
While this hypothesis may be valid in biochemical and membraneless muscle studies and in inhibiting initial force development, recent evidence suggests that MLCK phosphorylation may not be necessary for cAMP-mediated *relaxation* of tracheal and arterial smooth muscle [271,277,278]. Relaxation induced by cAMP-increasing agents occurs readily after the transient in myosin P-light chain phosphorylation occurs and when P-light chain phosphate content is low [271]. Thus, no change in phosphorylation is necessary for β-adrenergic-dependent relaxation. Moreover, no change in MLCK affinity for Ca^{2+}-calmodulin is apparent in tracheal smooth muscle which is treated with concentrations of isoprenaline that produce relaxation and activate phosphorylase kinase [271]. Phosphorylation of MLCK in intact tracheal smooth muscle was recently reported; however, no index of effects on MLCK activity was provided [279].

It is clear that MLCK phosphorylation by cAMP-dependent protein kinase may not be the dominant mechanism by which cAMP brings about relaxation in smooth muscle. Effects at various loci of membranous Ca^{2+} flux and translocation have been proposed, including equivocal results with respect to increased uptake of Ca^{2+} into microsomal vesicles [280,281], decreased influx of Ca^{2+} through PDC [282], increased Ca^{2+} efflux [283], changes in K^+ fluxes and membrane conductance [278,284], or effects on $(Na^+ + K^+)$-ATPase activity [285]. Clearly, multiple mechanisms may play a rôle in the cAMP-mediated relaxation of airway smooth muscle.

PHARMACOLOGICAL REGULATION OF Ca^{2+}

Within the past few years, a new class of cardiovascular agents, termed Ca^{2+} entry blockers, has emerged. These structurally distinct compounds characterized by the dihydropyridines (such as nifedipine (18), verapamil (19) and diltiazem (20)) act primarily by inhibiting influx of Ca^{2+} through the PDC [286,287]. Other weakly basic, lipophilic Ca^{2+} entry blockers characterized by prenylamine (21), flunarizine, cinnarizine (22), bepridil (23), fendiline and

perhexiline may have additional loci of action [288,289], quite possibly related to calmodulin antagonism [290].



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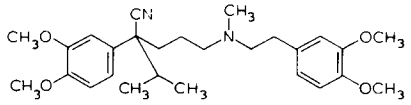

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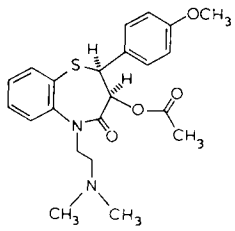

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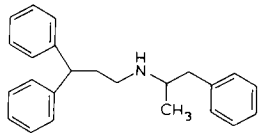

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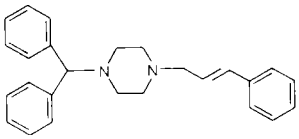

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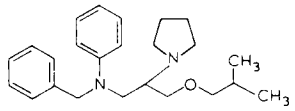

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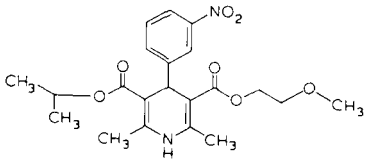
As with other types of smooth muscle, Ca^{2+} entry blockers are potent inhibitors of the PDC in airway smooth muscle. However, for the most part, therapeutic concentrations (micromolar or less) of Ca^{2+} entry blockers are largely ineffective in inhibiting contractions elicited by mediators such as LTD_4, acetylcholine, histamine or serotonin [234,291–295]. This is consistent with the previously discussed primary mechanisms of mobilization of Ca^{2+} influx through ROC and from release of intracellular Ca^{2+} stores for most of these mediators.

In certain instances, Ca^{2+} entry blockers have been shown to inhibit the release of various mediators. Verapamil and nifedipine inhibit antigen-IgE-mediated histamine release from rat peritoneal mast cells [296,297] and human leucocytes [298] as well as IgE-mediated secretion of SRS-A from sensitized

human lung fragments [256,299,300]. However, these latter effects were obtained with relatively high concentrations of the antagonists and no effect on histamine release was apparent. Nimodipine (24) and verapamil were also without effect on histamine release from human lung fragments [301]. However, nifedipine inhibited release of PAF-acether from human polymorphonuclear neutrophils at micromolar concentrations [302].

Compounds proposed to modulate the release of intracellular stores of Ca^{2+} in smooth muscle have also been identified. TMB-8 (25) and the methylenedioxyindenes (26) are agents which have been proposed to function



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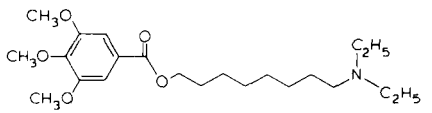

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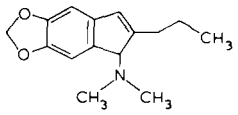

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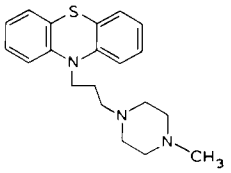
at this level [303,304]. However, precise biochemical-mechanistic studies are only now emerging [305], so the pharmacologically relevant site of action of these compounds is not known. It may also be possible to stimulate reuptake of Ca^{2+} from the sarcoplasm into membranous vesicles or to stimulate Ca^{2+} extrusion through the sarcolemma, primarily through the Ca^{2+}-stimulated sacrolemmal Mg^{2+}-ATPase [306]. Na^{+}–Ca^{2+} exchange also occurs in smooth muscle, but is seemingly secondary in importance to the sarcolemmal Mg^{2+}-ATPase for extrusion of sarcoplasmic Ca^{2+} [307]. Compounds which specifically act at any of these sites in smooth muscle have not been identified.

Pharmacological modulation of the mechanism(s) which regulate contractile protein function represents another potential site of action for antiasthmatic agents. Thus, inhibition of myosin P-light chain phosphorylation, Ca^{2+}-sensitive latch bridge regulation, or the thin-filament regulatory mechanisms, offer variable sites for drug action. Within the more well-defined myosin light chain phosphorylation system, MLCK activity may be directly inhibited, either

through competition for the Ca^{2+}-calmodulin complex or by direct inhibition of kinase catalytic activity. Stimulation of myosin phosphatase activity offers another potential mechanism for development of inhibitors of myosin light chain phosphorylation. Agents which relax smooth muscle and function at either the phosphatase or MLCK catalytic site of action have not yet been identified. Identification and synthesis of the peptide site of phosphorylation of the P-light chain [308] offer promise for further research in this area.

Competition for the Ca^{2+}-calmodulin complex is currently the most popular method for inhibiting smooth muscle myosin P-light chain phosphorylation. The phenothiazines (such as trifluoperazine, 27), naphthalene sulphonamides (such as W-7, 28), or the imidazolium derivative, calmidazolium (29) have all



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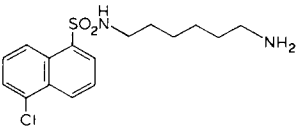

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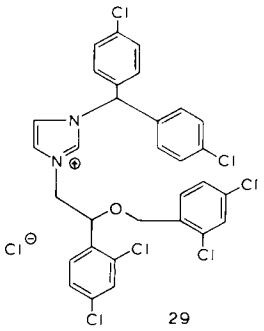

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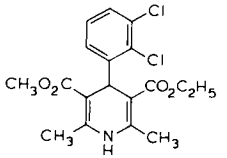

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been shown to antagonize Ca^{2+}-calmodulin-dependent phosphodiesterase activity or myosin P-light chain phosphorylation effectively [309–320]. However, calmidazolium (also known as R 24571), is a most potent inhibitor of myosin phosphorylation in purified contractile protein preparations, yet is ineffective in inhibiting smooth-muscle contraction [320].

Some Ca^{2+} entry blockers have also been proposed to bind to calmodulin and function as calmodulin antagonists. Felodipine (30) binds to calmodulin in the micromolar range [321], but functional inhibition of myosin P-light chain

phosphorylation occurs at much higher concentrations [322]. Moreover, this dihydropyridine completely inhibits force development in intact smooth muscle in the nanomolar range [321,322], suggesting that the pharmacologically relevant mechanism of Ca^{2+} antagonism is not through direct inhibition of phosphorylation, but rather is similar to other dihydropyridines (blockade of the Ca^{2+} entry channel) [322,323]. Bepridil and flunarizine inhibit calmodulin-stimulated phosphodiesterase activity [290] and prenylamine [316], perhexiline and cinnarizine [324] directly inhibit myosin P-light chain phosphorylation. Thus, the Ca^{2+} antagonism derived from these weakly basic, lipophilic compounds may be due to combined effects at the Ca^{2+} entry channel and myosin P-light chain phosphorylation system. Interestingly, calmodulin, or a calmodulin-like protein, may also be important in regulating Ca^{2+} channel function [325,326].

Another Ca^{2+} entry blocker, diltiazem, is capable of inhibiting force development in membraneless smooth muscle preparations [327], but does this without apparently affecting myosin P-light chain phosphorylation [328]. The effects of diltiazem on other proposed smooth muscle regulatory systems and the potential for future drug development at this level remain to be elucidated.

Calmodulin inhibitors may also diminish release of histamine and other mediators in various cell types [255]. However, a recent study [301] shows that calmidazolium inhibits SRS-A release, but not histamine release, in human lung fragments. Although preliminary, these studies indicate that inhibition of Ca^{2+}-calmodulin would be highly beneficial in the treatment of asthma through both a direct, bronchodilatory effect (inhibition of myosin light chain phosphorylation) and an additional site of action via inhibition of release of bronchoactive mediators.

USEFULNESS OF Ca^{2+} ENTRY BLOCKERS IN ASTHMA

In general, Ca^{2+} entry blockers such as nifedipine and verapamil are effective in inhibiting exercise-induced asthma [329–332] or cold air bronchoconstriction [333], but equivocal reports are evident with respect to the relative effectiveness of Ca^{2+} entry blockers in chronic asthma [235,255,256,334]. A notable exception has been cinnarizine [335], which, as previously discussed, may have other loci of action in addition to Ca^{2+} entry blockade. In other studies [336], nifedipine was effective in preventing histamine-induced bronchoconstriction in asthmatics, while others [337] found no effect of verapamil on either histamine- or methacholine-induced bronchospasms in non-asthmatic or in asthmatic subjects. Verapamil was also unable to inhibit bronchospasm induced by either histamine or carbachol in sheep which were

allergic to *Ascaris*, yet did prevent bronchoconstriction in response to subsequent antigen challenge [338]. Thus, a preferential effect of verapamil on mediator release from mast cells was implied. Others [339] have reported that verapamil, but not nifedipine, inhibited constrictive responses to this allergen in beagles after aerosol administration, while both were effective following intravenous administration.

Verapamil, at high concentrations, inhibited antigen-induced contractions of sensitized guinea-pig trachealis strips [340]. Similarly, high concentrations of nifedipine did not inhibit contractions elicited in actively-sensitized guinea-pig trachealis strips, but did reduce contractions in passively-sensitized human bronchial smooth muscle [341]. Also, micromolar concentrations of nifedipine significantly inhibited contractions elicited by acetylcholine and histamine in human bronchial strips [342].

The effectiveness of verapamil, nifedipine, flunarizine and diltiazem in several models of immediate hypersensitivity was recently reported [343]. Again, high doses of verapamil, diltiazem or flunarizine were necessary to block antigen-induced bronchoconstriction in the guinea-pig. Nifedipine was ineffective in the latter model; furthermore, all four antagonists were without effect on SRS-A-mediated bronchospasm in the guinea-pig. *In vitro*, flunarizine, verapamil and diltiazem were unable to inhibit antigen-induced histamine release in rat peritoneal mast cells. These data support the notion that Ca^{2+} entry blockers have little potential use in immediate hypersensitivity reactions.

Conflicting reports are apparent in the literature regarding the effectiveness of Ca^{2+} entry blockers in allergen-induced bronchospasms in humans. A significant effect of nifedipine (sublingual) in grass pollen-induced bronchoconstriction has been reported [341]. However, a similar dose and route of administration for nifedipine and, also, inhaled verapamil was not effective against bronchoconstriction elicited by *Dermatophagoide pteronyssimus* [344]. In a separate study using a similar challenge, no protection with low doses of inhaled verapamil and actual bronchoconstriction in some patients with higher doses were reported [345].

FUTURE DIRECTIONS FOR NEW DRUG DEVELOPMENT OF MODULATORS OF Ca^{2+} AND/OR cAMP (*Table 7.5*)

Although some efficacy is apparent in the treatment of certain types of asthma with voltage-dependent Ca^{2+} entry blockers, it is readily apparent that high concentrations, which probably indicate a nonspecific site of action, are necessary to achieve some of these beneficial effects. This probably relates to differences in Ca^{2+} channels and preferential activation of ROC (voltage-

Table 7.5. CELLULAR REGULATION OF AIRWAY SMOOTH MUSCLE CONTRACTION AND/OR MEDIATOR RELEASE. LOCI FOR PHARMACOLOGICAL MODULATION OF CALCIUM AND CYCLIC AMP SYSTEMS

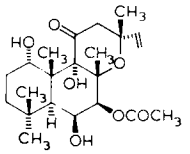
Site	*Representative modulator*
I. Ca^{2+}	
1. Ca^{2+} influx	
1.1. Potential-dependent Ca^{2+} channel	Verapamil; nifedipine
1.2. Receptor (mediator) operated Ca^{2+} channel	?
2. Intracellular Ca^{2+} storage (SR)	?
3. Contractile protein modulators	
3.1. Myosin light chain phosphorylation	
a. Calmodulin inhibitor	W-7, calmidazolium?
b. Kinase catalytic inhibitor	?
c. Phosphatase stimulator	?
3.2. Latch bridge inhibitor	?
4. Ca^{2+} extrusion mechanisms (i.e., sarcolemmal Ca^{2+}-ATPase)	?
II. cAMP	
1. Adenylate cyclase stimulators	β-adrenergic agonists, forskolin
2. Specific phosphodiesterase inhibitors	Milrinone, MDL-17,043 CI-914
3. cAMP protein kinase modulators	?

independent) by most bronchoconstrictor mediators. Moreover, it is evident that release of all mediators is not greatly affected by Ca^{2+} entry blockers. Therefore, an ideal therapeutic agent would be one which interacts at either (1) the ROC, (2) the site of release of intracellular Ca^{2+} or (3) the contractile proteins (myosin light chain phosphorylation). Given the myriad of mediators, one would have to postulate a common ROC and/or site of intracellular Ca^{2+} release for efficacy at this level by a single agent. Another site for inhibition would be the contractile protein-myosin light chain phosphorylation system, as this would be distal to any site of Ca^{2+} flux. The relative importance of the P-light chain and other Ca^{2+} regulatory systems has been previously demonstrated in trachealis muscles from various species. However, the importance of these systems in the acute and chronic phases of asthma, and the specific characteristics of these systems (such as the transient myosin P-light chain phosphorylation) in pathological smooth muscle remain to be elucidated. Acute bronchospasm is conceivably directly related to an increase in myosin P-light

chain phosphorylation, while the maintenance of this constriction might be related to another Ca^{2+}-regulated event. Confirmation of these speculations is critical for the future development of therapeutic agents working at this level.

Since calmodulin has been implicated in mediator release, a calmodulin inhibitor at this level would not only serve to inhibit Ca^{2+}-calmodulin-dependent myosin light chain phosphorylation, but might also be predicted to inhibit mediator release. Since extracellular and intracellular pools of Ca^{2+} have been implicated in the release of various mediators, an agent modulating both sites would seem to be optimal.

Modulation of the cyclic AMP system in airway smooth muscle offers another therapeutic approach to the treatment of asthma. The relatively recent advancement of forskolin (31), a non-receptor-dependent activator of adenylate cyclase [346] which possesses bronchodilator activity [347], offers the possibility for development of agents which might directly activate adenylate cyclase activity and stimulate cyclic AMP formation. Also, recent development of agents such as milrinone (32), MDL 17,043 (33) and CI-914 (34), which are specific inhibitors of the high cAMP affinity form (peak III isozyme) of phosphodiesterase, offers another approach towards the attainment of compounds which increase cAMP formation. Finally, a more novel approach would be development of modulators of the activity of cAMP-dependent protein kinase. This approach is particularly attractive, since a diminished activity of this kinase may be a locus of subsensitive β-adrenergic responsiveness which exists in smooth muscle from asthmatic dogs [267].



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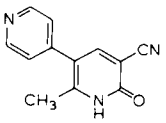

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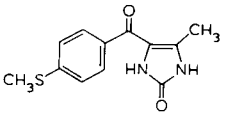

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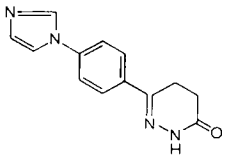

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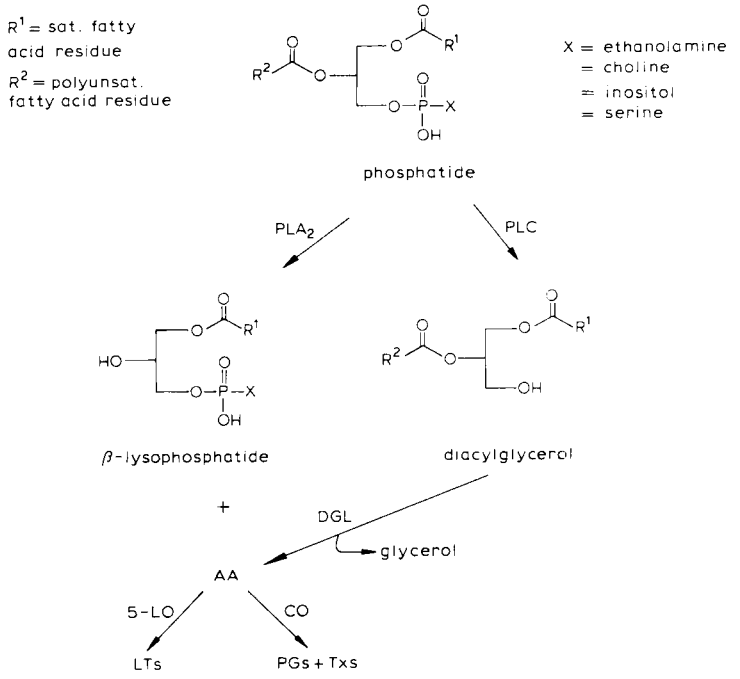
MODULATION OF THE METABOLISM AND ACTION OF PHARMACOLOGICALLY ACTIVE LIPIDS

PHOSPHOLIPASE INHIBITION

In most mammalian cells, the phospholipid fraction constitutes the major source of arachidonic acid (AA) used in the biosynthesis of leukotrienes (LT), prostaglandins (PG) and thromboxanes (Tx). All three classes of compounds are implicated as mediators of anaphylaxis and inflammation. Phospholipase A_2 (PLA_2) is considered the primary hydrolytic enzyme in the degradative pathway to lysophosphatides and AA (*Scheme 7.1*) and therefore a tempting site for chemical inhibition.



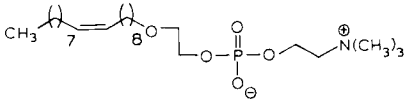
Scheme 7.1. Biosynthesis of arachidonic acid.

Most well-known inhibitors of PLA_2 are nonspecific and can be divided into agents which inhibit the enzyme: (a) by interacting directly with the enzyme (e.g., bromophenacyl bromide) [348]; (b) by interfering with the binding of the

substrate (e.g., mepacrine, chlorpromazine, propanolol) [349]; or (c) by interfering with calcium ion binding (e.g., procaine, indomethacin) [350]. Although these compounds are valuable as research tools, they possess other actions that preclude their use in the treatment of asthma.

Corticosteroids are reported to inhibit PLA_2 through the induction of antiphospholipase proteins. Apparently, they induce the synthesis and release of the proteins macrocortin, M_r 15,000, found in macrophages [351], and lipomodulin, M_r 40,000, found in neutrophils [352]. These proteins interact directly with the PLA_2 and there seems to be a stoichiometric relationship between inhibitor and enzymes. If the early results of antiphospholipase activity are confirmed for these proteins, it is intriguing to speculate that their synthesis by recombinant DNA methodology or the synthesis of active fragments or small molecule peptide substitutes by traditional organic chemical methodology may provide valuable PLA_2 inhibitors with potential as antiasthma drugs. It remains to be seen whether these proteins or their fragments also produce the side-effects characteristic of corticosteroid administration.

Recently, non-hydrolyzable, rationally designed porcine-pancreatic PLA_2 inhibitors (35) were described. These inhibitors were site-specific inhibitors, but



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did not appear to possess *in vivo* activity [353]. It is encouraging that the design of specific PLA_2 inhibitors based on conformation and active site is possible. However, the challenge will be to employ a source of PLA_2, such as human alveolar macrophages, circulatory basophils or chopped lung, which is relevant to the disease, in order to study these inhibitors.

There may be additional problems in developing PLA_2 inhibitors. During chronic exposure, nonspecific inhibitors of phospholipid turnover are reported to cause lipid storage pool disease [354]. On the other hand, with specific PLA_2 inhibitors, the liberation of AA through the actions of phospholipase C (PLC) and diglycerol lipase (DGL) remains; however, the importance of this metabolic pathway in the generation of LTs and PGs is not clear. Furthermore, specific PLA_2 inhibitors theoretically would prevent the formation of all products of the AA cascade, including endogenous PG bronchodilators such as PGE_2 and PGI_2. Since products of the AA cascade interact on many levels with other systems, the effect of specific PLA_2 inhibitors on asthma or other allergic diseases can not be predicted.

MODULATION OF PROSTAGLANDIN METABOLISM AND ACTION

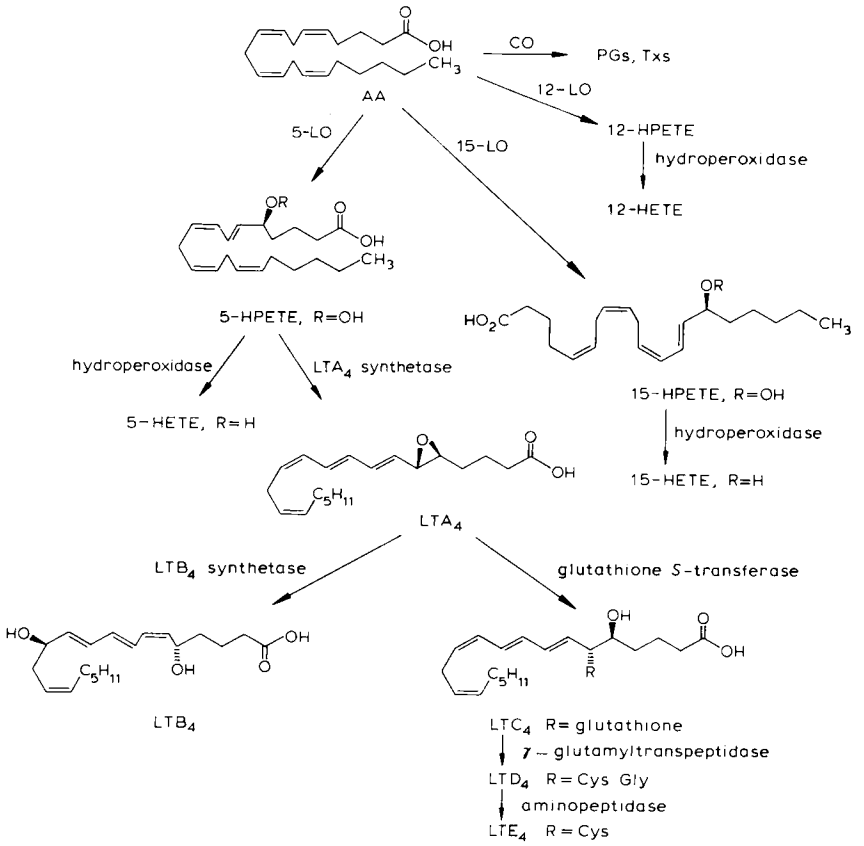
The biosynthetic pathway of prostaglandins (PG) from arachidonic acid (AA) via cyclooxygenase (CO) is well established [355] and the inhibition of cyclooxygenase by non-steroid anti-inflammatory drugs (NSAIDs) partly explains their therapeutic effectiveness in rheumatic diseases [356]. With respect to the treatment of asthma or allergic diseases, however, the modulation of PG metabolism and action is of limited value. In fact, NSAIDs may exacerbate asthmatic conditions in certain patients [357]. Furthermore, PG analogues have been thoroughly explored as bronchodilators, but they lack clinical efficacy and have undesirable side-effects such as bronchial irritation and cough [358].

INHIBITION OF LEUKOTRIENE BIOSYNTHESIS

Leukotrienes, products of the 5-lipoxygenase (LO) biosynthetic pathway, may play an important rôle in the pathophysiology of asthma. The sulphidopeptide leukotrienes, LTC_4, LTD_4 and LTE_4 previously referred to as slow-reacting substance of anaphylaxis (SRS-A), contract smooth muscle and evoke bronchoconstriction in several species, including man. These LTs are also potent stimulators of mucus production and secretion, are potent vasodilators and produce wheal and flare response in man [359–361]. 5-Hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosatetraenoic acid (5-HETE), also products of the 5-LO biosynthetic pathway, stimulate histamine release from basophils [367]. Clearly, chemical intervention of LT biosynthesis and action has clinical potential.

The biochemical steps in the synthesis of LTs from AA have recently been elucidated (*Scheme 7.2*). 5-HPETE derived from the 5-lipoxygenation of AA is converted by enzymatic dehydration into LTA_4 [368]. LTA_4, in turn, is converted by enzymatic hydration into LTB_4 [369] and by conjugation with glutathione to LTC_4, which is transformed by sequential peptidolysis to LTD_4 and LTE_4 [370–372]. Since each step in the biosynthesis of LTs involves a unique enzymatic reaction, all enzymes in the 5-LO cascade are potential candidates for inhibition.

In the first step in the biosynthesis of LTs, 5-LO catalyzes the formation of the C-5 hydroperoxide of AA by specifically abstracting the C-7 pro-S hydrogen [373]. Presumably, the mechanism involves a pentadienyl radical [374] and the rate of reaction is controlled by a complex set of factors based on the peroxide content and redox state. It is not surprising, therefore, that most of the reported 5-LO inhibitors are antioxidants or free-radical scavengers and are nonspecific in that they also inhibit other lipoxygenases such as 8-, 12- and

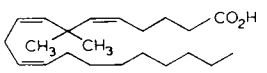


Scheme 7.2. Biosynthesis of leukotrienes.

15-LOs and cyclooxygenase. For example, nordihydroguairetic acid (NDGA), 3-amino-1-[3-trifluoromethylphenyl]-2-pyrazoline (BW755C), phenidone, quercetin, gossypol and butylated hydroxyanisole (BHA) are reported to inhibit 5-LO, 12-LO, 15-LO and cyclooxygenase [375–377].

Another group of 5-LO inhibitors can be classified as substrate analogues. Compounds such as 7,7-dimethyl-AA (36) and 7-ethano-AA (37) reversibly inhibit 5-LO by blocking C-7 proton abstraction [378,379].

In contrast, acetylenic substrate analogues are believed to irreversibly inhibit LO enzymes. The mechanism of inactivation was originally thought to involve a highly reactive allene hydroperoxide which reacts covalently with amino acid



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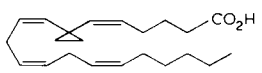

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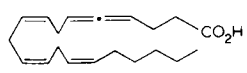

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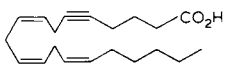

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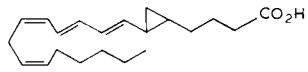

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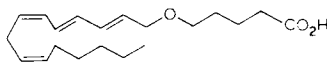

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residues of the active centre [380]. Later, in reports on the irreversible LO inhibitors 4,5-dehydro-AA (38) [381] and 5,6-dehydro-AA (39) [382], it was postulated that the allenic hydroperoxide intermediate could undergo facile oxygen-oxygen bond homolysis to radicals which inactivate the enzyme. This hypothesis is supported experimentally by the detailed kinetic studies suggesting that the oxygenation of a methionine residue at the catalytic site is the reason for inactivation [383]. It is interesting to note that the LTA_4 analogues 5,6-methano- (40) [384] and 5,6-seco-LTA_4 (41) [385] are inhibitors of 5-LO, although they are not, strictly speaking, substrates for 5-LO.

15-HETE is reported to be an endogenous inhibitor of 5-LO [386]. This fact was used by one of us in an attempt to rationally design specific inhibitors of 5-LO by replacing the relatively unstable double bonds of 15-HETE with aromatic rings [387]. When comparing certain computer-generated space-filling models of 15-HETE with aromatic-ring-stabilized analogues, several features become apparent. The 11,13-*cis,trans*-pentadiene of 15-HETE is easily accommodated with a phenyl ring. However, substituting a ring for the remaining C-5 or C-8 double bond while retaining a good isosteric fit is less straightforward. Our solution was to bridge C-2 to C-7 and to substitute a methylene oxygen group for the C-8 double bond. The isosteric relationship between compound (48) (see *Table 7.6*) and 15-HETE is shown in *Figure 7.2*.

The results obtained for a representative group of cyclic 15-HETE analogues as inhibitors of rat neutrophil 5-LO are listed in *Table 7.6*. In general, esters are more active than the corresponding carboxylic acids. The most potent non-nitrogenous compound (46) (see *Table 7.6*) has an ester moiety in the 2-position. Blocking the side-chain hydroxyl group with acetyl, methyl or

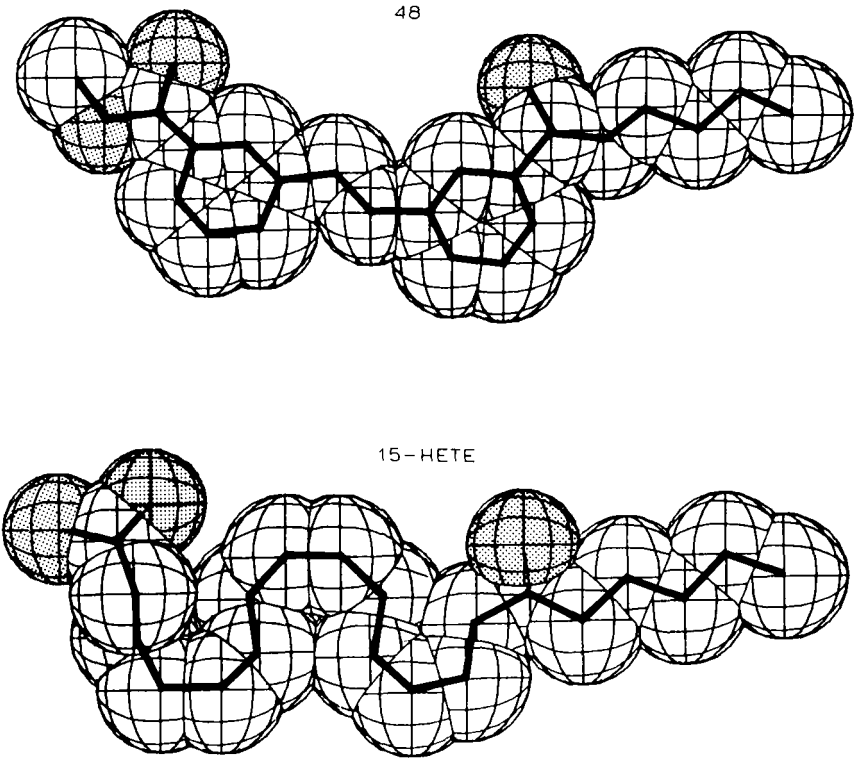


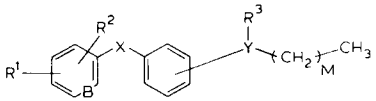
Figure 7.2. Isosteric relationship between compound (48) and 15-HETE.

tetrahydropyranyl lowered activity. Other modifications, such as substituting groups other than a carboalkoxy in the A ring, reversing the ether linkage, eliminating water from the alkyl side-chain and branching of the alkyl side-chain also reduced activity. However, substituting a carbonyl function for a carbinol moiety or a methyl for the methine of the carbinol moiety slightly increased activity.

The incorporation of nitrogen within the A ring increased 5-LO inhibitory activity. It is significant that, like the carbomethoxy phenyl analogues, the pyridyl isomer with nitrogen in the 2-position is more active than either the 3- or the 4-pyridyl isomer.

The *in vitro* profile of several 15-HETE cyclic analogues in the AA cascade enzyme assays is shown in *Table 7.7* [388]. These data demonstrate that

Table 7.6. 5-LIPOXYGENASE INHIBITORS



5-Lipoxygenase (5-LO) was assayed by incubating rat PMN with calcium ionophore (3 μg/ml) and [^{14}C]arachidonic acid (AA) for 3 min at 30°C, and determining 5-HETE produced.

No.	R^1	R^2	*B*	*X*	*Y*	R^3	*M*	*5-LO* I_{50} *values (μM)*
42	3-CO_2H	H	C	CH_2O	3-$CHCH_2$	OH	3	25
43	2-CO_2H	H	C	CH_2O	3-$CHCH_2$	OH	3	30
44	4-CO_2H	H	C	CH_2O	3-$CHCH_2$	OH	3	21
45	3-CO_2CH_3	H	C	CH_2O	3-$CHCH_2$	OH	3	1.2
46	2-CO_2CH_3	H	C	CH_2O	3-$CHCH_2$	OH	3	0.6
47	4-CO_2CH_3	H	C	CH_2O	3-$CHCH_2$	OH	3	3.2
48	3-CO_2CH_3	H	C	CH_2O	2-$CHCH_2$	OH	3	3.2
49	3-CO_2CH_3	H	C	CH_2O	3-$CHCH_2$	$OCOCH_3$	3	5.0
50	3-CO_2CH_3	H	C	CH_2O	3-$CHCH_2$	OCH_3	3	10
51	3-CO_2CH_3	H	C	CH_2O	3-$CHCH_2$	OTHP	3	23
52	2-CH_2OH	H	C	CH_2O	3-$CHCH_2$	OH	2	4
53	3-CO	H	C	CH_2O	3-$CHCH_2$	OH	2	4.0
54	3-CH_2NH_2	H	C	CH_2O	3-$CHCH_2$	OH	3	1.4
55	3-$CONH_2$	H	C	CH_2O	3-$CHCH_2$	OH	3	2.0
56	H	H	C	CH_2O	3-$CHCH_2$	OH	3	2.7
57	H	H	C	OCH_2	3-$CHCH_2$	OH	3	6.7
58	3-CO_2CH_3	H	C	CH_2O	3-$CH{=}CH_2$	CH_3	3	5.0
59	H	H	C	CH_2O	3-C-CH_2	O	3	2.0
60	H	H	4-N	CH_2O	3-$CHCH_2$	OH	3	2.7
61	H	H	2-N	CH_2O	3-$CHCH_2$	OH	3	0.5
62	H	H	3-N	CH_2O	3-$CHCH_2$	OH	3	2.7
63	3,4 		2-N	CH_2O	3-$CHCH_2$	OH	3	0.3
64	H	H	C	CH_2O	3-$CHC(CH_3)_2$	OH	3	2.5
65	3,4 	–	2-N	CH_2O	3-C-CH_2	O	3	0.15
66	H	H	C	CH_2O	3-$C(CH_3)CH_2$	OH	3	1.0
67	3,4 	–	2-N	CH_2O	3-$C(CH_3)CH_2$	OH	3	0.1
68	3-$CONH_2$	H	C	CH_2O	3-CCH_2	O	3	0.7

Table 7.7. *IN VITRO* PROFILE OF 15-HETE CYCLIC ANALOGUES

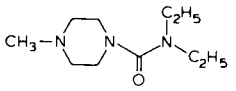
5-Lipoxygenase (5-LO) was assayed by incubating rat or human PMN with calcium ionophore (3 μg/ml) and [^{14}C]arachidonic acid (AA) for 3 min at 30°C, and determining 5-HETE produced. Cell-free 5-LO was from rat basophilic leukaemia cells (RBL), and 12-LO from rat platelets. Compounds were assayed at 100 or 50 (*) μM against 12-LO. Cyclooxygenase (CO) was from sheep seminal vesicles (compounds at 200 μM). Inhibition as percentages.

No.	*5-LO*			*12-LO*	*CO*
	Rat PMN	*Human PMN*	*Cell-free*		
56	6.0	20	–	8	100
45	2.5	7	100	43%	2%
55	1.4	8	23	80%*	30%
61	2.0	17	56	8%	−33%
62	0.5	8	140	0%	0%
63	0.16	6	80	−25%	−21%
67	0.15	14	20	−47%	–

compounds in this series differ from compounds such as BW755C in that they are potent inhibitors of cellular 5-LO while lacking inhibition against cell-free 12-LO and CO from sheep seminal vesicles.

The effect of specific 5-LO inhibition on asthma or allergic diseases is not easy to predict. Since 5-LO occupies a branch point in AA metabolism, inhibition of this enzyme may make more AA available to the CO pathway. It is established that CO inhibition increases LT production; therefore, it is reasonable to predict that 5-LO inhibitors will increase PG and Tx production. Whether this has a positive or negative effect on asthma depends on the cell system or tissue involved. The modulatory rôle of LTs in numerous physiological responses has already been documented, suggesting that LO inhibitors may potentially influence organs and physiological events other than those involving the lung. In asthma, aerosol inhalation of 5-LO inhibitors may be the preferred route in order to circumvent this potential problem.

Following 5-LO, the next enzyme in the LT cascade is LTA_4 synthetase. It is an ideal target to inhibit because effects due to shunting of AA to CO pathway would be minimized and only the synthesis of LTs would be inhibited. Little is known about the enzyme except that it specifically removes the 10-pro-R hydrogen in the dehydration of 5-HPETE to give LTA_4 [389]. Diethylcarbamazine (DEC) (69) is reported to be a specific inhibitor of LTA_4 synthetase [390]; however, the precise mechanism of inhibition is not known at present. DEC, initially developed as an antihelminthic agent, was observed to provide relief in patients with intractable asthma [391]. Later, DEC was



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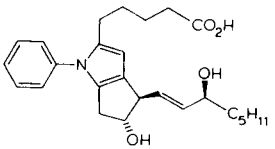
shown to inhibit the release of sulphidopeptide LTs in the rat [392] and from monkey [393] lung tissue. DEC and/or its analogues have not been further explored, as far as we are aware, as potential LT synthesis inhibitors or as agents for the treatment of asthma.

Of the remaining enzymes in the LT cascade, only glutathione *S*-transferase has attracted serious attention. U-60,257 (70) was originally reported to be a



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selective inhibitor of glutathione *S*-transferase [394]; however, it was later found to be active against 5-LO from human PMNs [395]. This dual action may explain why U-60,257 was more active *in vivo* when given by aerosol to *Ascaris*-sensitive monkeys than it was in the reported *in vitro* models [396].

Glutathione *S*-transferases are found in most cells and are important in detoxification reactions. The potential for specific glutathione *S*-transferase inhibitors as therapeutically useful agents depends on whether a unique glutathione *S*-transferase is involved in the biosynthesis of LTC_4 (i.e., whether a LTC_4 synthetase exists). Preliminary results in a rat basophilic leukaemic cell line are suggestive of an LTC_4 synthetase in the high-speed particulate fraction that may be different from the soluble glutathione *S*-transferases [397]. The existence of an LTC_4 synthetase in human leucocytes would help validate this approach to the development of antiasthma drugs.

LEUKOTRIENE ANTAGONISM

Another approach to the development of novel antiasthma agents and anti-allergy agents involves the search for sulphidopeptide LT antagonists. FPL 55,712 (71) (*Figure 7.3*) was the first compound to be described as an antagonist of sulphidopeptide leukotrienes [398]. As a pharmacological tool,

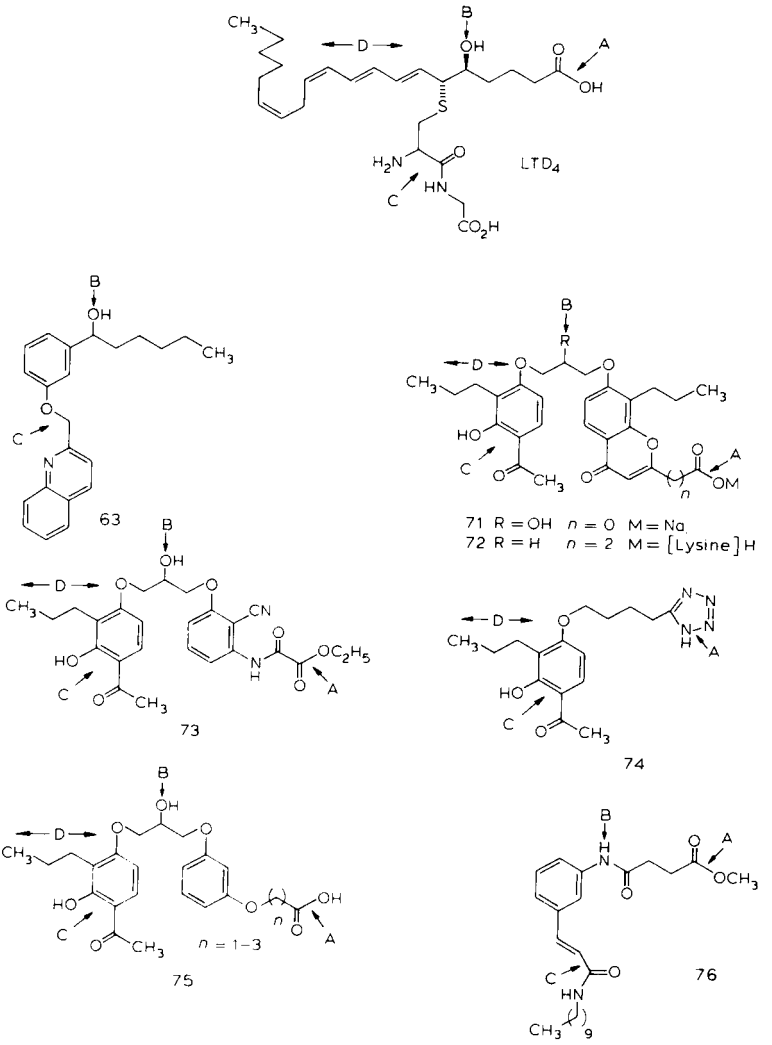


Figure 7.3.

it has been extensively used to define the rôle sulphidopeptide LTs play in the immunopathology of immediate hypersensitivity in man and animals [399]. However, FPL 55,712 has a short duration of action and is orally inactive, and as such has not been further developed as a drug.

Several analogues of FPL 55,712 have been reported as sulphidopeptide LT antagonists. For example, FPL 59,257 (72) is a longer-acting LTD_4 antagonist; however, it is still orally inactive. While FPL 55,712 competitively antagonizes LTD_4 using guinea-pig ileum, FPL 59,257 is a non-competitive antagonist [400]. Wy-44,329 (73) is a LTD_4 antagonist that is chemically related to FPL 55,712. This molecule has the left-hand portion of FPL 55,712 appended to a substituted ethyl oxanilate instead of the chromone found in FPL 55,712 [401]. Wy-44,329 has a much longer duration of action but, like the FPL compounds, it is orally inactive.

Recently, LY-171,883 (74) was reported orally active against LTD_4-induced bronchospasm in the guinea-pig [402]. It has the 2-propyl-3-hydroxyacetophenone of FPL 55,712 with the chromone heterocycle removed and the carboxylic acid moiety replaced by a tetrazole. Apparently, LY-171,883 is under development as an antiasthma drug. Because of the initial success of employing the 2-propyl-3-hydroxyacetophenone moiety with various right-hand systems, we expect many related compounds with this combination in the future. Several examples of incorporating the left-hand portion of FPL 55,712 have already appeared in the patent literature; a typical example is compound (75) [403].

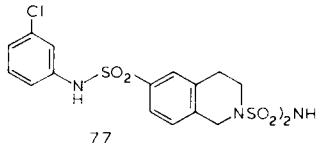
In contrast to the above compounds, some structurally novel sulphidopeptide LT antagonists are known. For example, REV 5901A (63), which contains a 2-methylquinolinyl heterocycle bridged to a 1-hydroxyhexylphenyl system, antagonizes LTC_4-induced contraction of guinea-pig lung *in vitro* and is also a potent 5-LO inhibitor, as indicated earlier. The fact that it inhibits 5-LO is not without precedent, since FPL 55,712 is a 5-LO inhibitor in a cell-free system [404] and Wy-44,329 inhibits the 5-LO in rat neutrophils (Chang, unpublished observations). When administered intraduodenally, REV 5901A inhibited sulphidopeptide LT-mediated bronchoconstriction in guinea-pigs [405]. Another structurally novel compound is a cinnamic acid derivative (76) [406]. Whether it is orally active is not clear from the patent literature.

The structural relationship among the above sulphidopeptide LT antagonists and LTD_4 deserves comment. All the compounds in *Figure 7.3* contain two or more of the following structural features: (A) a terminal carboxylic acid or equivalent, (B) a hydroxyl group or equivalent, (C) a potential metal-chelating moiety and (D) a lipophilic tail. The long lipophilic tail of LTD_4 may confer agonist properties because the antagonists either lack this portion or have it in truncated form. LY-171,883 contains a tetrazolyl moiety, which has a pK_a and electronic configuration similar to a carboxylic acid, in addition to functional groups (C) and (D). REV 5901A has only functional groups (B) and

(C). It would be interesting to examine a compound similar to REV 5901A with a carboxylic acid substituted for the hexyl-chain terminal methyl and an *n*-propyl group added on the phenyl 3-position to determine whether the LTD_4 antagonism potency would increase significantly. Both Wy-44,329 and FPL 55,712 have all four functional groups; however, the spatial relationships among the groups do not closely approximate the distances found in LTD_4. The amide group of compound (76) may act as a substitute for the hydroxyl group (B) because the pK_a values are similar for both groups. There is evidence that Ca^{2+}, Mg^{2+} and Mn^{2+} enhance binding of LTD_4 at receptor sites [407]. Therefore, it is intriguing to speculate that a metal divalent cation is involved with the above antagonists, because they all contain potential metal-cation-binding functional groups.

LT receptor sites are heterogeneous in nature. Schild analysis of FPL 55,712 antagonism provided evidence for two distinct receptors for LTD_4 in the guinea-pig trachea [408]. Separate receptors for LTC_4 and LTD_4 in guinea-pig lung parenchymal strip were also described [409]. Therefore, generalizations about LT receptors and compounds that antagonize LTs must be taken with caution.

Compounds have been reported to be sulphidopeptide LT antagonists because of their effects in smooth muscle preparations or because they block antigen-induced bronchoconstriction *in vivo*. However, the observed activity may be due to other causes. For example, SKF 88,046 (77), which was initially



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reported to be a selective antagonist of LTD_4, is now known to act indirectly as an end-organ antagonist of TxB_2, $PGF_{2\alpha}$ and PGD_2 [410].

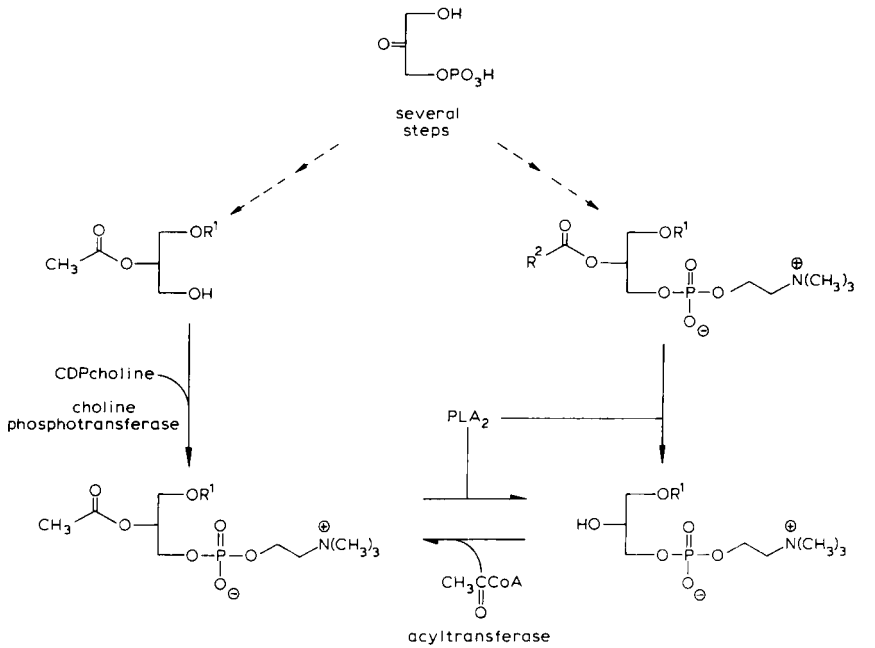
In spite of the above discussion, it might be argued that compounds with more than one mechanism of action may be desirable. For example, a mixed LT antagonist and 5-LO inhibitor might be beneficial in asthma. However, specific compounds are needed to validate the sulphidopeptide LT antagonist approach to the development of novel antiallergy and antiasthma drugs.

MODULATION OF PLATELET-ACTIVATING FACTOR SYNTHESIS AND ACTIVITY

Although the understanding of activities, biochemistry and pathophysiology of PAF is only at the descriptive stage, enough data are available to warrant drug

research in this area. PAF is currently being considered as a mediator of acute allergic and inflammatory responses. Tissues and a variety of cells from humans and other species release PAF when exposed to anaphylactic agents [411–413]. It has been shown that PAF is released into the circulation of rabbits during anaphylactic responses [414]. In addition, PAF contracts lung tissue from several species, including man, *in vitro* [415,416] and induces bronchospasm in experimental animals [417–419]. PAF also increases vascular permeability [420], neutrophil infiltration [421] and causes vasodilation [422]. Specific receptor sites for PAF on rabbit platelet and guinea-pig ileum have also been described [423].

The biosynthetic pathway of PAF has not been completely described; hence, *Scheme 7.3* represents the current information on the formation and degradation of PAF. Apparently, the intermediates in the biosynthesis of PAF originate from dihydroxyacetone monophosphate after several transforma-



R^1 = $C_{16:0}$, $C_{18:0}$, $C_{18:1}$

R^2 = C_{18}–C_{20} sat. and unsat.

Scheme 7.3. Biosynthesis of platelet-activating factor.

tions; the critical step is the formation of the ether linkage with a reduced long-chain fatty acid. Two independent pathways have been identified for the final step in the biosynthesis of PAF. One involves a specific acyl transferase that is not calcium-dependent and uses lyso-PAF as a substrate [424]. The other pathway is also independent of calcium and involves the action of choline-phosphotransferase on 1-*O*-alkyl-2-acetylglycerol and cytidine diphosphate choline [425]. Both enzymes are found in greatest abundance in lung and spleen. In addition, PLA_2 may be involved in both the formation and degradation of PAF, since inhibitors of this enzyme prevent the release of PAF [426].

There has been a recent suggestion that DSCG is effective in the treatment of asthma because it inhibits the late bronchoconstrictor onset response due to PAF [427]. If this speculation gains experimental support, the search for novel PAF modulators should increase. However, others have failed to demonstrate effects of antiallergic drugs such as cromoglycate on PAF-induced bronchospasm in the guinea-pig [180]. Therefore, the rôle of PAF in asthma remains to be elucidated.

AEROSOL VERSUS SYSTEMIC ADMINISTRATION OF BRONCHODILATORS AND ANTIALLERGICS

Inhalation therapy has been used for several thousand years in the treatment of lung disorders and is well accepted because of its effectiveness and rapid onset of action. It provides a special advantage over parenteral or oral routes of administration, since the primary event in allergic asthma occurs at the airway lumen or bronchial epithelium. The inhalation route of administration of bronchodilators and antiallergics which is usually achieved by metered dose inhaler (MDI), drug powder inhaler or nebulizer also minimizes the risk of systemic side-effects.

For example, β_2-adrenoceptor agonists administered by inhalation give better bronchodilatory effects, with less muscle tremor and tachycardia, than when given parenterally or orally. Only about 10% of the dose of bronchodilator generated by an MDI is retained in the lung, the majority being deposited in mouth and throat [428]. Some of this is absorbed through the buccal mucosa, although most is swallowed and in the case of β_2-agonists such as isoprenaline, metabolized during its passage through the wall of the intestine or the liver. About 75–90% of the oral dose of the same drug is metabolized, so that the oral dose is often greater than the inhaled dose by a factor of 10. These pharmacokinetic factors contributed greatly to airways selectivity; for

example, β_2-agonists such as terbutaline are not metabolized in the lung by first-pass *O*-methylation by COMT and may be expected to reach the systemic circulation.

Potential problems with aerosol therapy are not thought to be serious. It is important that the aerosol drug be potent, since the drug is dispersed into a large volume and undergoes substantial loss due to mucociliary clearance. Also, during a severe acute asthma attack, nebulized bronchodilators are effective only after expectoration of mucus, which necessitates the use of parenterally administered bronchodilators. Regular intake of bronchodilators, antiallergics or corticosteroids does not result in addiction, as was sometimes thought to be the case. The chlorofluorocarbon propellants used in MDIs cause palpitations, but only if they are inhaled in excess [429].

The method of inhalation affects drug delivery. Nebulized bronchodilators are widely used but they are generally not as good as the MDIs. However, the MDIs are often misused, resulting in inefficiency. A new technological development is the use of the spacer tube or cone between the mouth and MDI which enhances pulmonary deposition of particles by facilitating evaporation of the chlorofluorocarbon propellant and reducing impaction of the drug in the oropharynx [430].

ADDENDUM

During the preparation of this chapter, it was proposed that macrocortin and lipomodulin, the anti-PLA_2 proteins, be known as lipocortin (M. DiRosa et al., Prostaglandins, 28 (1984) 441); furthermore, this material has been cloned by Biogen. A number of specific PAF antagonists have also been reported and one of them, BN 52021 (9*H*-1,7a(epoxy-methano)-1*H*,6a*H*-cyclopenta[*c*]furo[2,3-*b*]furo[3′,2′:3,4]cyclpenta[1,2-*d*]furan-5,9,12-(4*H*)-trione,3-*tert*-butyl-hexahydro-4,-7b,-11-hydroxy-8-methyl), inhibited both PAF-induced and anaphylactic-bronchospasm in guinea-pigs (C. Touvay et al., Int. J. Immunopharmacol., 7 (1985) 385).

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